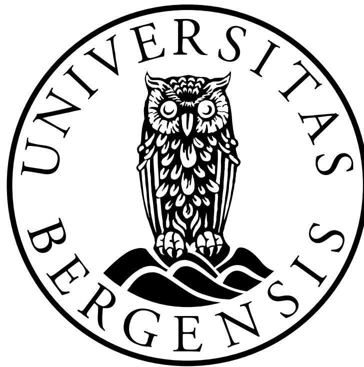
Biological Characteristics of Stem-Like Cells in Oral Squamous Cell Carcinoma

Xiao Liang



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Summary

It has been shown that many solid malignancies, including oral squamous cell carcinoma (OSCC), contain a small population of tumorigenic cells referred to as cancer stem-like cells (CSCs) or tumor initiating cells (TICs). The concept of stem cell hierarchy in cancers, coupled to the phenomenon of epithelial-to-mesenchymal transition (EMT), seems to explain well the biology of most cancers, as well as the failure of the currently used therapeutical methods. Nevertheless, more evidence is needed for understanding of both these concepts as well as the relationship between them in cancer in general, and in OSCC in particular.

By using marker-based fluorescence-activated cell sorting (FACS), the most commonly used method for isolation of CSCs, we aimed to investigate whether there is a link between CSCs and the EMT phenomenon in OSCC-derived cell lines and primary cells (Paper I). Subpopulations of cells were sorted by FACS using their differential expression of the CD44 adhesion molecule and epithelial specific antigen (ESA), and then investigated for stem cell and EMT properties both *in vitro* and *in vivo*. Our findings showed that the CD44^{high} fraction of CSCs contained (1) an ESA^{high} fraction which was the holoclone-forming population and had more epithelial characteristics (EPI-CSCs), and (2) an ESA^{low} fraction which was more migratory and formed more spheres (EMT-CSCs). These two subpopulations of CSCs were able to swift between each other, although the EPI-CSC fraction was highly bipotent (~100%), while only a proportion of the EMT-CSC fraction was bipotent (≤ 50%).

We further investigated whether the CSCs phenotype and the switch between the two phenotypes (EPI-CSCs and EMT-SCSs) can be modulated by external signals from stem cell niches such as neighboring stroma (Paper II). OSCC-derived cell lines and primary cells were FACS sorted into a subpopulation enriched for CSCs (CD44^{high}) and a subpopulation depleted in CSCs (CD44^{low}), and the effect of carcinoma associated fibroblasts (CAFs), one of the major components of tumor stroma in carcinomas, on self-renewal and tumorigenic abilities of these subpopulations was investigated. The results demonstrated that CAFs were capable to enhance the self-

renewal and tumorigenicity of OSCC cells, and that TGF-β1 was the key regulator for this effect. Of most interest was the finding that CAFs induced clonogenic and tumorigenic abilities in the subpopulation of cancer cells depleted for CSCs, pointing to the need for all cell subpopulations to be targeted in cancers for successful therapy.

Although FACS is used extensively for CSC sorting, the method might be prone to certain inconsistencies and a subjective way of gate setting, in addition to the fact that the antibody used for staining might modify the phenotype of cells when binding to them. Therefore we aimed to establish more functional and robust methods for isolation of CSCs (Paper III). The potential of a previously reported method for isolating normal epithelial stem cells using their differential adhesiveness to one of the main components of basement membrane, collagen IV, for CSCs enrichment was tested for OSCC-derived cells. Assessment of the biological properties of the subpopulations separated over different time according to their adhesiveness to collagen IV (RAC-10 min, MAC-30 min, and LAC-4 h) indicated that the rapid adherence to collagen IV can be used as a non-invasive and functional method to enrich for OSCC cells with increased colony and sphere forming ability, tumor formation ability and with high expression of stem cell-related markers (RAC). We also characterized these cells for their electrophysiological properties utilizing the technique of dielectrophoresis (Paper IV). The results showed that the subpopulation of RAC cells enriched for CSCs possessed differential electrophysiological properties when compared to the other subpopulations (MAC and LAC), and this was mainly due to their particular cell membrane morphology, very rich in cellular protrusions. In addition, this work suggested that CSCs in general might exhibit unique dielectrically features that can be putatively used in the future for their isolation, as a label-free and non-invasive method.

List of publications

Paper I

Biddle A, ***Liang X***, Gammon L, Fazil B, Harper LJ, Emich H, Costea DE and Mackenzie IC. Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. Cancer Res. 2011 Aug 1;71(15):5317-26. Epub 2011 Jun 17.

Paper II

Osman AH, ***Liang X***, Bilal F, Biddle A, Neppelberg E, Lybak S, Liavaag PG, Jacobsen H, Førde H, Vintermyr OK, Johannessen AC, Mackenzie IC, Wang J, Enger PO and Costea DE. Carcinoma associated fibroblasts promote a cancer stem-like cell phenotype through TGF-β1 secretion. (submitted manuscript)

Paper III

Liang X, Osman T, Sapkota D, Neppelberg E, Lybak S, Liavaag PG, Johannessen AC, Jacobsen H, Enger PO, Wang J and Costea DE. A functional approach for isolation and enrichment of oral cancer cells with tumor-initiating properties. (submitted manuscript)

Paper IV

Liang X, Graham K, Johannessen AC, Costea DE and Labeed FH. Human oral cancer cells with stem-like properties exhibit high effective membrane capacitance due to rich cell membrane surface protrusions. (submitted manuscript)

Abbreviations

OSCC	Oral squamouscell carcinoma
CSCs	Cancer stem-like cells
TICs	Tumor-initiating cells
EMT	Epithelial-to-mesenchymal transition
MET	Mesenchymal-to-epithelial transition
CAFs	Carcinoma associated fibroblasts
FACS	Fluorescence-activated cell sorting
ESA	Epithelial specific antigen
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
HNC	Head and neck cancers
TA	Transit amplifying
EGFR	Epidermal growth factor receptor
COX-2	Cyclooxygenase-2
AML	Acute myeloid leukaemia
HIFs	Hypoxia inducible factors
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
PDGF	Platelet-derived growth factor
TGF-β	Transforming growth factor-β
ALDH1	Aldehyde dehydrogenase 1
bFGF	Basic fibroblast growth factor
LRCs	Label-retaining cells
BrdU	Bromodeoxyuridine
3HTdR	Tritiated thymidine
SP	Side population
ABC	ATP binding cassette
HNSCC	Head and neck squamous cell carcinoma

ALDH1A1	Aldehyde dehydrogenase 1 family member A1
NOD-*scidIL2R*γ^{null}	NOD/SCID interleukin-2 receptor gamma chain null
BM	Basement membrane
DEP	Dielectrophoresis
KSFM	Keratinocyte specific medium
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal bovine serum
BPE	Bovine pituitary extract
PBS	Phosphate-buffered saline
RAC	Rapid adherent cells
MAC	Middle adherent cells
LAC	Late adherent cells
CM	Conditioned medium
pHEMA	Poly (2-hydroxyethyl methacrylate)
H&E	Hematoxylin and eosin
PFA	Paraformaldehyde
PI	Propidium iodide
PE	Phycoerythrin
TBST	Tris-buffered saline with Tween
BSA	Bovine serum albumin
PVDF	Polyvinylfluoride
4-MU	4-methylumbelliferone
StEM	Standard error of the mean
NOFs	Normal oral fibroblasts
CAF-CM	Conditioned medium collected from Carcinoma associated fibroblasts
SEM	Scanning electron microscopy

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1. INTRODUCTION

1.1 Oral squamous cell carcinoma

1.1.1 Epidemiology

Head and neck cancers (HNC) are defined as malignant tumors of the upper aerodigestive tract consisting of lip, oral cavity, nasal cavity, paranasal sinuses, pharynx and larynx [1, 2]. More than 650 000 new cases of HNC are diagnosed every year all over the world [3]. In Europe, there are approximately 143 000 new cases and more than 68 000 patients die due to this disease every year [3].

Oral squamous cell carcinoma (OSCC) is the most frequent type of tumor among HNC. It accounts for more than 90% of the cancer types occurring in the oral cavity [1, 4]. On a global scale, combined with oropharyngeal cancer, oral cancer ranks the tenth most common cancer, with the seventh most common cause of cancer induced mortality [1]. Over the past decade, OSCC has been shown to have a significant increased incidence, and a striking geographical variation with high incidence in South and Southeast Asian (*e.g.* China, India, Pakistan and Sri Lanka), as well as parts of Western and Eastern Europe (e.g. France, Hungary, Slovakia and Slovenia) (Figure 1) [5].

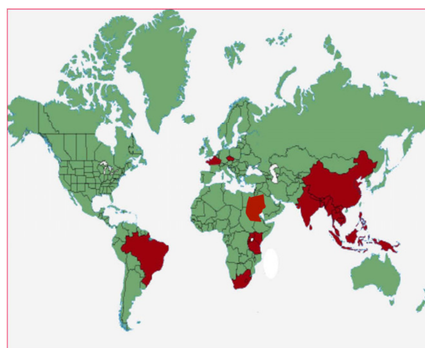

Figure 1. Countries with high incidence and mortality from oral cancer

(Source: Warnakulasuriya, S.; 2009 [5])

OSCC originates from the epithelial lining the oral cavity. Although the oral cavity is easily accessible, and oral cancer itself can be early detected, mortality from this disease remains high due to the late-stage diagnosis, therapy-resistant local and regional recurrence, and distant metastases [6-8]. Despite advances in surgical resection in combination with radiation and chemotherapy, OSCC patients still have poor life expectancy with a high mortality, and a 5-year survival rates ranging from 50-55%, unchanged over the past several decades [9]. Furthermore, rates of secondary oral malignancies are also high with up to a third of patients suffering from tumor recurrence or secondary primary tumors [10].

1.1.2 Malignant transformation of oral mucosa

The development of cancer in the oral cavity is a highly complex multi-step process known as oral carcinogenesis (Figure 2) [11]. There are molecular studies proving that at least some of the cancers in the oral cavity progress from preneoplastic/precancerous lesions developing into carcinoma *in situ,* and then into invasive squamous cell carcinoma (Figure 2) [11, 12].

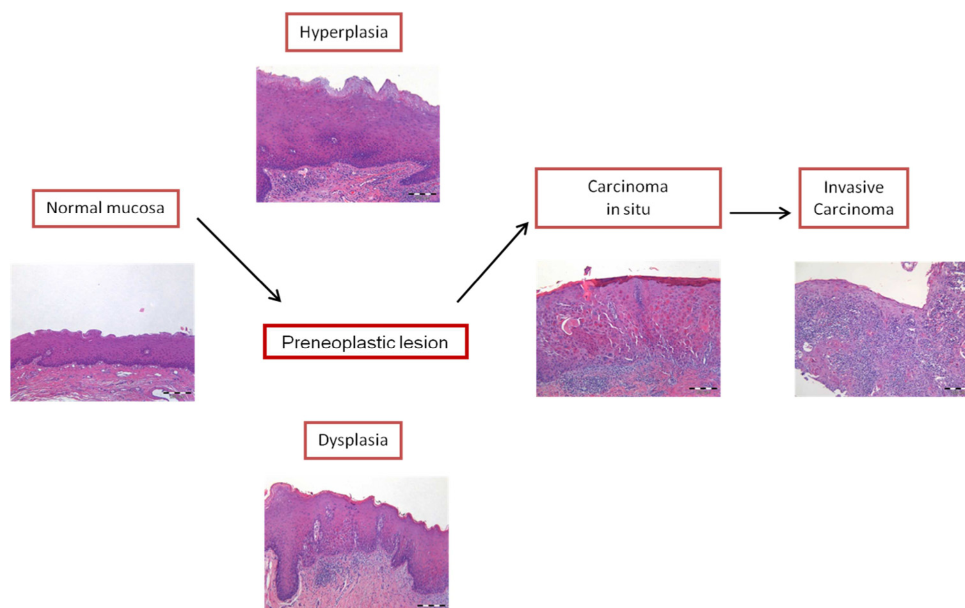

Figure 2. Oral carcinogenesis

Many OSCCs are thought to develop within a field of a disordered oral mucosa (the field cancerization theory) [11, 13, 14]. There are many terms used for the initial lesions, such as ‘pre-cancer’, ‘precursor lesions’, ‘pre-malignant’, ‘intra epithelial neoplasia’ and ‘potentially malignant disorders’, describing the same clinical appearance of a family of morphological alterations with increased potential for malignant transformation [15].

Potentially malignant epithelial lesions include oral leukoplakia, erythroplakia, palate lesion of reverse cigar smoking, oral lichen planus, oral submucous fibrosis, discoid lupus erythematosus, and hereditary disorders (such as congenital dyskeratosis and epidermolysis) [15]. Among them, oral leukoplakia and erythroplakia are the most recognized precursor lesions for malignant transformation in oral mucosa [16, 17]. It is desirable to find ways to predict malignant progression in an epithelial precursor lesion. The presence of epithelial dysplasia seems to be the most important indicator of a malignant progression in a precursor lesion [17, 18]. Nevertheless, the clinical or histological appearance alone does not predict very accurately the risk of malignant transformation [19]. In spite of tremendous progress in molecular oncology, none of the molecular markers alone, or in combination, is capable to predict malignant transformation in an individual patient [17].

1.1.3 Molecular pathogenesis of OSCC

Many hallmarks of cancer, consisting of several essential capabilities acquired during the multistep development of human tumors, have been identified and characterized, providing a solid ground for understanding the biology of cancer. When they were first described, these hallmarks included sustained proliferative signalling, evading programmed cell death (apoptosis), limitless replicated potential, evasion of growth suppressors, sustained angiogenesis as well as activating the invasion and metastasis (Figure 3) [20, 21]. More recently, two more hallmarks of major importance for tumorigenesis were described, namely the reprogramming of cellular metabolism, evading immune destruction (Figure 3) [22]. In addition, two newly emerging enabling characteristics: genomic instability and mutations, as well as tumor-

promoting consequences of inflammatory responses, were recognised a playing an important role in survival, proliferation, and dissemination of tumor cells (Figure 3) [22].

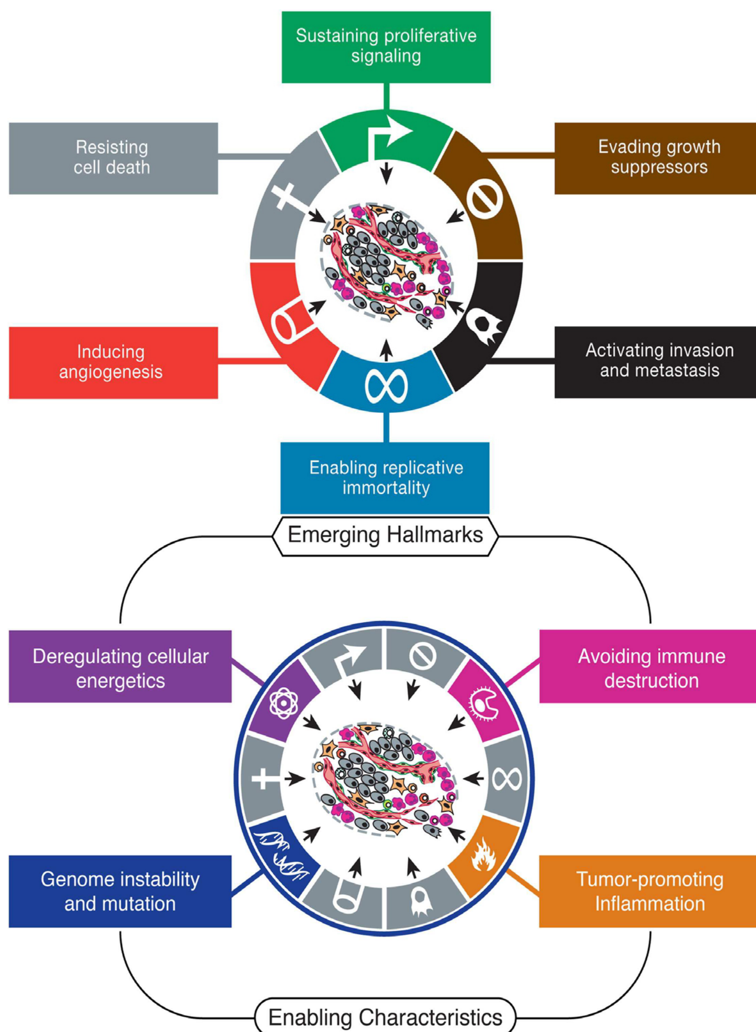

Figure 3: Hallmarks of cancer with emerging hallmarks and enabling characteristics (Source: Hanahan, D. and Weinberg, R. A.; 2011 [22])

Like other cancers, the multistep process underlying oral cancer evolves from the combined influences of individual's genetic predisposition, and chronic exposure to environment carcinogens (such as tobacco, alcohol, oncogenic virus and

inflammation) which can lead to genetic, epigenetic, and metabolic changes (Figure 4) [23]. Recent studies suggest that both precursor lesions and invasive OSCC share similar genetic abnormalities, loss of cellular control, and phenotypic characteristics that give to the transformed cells the ability to invade and metastasize [24, 25]. The multiple genetic alterations are able to activate mutations or amplify oncogenes and inactivate tumor suppressor genes, driving the progressive transformation of normal cells into highly malignant derivatives [20, 26]. In addition, it seems that the molecular targets relevant to cancer are most likely present in precursor lesions as well [24].

Concerning the cell of origin of precursor lesions or OSCC, the normal basal stem cells have been considered the key target for alterations of their genotype, phenotype and physiology, and the starting point for malignant transformation in oral carcinogenesis [27]. In this view, the molecular mechanism underlying development of OSCC includes multistep genetic and epigenetic changes in basal stem cells, such as loss of cell cycle inhibitors p16, p14, and overexpression of epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2). These changes would result in accumulating abnormalities in the relatively quiescent normal basal stem cells, most likely due to exposure of mucosa to carcinogens. Deletion of 9p21and 3p21 are related to the very early transformation from benign hyperplasia to dysplasia. Mutations in *TP*53 gene were also shown to precede transformation from premalignant stages to the invasive stage. Thus, losses of chromosomal material at 9p, 3p, and 17p are considered as early changes of oral carcinogenesis, whereas losses at 13q and 8p are associated with late stages of carcinogenesis (Figure 4) [28].

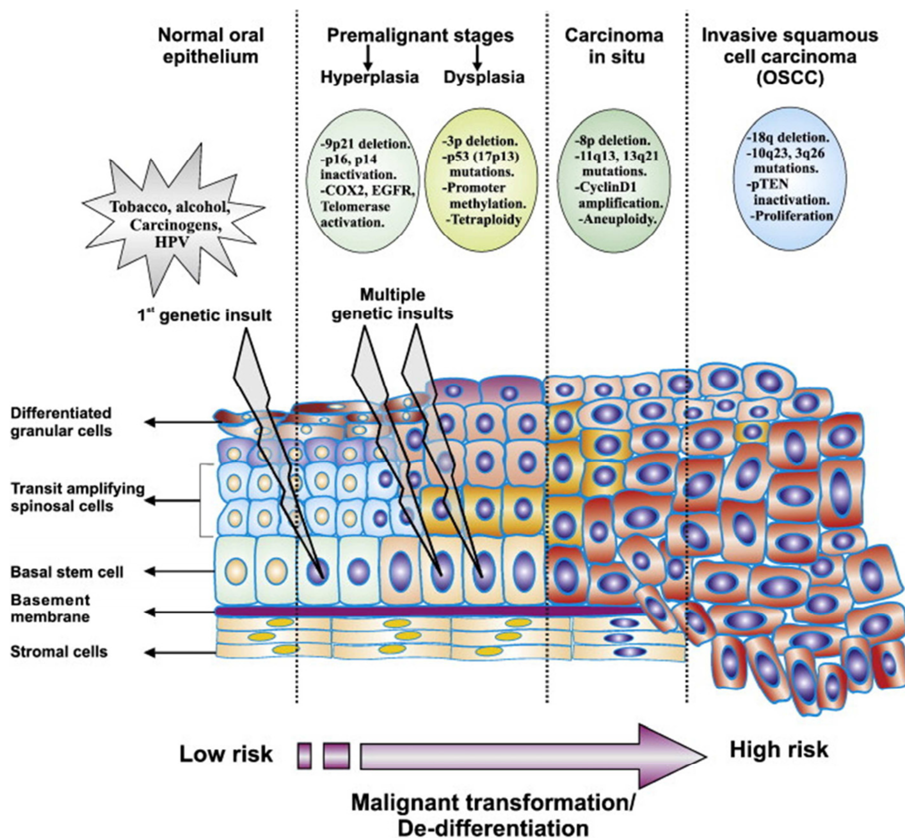

Figure 4: Classic histopathological and genetic tumor progression model of OSCC

(Source: Richard, V., et al ; 2010 [28])

1.2. Cancer stem-like cells in OSCC

1.2.1 Cancer stem-like cells

Tumors are composed of a heterogeneous combination of various subpopulations of cells with different phenotypic characteristics and different proliferative and differentiation capabilities, and this is referred to as tumor heterogeneity [29].Two general models are thought to account for tumor heterogeneity: (1) the "stochastic" model or "clonal evolution", and (2) the "cancer stem cell" (CSC) model [28, 30, 31]. The "stochastic" or "clonal evolution" model considers that most, if not all, cancer cells, although displaying different phenotypes, have proliferative and tumor

generating potential, despite of each single tumor cell having a low probability of exhibiting this potential (that can be assessed by clonogenicity or tumorigenicity assays). On the other hand, the "CSC" model postulates that tumor growth relies exclusively on only a distinct subset of cancer cells that show a consistent high proliferate potential, and that are ultimately responsible for tumor formation and growth, whereas most of the bulk tumor cells are depleted of this ability (Figure 5) [31].

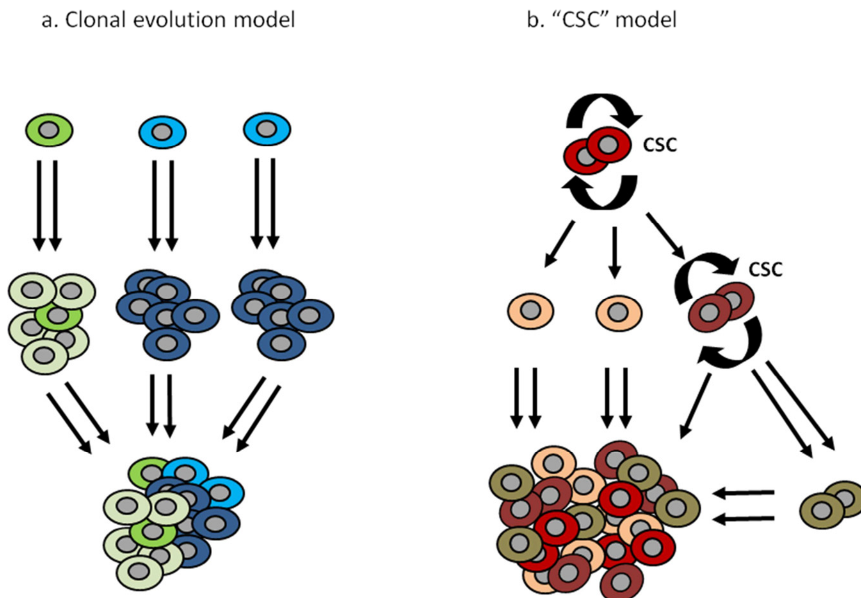

Figure 5: The two models of tumor evolution and heterogeneity

The "CSC" model also postulates that this small group of cancer cells responsible for tumor maintenance, defined as "cancer stem-like cells" (CSCs) or "tumor initiating cells" (TICs), share striking similarities with normal stem cells, including the capacity for self-renewal, the ability to differentiate, active telomerase expression, activation of anti-apoptotic pathways, increased membrane transporter activity, and the ability to migrate and metastasize, and several studies have proven these as being indeed characteristics of CSCs [32]. CSCs are thought to maintain their low numbers by slow self-replication, and could undergo both symmetric and asymmetric divisions. In a state of tumor equilibrium, it is conceived that most of the CSCs are

able to generate another CSC and a transformed "progenitor-like" cell by asymmetric cell division. The progenitor-like cells have limited self-renewal ability but are highly proliferative, similar to a transit amplifying (TA) population in normal tissue, and give rise to partially differentiated bulk tumor cells through a combination of proliferation and abortive differentiation (Figure 6) [33].

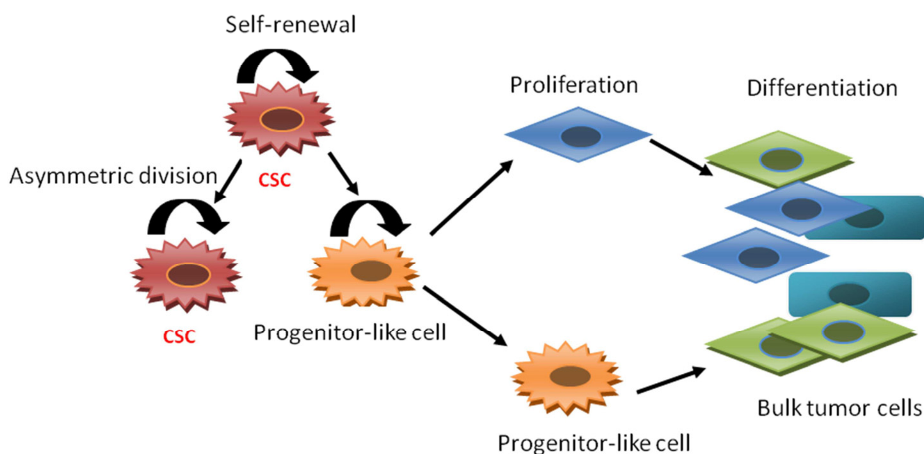

Figure 6: Cancer stem-like cells (CSCs) hypothesis.

Although the concept of CSCs remains a matter of controversy, prospective identification of cells that are able to self-renew and form new tumors can be performed in various ways, but the most common one is based on the differential expression of various cell-surface markers and fractionation of tumors into subpopulations of cells. The first evidence for the presence of CSCs came from acute myeloblastic leukemia, in which only a small subset of cancer cells was found to be clonogenic in culture [34]. CSCs could be then experimentally defined in human acute myeloid leukaemia (AML), where a rare subset of cells identified by its $CD34^{+}/CD38^{-}$ phenotype were capable of initiating human AML after transplantation to immunocompromised mice [35]. Recently, more evidence suggests that CSCs exist in many different solid tumor types, including breast [36], colon [37], brain [38], lung [39], prostate [40], liver [41], and melanoma [42].

With respect to oral lesions, very early studies showed that only a subpopulation of tumor cells could form expanding tumor colonies, suggesting that human OSCC may contain some form of stem cells [43]. Later on, it has been shown that only a small subpopulation of the cells in OSCC corresponds to tumor-initiating cells [44], and that cells with self-renewal ability persist in OSCC cell lines even after long time *in vitro* propagation, and therefore appear to possess the essential defining properties of CSCs [45].

1.2.2 Stem cell niche

Normal stem cells rely on a specialized microenvironment or niche for their survival and function [46]. Similar to normal stem cells, CSCs are considered to reside in a similar tumor microenvironment, termed "CSC niche", which contains inflammatory cells, stromal cells such as fibroblasts, blood vessels, and extracellular matrix components (Figure 7) [47]. CSCs depend on their niche for support and maintenance of their stem characteristics. The niche allows CSCs to stay in an undifferentiated state by regulating the proliferative, self-renewal signaling, and the development of differentiated progenitor cells [48, 49].

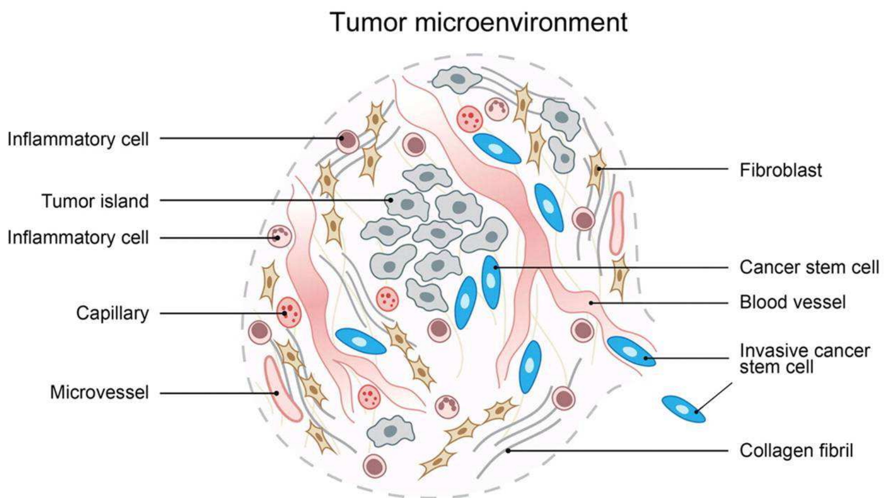

Figure 7. The CSC niche

(Source: Krishnamurthy, S., and Nor, J. E.; 2011 [47])

Furthermore, recent evidence have also demonstrated that the CSC niche play an important role in tumor metastasis by de-differentiating non-CSCs into CSCs, inducing epithelial-mesenchymal transition (EMT) and finally evolving in a pre-metastatic niche at the new site, which contributes to the formation of metastasis (Figure 8) [46].

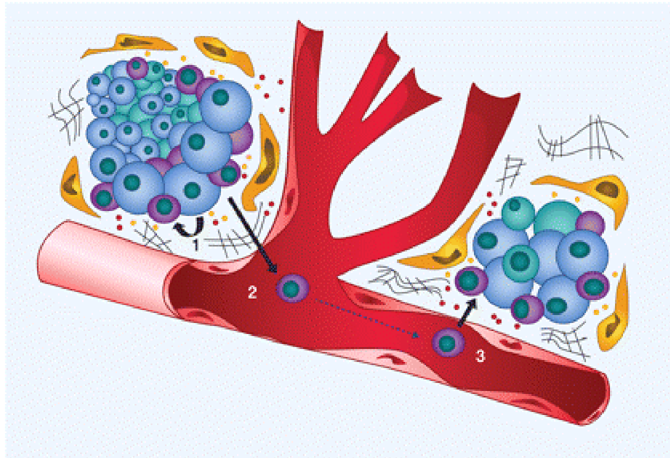

Figure 8. The CSC niche in tumor growth and metastasis
(Source: Borovski, T., et al; 2011 [46])

Several studies indicated that fibroblasts, the predominant cell type in the stoma, play an important role on oral epithelial cell behavior and survival, including malignant tumor invasion [50, 51], and thus it has been theorized that fibroblasts would also play a major role for CSC maintenance, and be a part of the CSC niche [52]. In OSCC, microenvironmental alterations that are related to fibroblast activation or conversion to an active phenotype appear to be involved in the malignant progression of oral epithelial cells [50, 51, 53]. Either through stimulation from cancer cells or by directly stimulation of the same exogenous agents that induce genetic alterations in the adjacent epithelial cells, fibroblasts could get activated or phenotypically change, and these activated/changed fibroblasts were generically called carcinoma associated fibroblasts (CAFs). The characterization and markers of CAFs are still controversial, but many studies indicated that CAFs share a similar morphology and phenotype with myofibroblasts [54, 55].

In the past decade, reports have shown that hypoxic niche contributes to tumor progression by activating adaptive transcriptional programs that promote cell survival, motility, and tumor angiogenesis [56]. Tumor cells were shown to accomplish the adaptation to microenvironment changes by expressing hypoxia inducible factors (HIFs), which may drive tumor growth through the generation or expansion of CSCs [57], and it has been shown that fluctuating hypoxia can indeed induce malignant progression and maintain CSC phenotype [57].

Endothelial cells present in the perivascular niche have been demonstrated to deliver critical cues for survival and self-renewal of stem cells in brain tumor [49]. Recently it has also been observed in OSCCs that the majority of the CSCs is localized in close proximity (within 100-μm radius) to blood vessels, and interacts with the components of the niche for their survival. Vascular endothelial cells that are surrounding and infiltrating tumors can secret factors to promote and enhance the survival and self-renewal of CSCs [58].

1.2.3 Epithelial-mesenchymal transition and mesenchymal-epithelial transition

Epithelial-mesenchymal transition (EMT) has been defined in embryogenesis as the process in which polarized epithelial cells with regular cell-cell junctions and adhesion lose their cell-cell junctions, and convert from epithelial into individual mesenchymal or into elongated fibroblastic phenotypic cells [59]. Conversely, mesechymal cells can undergo reverse transition, termed mesenchymal to epithelial transitions (MET). EMT can also occur as a response to injuries, where epithelial cells nearby the injury site move towards the lesion to contribute to the healing process [60]. However, inappropriate reactivation of EMT seems to contribute to the development of a variety of human pathologies such as tissue fibrosis and cancer [61]. EMT has been observed in many epithelial cancers including OSCC [62]. During EMT, tumor cells acquire a more invasive phenotype that would allow them to migrate in extracellular microenvironments, and settle in the areas where new organ formation happens. Subsequently, the tumor cells that were of a more

mesenchymal phenotype while disseminating, are supposed to undergo the reverse transition, MET, at the secondary site and this would explain how most metastases preserve the pathology of their corresponding primary tumors [63].

Increasing evidence is gathering to demonstrate that acquisition of EMT characteristics by carcinoma cells plays a critical role in cancer progression and metastasis, which leads to tumor recurrence and poor clinical prognosis [64-67]. In carcinomas, dissemination of local carcinoma cells from primary tumor through the process of EMT is considered as the main mechaninsm for tumor cells to penetrate the fragmented basement membrane, intravasate into lymph or blood vessels, and then be passively transported by blood or lymph to distant organs. The solitary carcinoma cells at the secondary sites can then extravasate, and either remain solitary and dormant (micrometastasis) or form a new carcinoma (macrometastasis) through a reverse MET (Figure 9) [63].

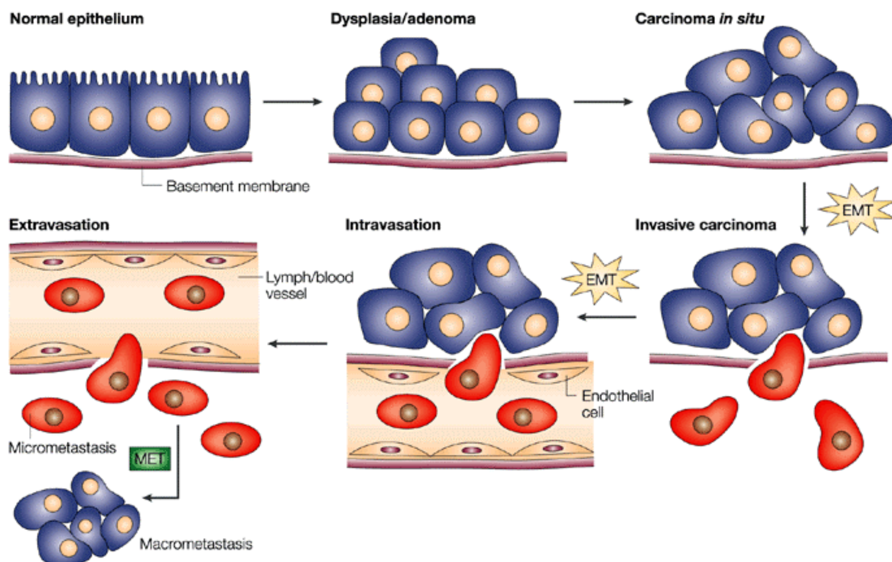

Figure 9. EMT and MET in the emergence and progression of carcinoma.
(Source: Thiery, JP, et al; 2002 [63])

As a caretaker of the epithelial phenotype, E-cadherin plays a vital role in the process of EMT and the reverse MET. Loss of E-cadherin can disrupt the mutual cell

connection and reduce the epithelial phenotype in epithelial cells through the regulation of a number of transcription factors such as Snail, Twist, and ZEB1 [68]. In addition to modulating cell-cell junctions, EMT can modulate other adhesion systems and induce actin cytoskeleton reorganization, leading to the mesenchymal phenotype and the scattering of carcinoma cells [59]. Controversial evidence shows, on the other hand, that up-regulation of E-cadherin in ovarian squamous cell carcinoma is highly related to malignant transformation and cancer growth although it induces epithelial features, being one of the few studies that suggests that MET plays a critical role in ovarian tumorigenesis [69].

Recent reports have shown that EMT-phenotypic cells have stem cell-like features [70-72]. In human mammary carcinomas, $CD44^{high}/CD24^{low}$ cells which have undergone EMT displayed many of the properties of CSCs, including forming mammospheres, soft agar colonies, and tumor formation ability, suggesting a direct link between the EMT and the gain of epithelial stem cell properties [73]. Various factors, including epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF)-B, and PDGF-D as well as transforming growth factor-β (TGF-β), Notch-1and Wnt could induce EMT, while miR-200 family could inhibit EMT by regulating the expression of transcription repressors ZEB1 and ZEB2 [59]. TGF-β pathway is one of the most common pathways of activating EMT in both neoplastic [74], and non-neoplastic tissues [75]. However, despite various studies that have investigated different aspects of EMT and carcinogenesis, this process is still not completely understood, and the newly suggested link to the maintenance of stem cell population in cancer made it more complicated [65].

1.2.4 Putative isolation and identification of CSCs

As mentioned already, it has been recognized that many similar features exist between normal stem cells and CSCs, suggesting that the strategies employed to normal stem-cell may be usefully applied to the identification of CSCs in solid malignancies. Approaches commonly applied in isolation and identification of stem

cells are based on clonal sphere formation, expression of specific surface markers or dye exclusion, and label retention.

Clonal sphere assay

Clonal tumor spheres are non-adherent colonies of cells derived from single tumor cells when cultured in serum-free medium supplemented with growth factors, such as the basic fibroblast growth factor (bFGF) and EGF [76]. Studies on human neural tumors showed that after long-term cell culture, tumor spheres remain non-adherent, maintain the potential for proliferation and expand, whereas the remaining tumor bulk exhibits adherence, loss of proliferation, and subsequent differentiation, suggesting thus that neurospheres could identify the subpopulation with stemness properties of a marked ability for proliferation, self-renewal, and differentiation [76]. Corroborative studies have confirmed tumor sphere formation as an efficient assay in separating CSCs from a range of solid tumors [76-78]. The sphere-forming cell subpopulation derived from human liver cancer cell lines has been also reported having CSCs characteristics, including self-renewal ability, extensive proliferation, drug resistance and increased expression of liver CSCs-related markers including Oct3/4, OV6, EpCAM, CD133 and CD44 [77]. Tumor spheres in human sarcoma, termed sarcospheres, have been demonstrated to contain cells with stem-like properties with increased self-renewal capacity, over-expression of the stem cell-related genes such as Nanog, OCT3/4 SOX2 and DNA repair enzyme genes, MLH1 and MSH2 and strong drug resistance [79]. In HNSCC, using the non-adhesive culture system, tumor spheres, which formed in 5 or 7 days, have been proven to express putative stem cell markers and exhibited chemoradiotherapeutic resistance, in addition to tumor-initiating and self-renewal abilities [80]. Taken together, these findings suggest that tumor sphere cells represent a relatively rare cell type that has self-renewal ability and increased tumorigenicity, thus they should be considered to represent a stem cell population.

Label retention assay

Label retention assay is based on the theory that normal stem cells or CSCs either spend long periods without cycling (proliferative quiescence) or undergo an "immortal strand" DNA replication, and therefore would retain a labeled DNA for an extended long term [81]. The conventional explanation for label retention is that the label-retaining cells (LRCs) are slow-cycling and divide infrequently in undamaged, steady-state tissues [82]. Based on this explanation, label retention assay could exploit the slow-cycling property of stem cells, and therefore retain their labels, whereas TA progenitors are rapidly dividing, and dilute their labels [83]. Based on their ability to retain a nucleoside analog, label retention assay has been widely used for identification of stem cells in both normal and tumor tissues [84-86]. It has been reported, by using fluorescent membrane-labeling dyes such as PKH67/PKH26 label-retaining cells, that cells with stem-like characteristics were enriched in the quiescent fraction of ovarian tumors [83]. In pancreatic adenocarcinoma, a label retention technique with lipophilic labeling dye DiI was used to identify a subpopulation of stem-like, slow cycling tumor cells which had increased invasive and tumorigenic potential [87]. By labeling with bromodeoxyuridine (BrdU), nasopharyngeal carcinoma stem cells could be detected both *in vitro* cell culture and *in vivo* subcutaneous inoculation in nude mice [88]. A subpopulation of basal epithelial cells which retains 3HTdR label for extended periods has been demonstrated in murine skin and oral mucosa [89]. So far, no reference have shown the distribution of LRCs in OSCC and whether CSCs in epithelial tumors are actually slowly cycling, but transplanted OSCC based on label-retaining may be useful to solve such questions [52].

However, recent studies reported that label retention may also be produced by unusual patterns of DNA segregation, as another explanation for label retention [52]. The studies using double labeling with Tritiated thymidine (3HTdR) and BrdU in normal intestinal mucosa suggested that stem cells segregate newly replicated DNA to the daughter cells expected to leave the stem cell compartment [90].

Flow cytometry using Hoechst dye exclusion

A distinct, minor cell population can be detected by dual-wavelength flow cytometry on the basis of the ability to efflux the fluorescent DNA-binding dye Hoechst 33342, referred to as side population (SP) as they fell to the ‘‘side” of the bulk of the positively stained cells in flow cytometry analysis plots [91]. The exclusion of Hoechst dye is mediated by the expression of ATP binding cassette (ABC) protein transporters, such as MDR1 and ABCG2 [91, 92]. SP cells were first identified in mouse bone marrow to highly enrich for cells expressing hematopoietic stem cell markers and with a long-term repopulating capacity [93]. After this original report, SP cells have also been characterized as stem cell populations in epidermal cells based on their high colony-forming efficiency, great proliferative potential and regenerative capacity of epidermal cells [94]. In normal tissues, it has been shown that SP cells possessed multi-potent differentiation capability and had increased expression of stem-like genes, thus, they were thought to have characteristics similar to stem cells [91].

More recent work demonstrated that SP cells were contributors to the maintenance of CSCs characteristics in many tumors [95, 96]. For example, in U373 glioma and MCF7 breast cancer cell lines, as well as LAPC-9 xenograft prostate tumor, SP cells were capable to generate non-SP cells *in vivo*, suggesting that SP cells have the capacity to differentiate. In addition, they were more tumorigenic than the corresponding non-SP cells indicating that they were also able to self-renew, fulfilling thus the two essential defining criteria of CSCs [95]. Similar observations have also been demonstrated in human lung cancer cell line NCI-H460[96] and gallbladder carcinoma cell line SGC-996 [97]. Concerning human oral cancer cells, SP cells isolated from SCC25 tongue cancer cell line were able to enrich for the CSCs as they showed great proliferation ability with high mRNA expression of stem cell markers including ABCG2, Oct-4 and EpCAM and drug resistance [98]. Taken together, these data indicate that SP cells do have similar properties to stem cells. However, it has to be noted that not all cell lines contain SP cells, hence this

population is not exclusively responsible for the prolonged *in vitro* lifespan of cell lines [91].

Flow cytometry using cell surface markers

Stem cells from normal adult tissues can be identified by expression of specific stemness protein markers and other stem-cell specific epitopes that are not expressed by somatic cells [99]. Similar to the normal stem cells, measurements of specific cluster of differentiation surface markers and stem cell-specific metabolic activities have been widely used for the characterization of CSCs [100]. The first evidence for identification of CSCs by using flow cytometry has been demonstrated in human AML by showing that isolated CSCs based on a specific phenotype were able to initiate leukaemia in transplanted immunodeficient mice [35]. Since the first study, this method has been applied to isolate CSCs by other researchers from various tumors, such as malignant glioma [101], osteosarcoma [102], breast cancer [103] and lung cancer [104]. Studies on both OSCC derived cell lines and OSCC primary cells have identified some markers which could be used for the isolation of stem cells from neoplastic oral mucosa. In OSCC-derived cell lines, consistently higher levels of expression of stem cell-related molecules such asβ1 integrin, E-cadherin, β-catenin, ESA and CD44 have been found in colonies with holoclone morphologies which were considered to be derived from stem cells [45].

CD44

CD44 protein, a cell-surface glycoprotein, involved in cell-cell interactions, cell adhesion and migration, displays a large array of cellular functions including hematopoiesis, lymphocyte activation, recirculation and homing. Numerous studies have showed that CD44 plays an important role in cell migration and tumor progression [105], and that its expression levels have prognostic value in certain malignancies [106-109]. CD44 was at first found to identify a population with stem cell properties in breast cancer, in which only a small subpopulation of cells marked with $CD44^{+}CD24^{-/low}$ $lineage^{-}$ had the ability to form new tumor *in vivo* [36]. Further evidence showed that CD44 was a potential cell surface marker for HNSCC CSCs

[44]. By using the mouse xenograft model, it was demonstrated that a minor population of CD44+ cancer cells, but not the $CD44^{-}$ cancer cells, gave rise to new tumors, and the tumors that arose from purified $CD44^{+}$ cells reproduced the original tumor heterogeneity and could be serially passaged [44]. In addition, $CD44^{+}$ was shown to be constitutively expressed on the surface of permanent HNSCC cell lines, and it was indicated that it may drive the progression and metastasis of HNSCC [110].

Enzymatic activity detection of Aldehyde Dehydrogenase

Aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme, plays a role in early differentiation of stem cells through catalyzing the conversion of retinol to retinoic acid in normal and malignant stem cells [100, 111]. High ALDH activity has been shown in normal hematopoietic and central nervous system stem cells [112-115]. It has also been found that stem cell populations in multiple myeloma [115] and AML [116] had increased ALDH activity. In addition to be a marker for normal breast stem [111], ALDH1 has been found also active in stem cells of several tumors like human breast cancer [117], human pancreatic adenocarcinoma [118] and lung cancer [119]. Its activity might be essential for both longevity of stem cell and the resistance of CSCs against chemotherapy [100]. ALDH 1 family member A1 (ALDH1A1) has been suggested as a marker in HNSCC for distinguishing premalignant cells, and also a potential target for vaccination therapy for HNSCC patients [120]. Recently, ALDH1 expression has been reported to be a useful marker for sorting CSCs in HNSCC and ALDH1+/lineage- cells were shown to have increased self-renewal ability, tumorigenicity, and radioresistance [121].

1.2.5 Mouse models in CSCs research

Injection of tumor cells into immunodeficient mice is still considered to be the gold standard for measuring tumor initiating potential, and assessing the tumorigenicity of human tumor cells [40]. When xenografted, only a small fraction of the human cancer cells form tumors, with one of the possible reasons being that they will be captured in an adverse mouse microenvironment or niche that would kill most of

them, the other possible explanation being that only a small, rare subset of tumor cells have the capacity to self-renew, differentiate, and propagate tumor growth, which are the defined features of CSCs [40].

Up to date, human CSCs have been studied upon xenotransplantation of neoplastic cells into mice with a compromised immune system (typically NOD/SCID mice) in a number of human malignancies, including leukemia [35], brain tumors [101],breast cancer [36], prostate cancer [40], ovarian cancer [122], and colon cancer [123]. Also for HNSCC, a cellular subpopulation expressing the surface marker CD44 was firstly isolated and identified as exhibiting stem cell-like characteristics and initiating tumors in an immunodeficient mouse model [44].

Recently, more highly immunocompromised NOD/SCID interleukin-2 receptor gamma chain null (NOD-*scidIL2R*γ^{null}) mice were generated and found to have a remarkably high engraftment level for human cells [124]. Using this new model, a study showed that approximately 25% of unselected melanoma cells formed tumors, while only rare 0.1 - 0.0001% of human cancer cells formed tumors when transplanted into NOD/SCID mice [125]. This study was more in the favor of the adverse mouse microenvironment as an explanation for the low xenotransplantation rate of human cells in mice rather than the presence of a rare subpopulation of CSCs.

Thus, although mice models are very useful in generating knowledge on the biology of cancer cells, there are limitations for the interpretations of the xenotranslantation results in the light of human tumor pathogenesis, since these models can not accurately reflect the human microenvironment and immune response [126]. To minimize these pitfalls, some improved xenotransplantation models have been developed, such as genetically modified mouse models, or the mouse model with co-implanted human stromal cells with CSCs, both of which are able to provide a more human-like or humanized growth environment [127].

Nevertheless, despite of its limitations, mouse models is still being regarded as one of the leading model systems in studying tumor pathogenesis since its use is

tremendously increasing our understanding of stem cell biology in cancer research [126].

2. RATIONALE OF STUDY

Appropriate treatment of OSCC remains one of the most difficult challenges in oral cancer. Mortality from this cancer remains high due to the therapy-resistant local and regional recurrence, and distant metastases. Despite all attempts, no significant progress has been achieved by combining traditional surgery and radiotherapy treatment with chemotherapy put any ref you like, but have one. Aaccumulating evidence demonstrate that solid malignancies, including OSCC, contain a small population of tumorigenic cells referred as CSCs or TICs [37, 45, 94, 128-130]. Typically, CSCs exhibit stem-like cell properties of self-renewing, tumor-sphere forming, differentiating into heterogeneous populations of cancer cells, and initiating new tumors when xenotransplanted into compromised mice [131], as well as initiating metastasis [132]. CSCs have been shown to display resistance to various therapeutical drugs and methods, leading to high recurrence and metastasis of carcinoma [133-136], therefore specific strategies aimed at targeting CSCs are essential for developing of an effective treatment for these tumors. In addition, the importance of EMT as one of the central mechanisms in invasion and metastasis, and the newly suggested link between EMT and the phenomenon of CSCs, point EMT as another yet important target for studies understanding carcinoma cell biology and treatments possibilities [128]. Effectors of EMT have been suggested as potential targets for development of improved tumor diagnosis, prognosis and therapy methods [137]. In breast cancer it has been shown that a $CD44^{high}CD24^{low}$ subpopulation with CSC-phenotype has undergone EMT [73], but the link between EMT and CSC has not been studied in OSCC so far. The plasticity of CSCs and whether there is an established link between EMT and the phenomenon of CSCs in OSCC as well needed to be established.

CSC behavior has been shown previously to be regulated not only by intracellular regulators such as transcription factors, but also by external signals from stem cell niches such as neighboring stroma [138]. Furthermore, a more recent study reported that exogenous TGF-β, which has been shown to be an EMT inducer [139], played a

key role in CSCs maintenance also in breast cancer [73]. Again, the key regulators of CSCs in OSCC are not known; carcinoma associated fibroblasts (CAFs), one of the major components of tumor stroma in carcinomas, and their growth factor synthesis might affect self-renewal and tumorigenic abilities of oral malignant and premalignant-derived cells, but this needed to be proven.

Nevertheless, the understanding of the biology of CSCs in OSCC is dependent on robust methods for their identification and isolation for further test. AlthoughFACS cell sorting for surface antigens that typify stem cells, is the most common approach to isolate the CSCs, it has several disadvantages. For instance, FACS requires high-cost, high-speed sorters and high-quality antibodies [140]; the technique itself needs special skills and training to master. In addition, FACS profile is quite prone to certain external factors such as cell preparation (the cell type and concentration of the sorting cells), and/or a certain degree of subjectivity for gate setting in gradient staining. This brings forward a need to establish robust, less invasive methods for isolation of CSCs in OSCC, and functional approaches such as differential adhesiveness to a main component of basement membrane (BM), collagen IV, a previously reported method for isolating normal epithelial stem cell could be tested for this purpose [141, 142]. Another non-invasive, more functional technique, dielectrophoresis (DEP), based on an electrostatic phenomenon, has been recently shown to differentiate between stem cells and their progeny in neurogenesis [143]. Previous studies reported that DEP can separate and characterize different cell populations and measure electrophysiological properties in cancer, allowing it to be used as a characterization tool where differences exist in electrical properties between different cancer cell populations [144]. Up to now, there is no research performing DEP as a method to study the cells with stem cell properties in oral cancer and it will be promising if DEP can be used for future isolation of CSCs as a label-free and non-invasive method.

3. AIMS OF THE STUDY

To identify and biologically characterize the stem-like cells in human OSCC both *in vitro* and *in vivo*.

Specific aims:

1. To evaluate the plasticity of CSCs and whether there is a link between EMT and the phenomenon of CSCs in OSCC (Paper I).

2. To determine the effects of CAFs on self-renewal and tumorigenic properties of oral malignant and premalignant-derived cells and determine the role of TGF-β1 in stem cell maintenance, invasion and metastasis dissemination (Paper II).

3. To investigate the rapid adherence to collagen IV as a method for enrichment for CSCs in OSCC and to further characterize the cell populations obtained using this method (Paper III).

4. To investigate the differences in electrophysiological properties of the subpopulations of oral cancer cells with different stem-like cell properties isolated based on the different adhesiveness to collagen IV (Paper IV).

4. METHODOLOGICAL CONSIDERATIONS

4.1 The choice of *in vitro* and *in vivo* studies

The specific studies described in Papers I, II and III included both *in vitro* assay and *in vivo* xenotransplantation in order to investigate the biological characteristics and the behavior of cells with stem cell properties of OSCC. The study described in paper IV is a biophysiological study performed to determine the electrophysiological properties of cells with stem-like properties in OSCC derived cell lines.

One of the essential biological feature attributed to CSCs or TICs is the tumorigenic potential [145]. The golden standard for tumorigenicity assay is still the *in vivo* transplantation mice system, in which human cancer cells are xenografted into immunocompromised mice (typically NOD/SCID mice) which could be assayed for tumor formation at various time points [81]. The transplantation mice system has been widely used for many CSCs researches due to its advantage of allowing studying the role of stem cells during cancer development and progression in dynamic physiological systems. Although CSCs from different tumor types seem to share certain markers for prospective identification, CSCs isolated from different tumor types could display marked behavioral and plasticity differences from one another. Properties which are useful for the identification and characterization of stem cells in a type of tumor might not be shared by the stem cells in another tumor, thus it is of major importance the choice of the specific assays to appropriately characterize the CSCs in a certain type of tumor [81]. Furthermore, although the subcutaneous mice model is widely used for xenotranplantation of various tumor types due to its simpler logistics, different mice models should be used in studying different types of tumors. In our studies, tongue xenotransplantation mice model was specifically chosen to characterize the tumorigenic potential of CSCs derived from OSCC. In this model, human tumor cells isolated form OSCC patients were injected into the tongue of the NOD/SCID mice. The advantage for this model was that it could mimic the *in vivo* microenvironment of the human oral cavity where OSCC are arising, and the

evaluation of the loco-regional spread of cells into the lymph nodes, which is one of the crucial step in oral cancer progression, and the only clinical parameter correlated with survival and prognosis in OSCC patients [146]. In addition, although not so straightforward and easy as the SC model, the tongue model allows also a relatively easy operation, examination, measurement and harvesting.

In addition to the universally accepted *in vivo* assay for demonstrating the biological properties of stemness in tumors, *in vitro* assays and flow cytometry, have also been widely used. There are several *in vitro* assay systems that have been commonly performed for the analysis and characterization of CSCs, including colony formation assay and tumor sphere formation assay to test the self-renewal ability of CSCs; transwell migration/invasion assay and newly developed three-dimensional organotypic model to test the tumor cells invasive or migratory ability. Of importance, in addition to the fact that they can be performed with human cells only and thus being highly relevant for human pathobiology, the *in vitro* assays are also able to complement the other pitfalls of the *in vivo* assay, such as short experimental time, simple skills, and less cost. More important, cell lines derived from patients with tumors from different sites or from different stages of tumor progression offer the possibility to model the stepwise oral carcinogenesis and allow the study CSCs at different stages of transformation.

Taken together, the combination of both *in vitro* and *in vivo* assays for characterization of CSCs appeared to be an accurate model for identification and analysis of biological properties of CSCs, and thus a combination of these assays was chosen for our experiments.

4.2 Cell Culture

4.2.1 Cell lines and culture medium (Papers I - IV)

A panel of cell lines derived from oral malignancies and premalignancies were used in our papers.

- oral cancer cell lines: Uk1, Ca1, H357, 5PT, CaLH3.

- oral dysplastic / premalignant cell lines: POE9n and DOK

All of the oral cancer cell lines and DOK were routinely grown in FAD medium [147], which is 3:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St.Louis, USA) and Ham's F12 (Sigma), supplemented with 10% Fetal Bovine Serum (FBS), 25 µg/mL Bovine Pituitary Extract (BPE), 0.4 µg/mL hydrocortisone (Sigma), 5 µg/mL insulin (Novo Nordisk, Bagsværd, Denmark), 20 µg/mL transferrin (Sigma), 50 µg/mL L-ascorbic acid (Sigma), 20 µg/mL L-glutamine. All cells were grown under standard cell culture conditions: a humidified incubator with 5% CO_2 and 95% air at 37 °C. At 60-70% confluence, the cells were released using 0.25% trypsin-EDTA (Sigma). POE9n was grown in its growth medium, Keratinocyte Serum Free Medium (KSFM, Invitrogen, USA) supplemented with 10 ng/mL EGF and 25 µg/mL BPE (Invitrogen), and Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 5 µg/mL hydrocortisone.

4.2.2 Generation of cells from primary human samples (Papers I, II and III)

Fresh normal or OSCC tissue samples were collected following protocols after approval by the local Research Ethics Committee and after informed consent. Tissues were minced into approximately 1mm^3 pieces, and then allowed to adhere to tissue culture dishes with KSFM (epithelial cells) or DMEM (fibroblasts) in incubator. After 1-3 weeks, the outgrowths from the explants were assessed morphologically, and the cells with either epithelial or fibroblast looking morphology were detached separately from the dishes by clonal rings, FACS was used to collect fibroblast-free epithelial populations based on the expression of epithelial-specific marker (ESA-APC) (Biomeda), and to collect CAFs based on expression of PDGFRB (CD140b-PE, R&D,USA).

4.2.3 Adhesion of cells to collagen IV (Papers III and VI)

An early report has suggested that epidermal stem cells with high colony-forming ability could be separated by rapid adherence to basement membranes (BM), including collagen IV and fibronectin [148]. By adopting this method well characterized for isolating normal epithelial stem cells, we used rapid adherence to BM to enrich in CSCs in OSCC. Rapid adherence to collagen IV, or fibronectin-coated dished, as well as to the simple plastic was used for optimization of this method in OSCC.

Collagen IV coated dishes were prepared as following: collagen IV solution was made by 10μg/mL human collagen IV (BD Biosciences, USA) diluted with 10 mM acetic acid. Tissue culture dishes were then coated evenly with this solution at room temperature for 1 h, rinsed carefully with phosphate-buffered saline (PBS, Sigma) and sterilized by UV radiation overnight before using. Fibronectin was coated on dishes by adding 5mL FBS into culture dish in incubator overnight, as a method reported previously [45] and then washed once with PBS before using. Normal culture dish was used in our study as simple plastic. Single cell suspensions in medium were allowed to attach to coated-dishes or simple plastic in the incubator. Cells that attached within 10 min were trypsinized and collected as rapid adherent cells (RAC). The unattached cells within the first 10 min were then transferred to new coated-dishes or simple plastic and kept in incubator for the next 30 min. Cells that adhered within this period were collected as middle adherent cells (MAC), and again the floating cells remaining from this time period were allowed to attach in another new coated-dishes or simple plastic for the next 4 h and these attached cells were referred as late adherent cells (LAC).

The ratio of primary OSCC cells from one patient (P1) adhering to collagen IV, fibronectin and simple plastic as RAC, MAC or LAC was calculated and differences were found for different types of coating (Figure 10). From RAC to LAC, the proportion of adherent cell population decreased in collagen IV-coated dish but increased in fibronectin-coated dish. For simple plastic, the cells were mostly

attached within 30 min. Thus, among these models, coating with collagen IV seemed to be the most valid method to indeed enrich in the rapid adherent cell population.

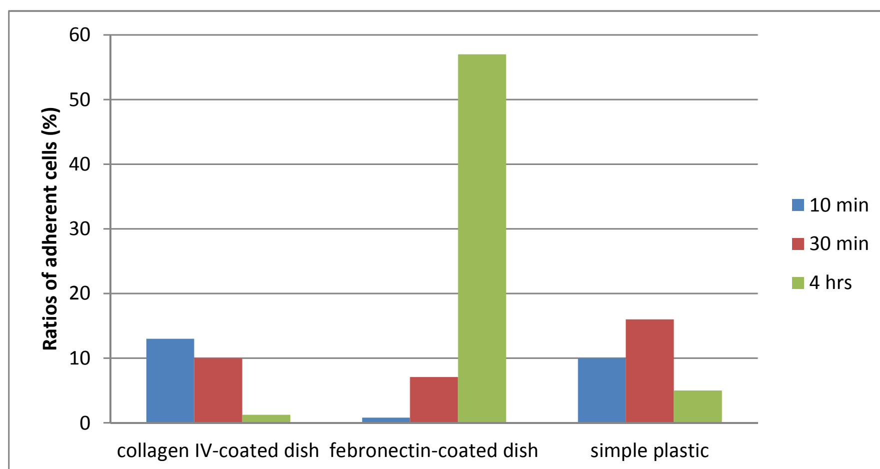

Figure 10.Ratios of OSCC cells from patient 1 adhering to collagen IV, fibronectin and simple plastic over different time.

The adhesion assay using rapid adhesiveness to collagen IV has been also chosen by many others previously, to enrich in stem cells in normal human [141], murine [21] and rabbit epithelium [142]. Therefore, we chose in our papers to sort different subpopulations based on adhesion to collagen IV over different time period, following the same procedure (Figure 11). Unfractionated cells that were not separated based on adhesive property were also used as control.

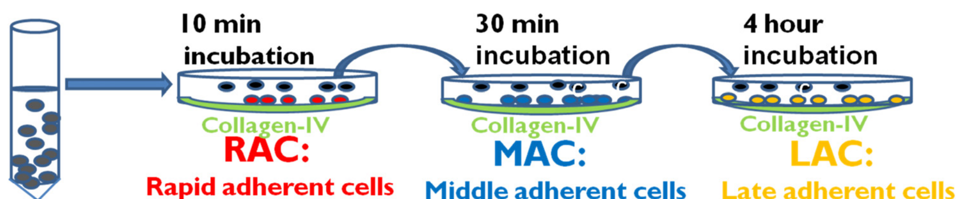

Figure 11. Flow chart of isolation of OSCC cell subpopulations based on adhesion on collagen IV

4.2.4 Collection of fibroblast conditioned medium (CM) (Paper II)

CAFs were grown in serum free DMEM medium overnight. CM was then collected and spun down to remove cellular debris. The supernatant was filtered through 0.45 μm filter. This CM was frozen down or used fresh 1:1 with fresh culture medium to culture the cells.

4.2.5 TGF-β1 exposure protocol (Paper II)

Previous reports showed that exogenous TGF-β was one of the key niche factors that regulated the stem cell behavior in breast cancer [149] and glioma [150]. To investigate the effect of TGF-β on self-renewal ability and tumorigenesis in oral premalignancy and malignancy, TGF-β1 was added to oral dysplastic or cancer cell lines using the following protocol:
Human TGF-β1 (BD biosciences, USA) was activated by PBS containing 0.001% HCl. TGF-β1 was then added (1ng/mL to 20ng/mL) into the serum free medium in different time periods (1h, 5h, 24h, 72h and 120 h) with the cells grown at clonal density.

4.2.6 Treatment with 4-methylumbelliferone (Paper IV)

The 4-methylumbelliferone (4-MU) stock solution (0.3 mM) was made by dissolving sodium salt 4-MU in distilled water. This stock solution was then diluted in cell culture medium and the cells were incubated in the presence of 4-MU for 48 hours.

4.3 Cell Morphology

4.3.1 Cell diameter measurement (Papers III and VI)

Cell size, as previously reported, was related to cell phenotype, cell differentiation and proliferative potential in human keratinocytes and fibroblasts [151, 152]. Furthermore, small cell size expressing stem cell-associated markers represented one

of the important stem cells properties and could enrich for putative stem cells in human corneal epithelium, while larger cells were more differentiated cells[153, 154]. To evaluate the cell size of different CSC populations in our study, the diameters of subpopulations were measured under a microscope at a magnification of × 200. A minimum of 50 cells were measured in each independent experiment.

4.3.2 Scanning Electron Microscopy (Papers III and IV)

Collagen IV was coated on poly-lysine coverslips in 24-well plates (Nunc. Denmark) following the same procedure as described above for the culture dishes. A total of 500 single isolated from cell lines were allowed to attach to poly-lysine and collagen IV-coated coverslips for 1.5 h at 37°. After fixation using 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH = 7.2) at 4° for 2 h, samples were then subjected to graded ethanol dehydration and critical point dried, mounted on aluminium stubs, coated with evaporated carbon and viewed using a JeolJSM-7400 field emission-scanning electron microscope.

4.4 Stem cell assays

Self-renew ability and tumorigenic potential are the hallmarks for CSCs. Thus, for a study aiming at characterizing CSCs should evaluate both these abilities. Clonogenic assay or colony formation assay was initially used to determine self-renewal capacity of CSCs in carcinomas, on the basis of the ability of a single cell to grow into a colony under adherent culture condition. More recently, a three-dimensional *in vitro* culture system, referred to as sphere forming assay, in which the cells grow in suspension/ non-adherent culture and form floating multicellular spheroids, which appears to mimic the *in vivo* condition was rather preferred to the colony formation assay [155]. Nevertheless, very recently it was pointed that the capacity to grown in suspension and form spheroids is more of an EMT ability than pure self-renewal trait, and thus the tumor initiation assay in animals models by transplantations of serially

diluted cell populations into mice with compromised immune system, typically NOD/SCID mice remained still the golden standard in studying CSCs [126].

4.4.1 Colony formation assay (Papers I, II, III and IV)

Many studies on normal and cancer stem cells have linked stem cell properties with the morphology of colonies developed from single cells [45, 156, 157]. In this sense, colony formation (clonogenic) assay was designed as an *in vitro* stem cell assay to functionally investigate cell populations for their ability to self-renew. In our studies, colony formation assay was performed using the following two methods.

1. Paper I- Tumor cells sorted by FACS were allowed to grow in culture before being resuspended, counted, and single cell cloned in 48-well plates by limiting dilution; or cells were seeded directly into 96-well plates by using the single cell plate sorting function of the FACS Aria sorter (BD biosciences). Wells were examined microscopically and those containing only a single clone were selected for analysis.

2. Papers II and III – A total of 500 cells were seeded in 6-well plates within 3 mL complete culture medium. After 7-10 days, wells were examined microscopically and stained with 0.5% crystal violet. Colonies were manually scored for analysis.

4.4.2 Sphere formation assay (Papers I, II, and VI)

Typically, stem cells form floating spherical structures when cultured *in vitro* under non-adherent conditions as spheres or in three-dimensional matrices relying on their property of anchorage independent growth [158]. In this sense, sphere-forming as an assay has been widely used in stem cell biology to evaluate both self-renewal and differentiation potentials. Sphere formation assay was initially generated from mammalian nervous system [159], and then used for the isolation and characterization of adult mammalian neural stem [160], prostate [161], and mammary

stem cells [161]. Increasingly, the application of sphere culture has been further used to isolate and maintain CSC subpopulations from various types of human tumors, such as brain [76], breast [162], pancreatic [163], ovarian cancers [122] and OSCC [164]. It is generally agreed that the tumor sphere-forming cells are able to proliferate, self-renew and possess *in vivo* tumorigenicity [77], which makes sphere formation as a functional assay for characterization of stem cell biology in tumors, and a surrogate for *in vivo* tumorigenesis assay. In our studies, sphere formation assay was used to determine *in vitro* self-renewal ability for various cell subpopulations isolated from OSCC. The protocol is described in detail below.

Non-adherent culture plates were made by dissolving Poly (2-hydroxyethyl methacrylate) (pHEMA, Sigma) in 95% ethanol and coating the 48-well plates. The plates were then air dried overnight before using. 500 single cells were allowed to seed into the non-adherent plates within 500 µl FAD medium containing 1% methylcellulose for 7 days to 3 weeks. Wells were then microscopically accessed and viable spheres were manually scored for analysis.

4.4.3 *In vivo* NOD/SCID mice model (Papers I, II, and III）

Xenotransplation mice model could mirror the phenotypic heterogeneity of the primary tumor. CSC populations showed greater tumorigenicity as compared to other tumor cells when xenografted into immunocompromised mice [126]. When studying oral cancer studying animal models, it has been reported that tumorigenicity of oral cancer cells was greater in the orthotopic xenotransplantation model system rather than after subcutaneous injection in immunocompromised mice, which was more commonly used but could not reproduce primary tumor site microenvironment or local-regional/distant metastasis [165, 166]. To establish a good model to determine *in vivo* tumor-initiating ability of OSCC-derived cells, we have initially tested different types of xenotransplantation mice models which were tongue xenotransplatation (Figure 12), tail vein injection (Figure 13) and intracardiac injection using NOD/SCID mice. Tongue xenotransplatation model was the most

sensitive mice model for OSCC-CSCs study since it could reproduce both the primary tumors and the local regional metastasis-cervical lymph node metastasis at a low dilution (100) of CSCs. In addition, the ease for establishment and measurement of tumors and its ability to recapture the microenvironments of the primary oral cancer made it our choice. Using the tail vein injection model, no metastasis was found even injecting high dilution (10,000) of cells over 9 months. Intracardiac injection system was also excluded from our studies not only because of the difficult technique, but also for the high number of cells needed for metastasizing. Therefore, the orthotopic tongue xenotransplantation mice model was adopted in our studies to characterize the different OSCC cell subpopulations for their tumorigenetic and metastatic ability. NOD/SCID mice with the age of 6-8 weeks were used and kept in an isolation facility under pathogen-free conditions and a 12-hour day/night cycle. All animal procedures were approved by the Norwegian Animal Research Authority. The oral cancer cell lines or primary cells from OSCC patients with p53 mutations (evident by sequencing of exons 4-8 of TP53 gene) were selected for the injection. Cells suspended in 50μl of matrigel (BD Biosciences) were injected into the tongue of mice. Tumor formation and cervical lymph node metastasis were assessed at the onset of symptoms. Tumor volumes were determined by bidirectional measuring with callipers and calculated by the following formula: volume = $(\text{width})^2 \times \text{length}/2$ [167].

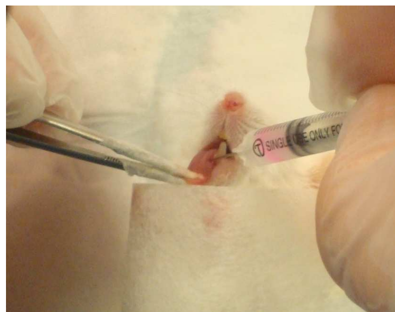

Figure 12. Tongue xenotransplantation model in NOD/SCID mice

Of note, we also used the more immunocompromised NOD/SCID ILγ2 deficient mice in order to be able to induce tumors by using DOK cell line (Paper II), a dysplastic cell line previously reported not to form tumors in nude mice, which

indeed did not form tumors when injected alone in any of the mice models we used. Nevertheless, when used in the NOD/SCID ILγ2 mice and co-injected with CAFs DOK cells formed tumors, as described in paper II.

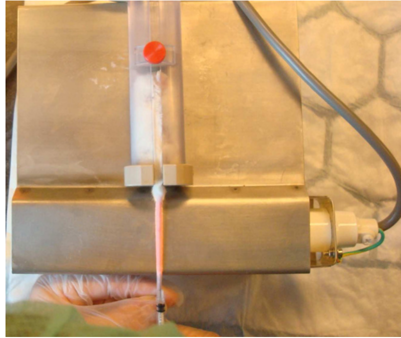

Figure 13. Tail vein injection model in NOD/SCID mice

4.5 Migration/Invasion assays (Papers I, II and III)

As a hallmark of tumor metastasis, tumor cells should acquire a migratory and invasive phenotype in order to metastasize [168]. Some *in vitro* assays have been developed to investigate the migration and invasive ability of tumor cells. The most commonly used assay is the transwell migration/invasion assay system, which is performed by placing cells on top of plastic inserts with a cell permeable membrane and a chemo attractant underneath in multi-well tissue culture plate. The migration or invasion potential was determined by counting the cells which traversed the membrane and migrated or invaded towards the higher concentration of chemoattractant [169]. For invasion assay, a gel containing extracellular matrix proteins, such as matrigel, was used to mimic the *in vivo* matrices [169]. It has been shown that, by using this assay, a strong correlation between *in vitro* migratory or invasion ability of tumor cells and there *in vivo* behavior was found. Thus, in our papers, we adopted the following transwell migration/invasion assay to investigate the potential of migration or invasion of different OSCC cell subpopulations.

For transwell migration/invasion assay in our studies (Figure 14), cells were placed in medium with low concentration of FBS in the upper chamber of the transwell tissue culture inserts with 8-um pore membrane(Corning Incorporated, USA) in 24-well plates, with the medium containing higher concentration of FBS at the bottom of the well. After 18-24 h incubation, the non-migrated/non-invaded cells on the top of the membrane were removed by using a cotton wool bud. The migrated/invaded cells on the underside of the membrane were fixed in 4% paraformaldehyde (PFA), stained with Hoechst 33342 (Paper I) or crystal violet (Paper III) and counted. For invasion assay, the inserts were firstly coated with 250 µl of 6 µg/µl of matrigel (Invitrogen) mixed with KSFM and then performed as described above.

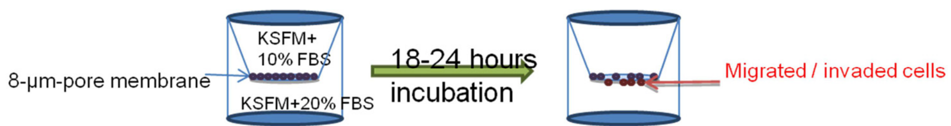

Figure14. Transwell migration/invasion assay

4.6 Three-dimensional organotypic model (Paper II)

The conventional two-dimensional (2D) monolayer cell culture models lack complex intercellular and cell-matrix interactions at their real physiological state [170]. To circumvent the limitation of 2D culture models, organotypic *in vitro* models have been elaborated. In these model systems the tissue is reconstituted in the cell culture by seeding the cells of interest in a 3D structure under the specific spatial arrangement and architecture of the primary tissue. The 3D organotypic cell culture system has been considered as the most appropriate *in vitro* model for epithelium-stroma interaction studies since they can restore in culture the 3D structure of environment and the *in vivo* microenvironment of the epithelial tissue is mirrored in these cultures [171]. For better understanding the biology of CSCs, it is essential for their optimal functionality to restore in culture their *in vivo* environment due to the close relationship between CSC populations and their niches including tumor stroma (such as fibroblasts). Thus, in our study on the role of stroma on CSC in OSCC we

used 3Dorganotypic culture models, as described in detail in the procedure below, where it was specifically used as an advanced *in vitro* model system for investigation of the role of epithelial-mesenchymal interactions on expression of stem-cell related markers.

3D cultures were performed by seeding dysplastic and neoplastic oral keratinocytes on top of collagen I biomatrices by using a protocol well-established in our laboratory [172].Two types of organotypic cultures were prepared and used in our study (Figure 15). In the first model (Figure 15a), namely organotypic monocultures, each different type of keratinocytes was grown on top of collagen simple collagen biomatrices without supplement of fibroblasts. In the second model (Fig. 15b), called the organotypic co-cultures, keratinocytes were seeded on the top of collagen biomatrices supplemented with primary fibroblasts. The organotypic cultures were grown in serum free culture medium [172] for 10 day and then lifted at air-liquid interface, harvested, formalin fixed and paraffin embedded or fresh-frozen [172].

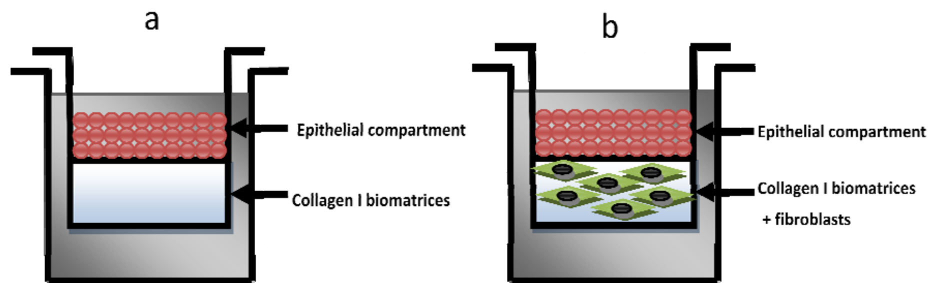

Figure15. Organotypic cultures

4.7 Flow cytometry

Tumors are heterogeneous and contain a diversity of phenotypically different cell subpopulations. Flow cytometry is a powerful technique which could simultaneously analyze multiple parameters of individual cells, and thus sort out various subpopulations from heterogeneous tumors. Furthermore, as one kind of specialized

flow cytometry, FACS is able to separate the living cells for further assays based on the expression of cell surface markers [100]. Besides, flow cytometry could be used to analyze DNA content including cell cycle analysis, as well as some functional tests for cells. It also allows analyzing data using its correspondent software. Flow cytometry was used in our papers for both cell sorting (BD FACS Arian) and the analysis of cell cycle and β1 integrin expression (BD Accuri C6).

4.7.1 FACS sorting (Paper I and II)

Cells were detached using either trypsin-EDTA (Sigma) or enzyme-free cell dissociation buffer (TrypLE™ Express, Invitrogen, USA) and then stained with antibodies (anti-CD44-PE, anti-ESA-APC antibody) at 1:100 dilution in PBS (Invitrogen, USA) prior to FACS. To test for ALDH1 activity, cells were stained with Aldefluor reagent (Aldagen, Uk) according to the manufacturer's instructions. The DAPI nuclear dye (1µg/mL, Sigma) (Papers I and II) or sytox blue (1µl/sample, Invitrogen) (Paper III) was used at to exclude dead cells.

4.7.2 Cell cycle analysis (Papers I and III)

Previous studies showed that CSCs in malignant human epithelium spend a longer time in G2 cell cycle phase which allowed for escapement of apoptosis by activating G2/M checkpoint proteins to provide more time for reparation of DNA damage [173]. Based on this, we performed the G2 cell cycle analysis by using flow cytometry in order to investigate whether there was a difference in the proportion of cells in the G2 phase among different subpopulations of OSCC cells.

Cells were fixed with 70% ice-cold ethanol and kept in 4 °C overnight. Before FACS analysis, the cells were washed with ice-cold PBS and then stained with propidium iodide (PI, 50 µg/mL in PBS) mixed with 125 µg/mL of RNAse A (Sigma) in dark at 37 °C for 30 min. Then cells were washed with cold PBS, DNA contents were measured by using AccuriC6 Flow Cytometer (Accuri Cytometers, Inc., USA).

4.7.3 β1 integrin expression (Paper III)

Apart from being a stem-cell marker, β1 integrin also functions as the major receptor for BM components such as collagen IV [148]. A relationship between the expression level of β1 integrin on the cell surface and proliferative capacity has been found in an early study [148]. In addition, it has been reported that FACS sorting cells with high ß1 integrin expression can be used to isolate normal human epidermal stem cells from TA cells, since the cells highly expressing β1 integrin showed the greatest colony formation efficiency [148]. Based on these previous findings, our study applied the flow cytometry analysis based on the following staining protocol for β1 integrin in order to investigate the different OSCC subpopulations in term of their different expression level of β1 integrin.

Prior to flow cytometry analysis, cell were detached and stained with Phycoerythrin (PE)-conjugated mouse monoclonal anti-human β1 integrin (R&D Systems) in 1:10 dilution. Isotype mouse IgG1-PE (BD Bioscience) was used as control. β1 integrin expression level was then measured by using AccuriC6 Flow Cytometer (Accuri Cytometers, Inc.,).

4.8 Immunostaining

4.8.1 Immunohistochemical staining (Papers I, II and III)

Cells were grown on cover slips in 12 wells plates and then fixed in 4% PFA. The cover slips were attached to glass slides using loctite glue, and rinsed twice with 1xTris-buffered saline with Tween (TBST). Heat induced epitope retrieval was done in 1x target retrieval solution (pH 6.0) in a pressure cooker or microwave treatment. Endogenous peroxidase activity was quenched with peroxidase block solution either before or after the addition of primary antibody. Ten percent solution of normal goat serum was made in a 3% Bovine Serum Albumin (BSA) in TBST to block the unspecific binding sites of the primary antibody. After discharging the solution of normal goat serum, the slides were incubated with the primary antibody at room

temperature in a humidified chamber for 60 min. Antibody diluent only was used as negative control. The slides was then rinsed and incubated for 30 min in room temperature with the secondary antibody conjugated with horseradish peroxidase labelled polymer (En vision kit, DAKO). The presence of antigen was visualized with DAB (3, 3’ diaminobenzidine, DAKO). The slides were then counterstained with haematoxylin (DAKO), dehydrated by immersing in increasing concentrations of alcohol followed by pure xyleneand mounted with an alcohol soluble mounting medium (Eukit, DAKO).

In tongue xenotransplantation mice model, all tongue tumors and cervical lymph nodes were 5µm formalin-fixed, paraffin-embedded and stained for H&E or p53 protein with a monoclonal specific antibody (DO-7 clone, titration 1: 50; DAKO) to identify the human cancer cells p53 mutated.

4.8.2 Immunofluorescence (Paper II)

For immunofluorescence, cells were fixed in 4% PFA and stained with various antibodies (1:100) in PBS with 0.25% BSA. Prior to imaging, cells were permeabilised with 0.1% Triton-X to allow the nuclear staining with DAPI (1 ug/mL).

4.9 Molecular assays

4.9.1 RNA extraction and Quantitative-PCR (Papers I, II and III)

Total RNA was extracted by using the RNeasy micro kit (Qiagen Inc., Valencia, CA, USA) (Papers I and III) or Cells Direct One-Step qRT-PCR kit (Invitrogen) (Paper II). Quantity and purity of the extracted RNA was checked using NanoDrop spectrophotometer (NanoDrop technologies, Inc., Wilmington, DE, USA). Total RAC reverse transcribed into cDNA using the Superscript III first strand synthesis supermix (Invitrogen) (Paper I and II) or High-Capacity cDNA Archive Kit system (Applied Biosystems, Foster City, CA, USA) kit (Paper II and III). QRT-PCR was then performed for the TaqMan assays.

4.9.2 Human stem cell RT² Profiler™ PCR Array (Paper III)

The human stem cell RT² Profiler™ PCR Array (PAHS-405, SABiosciences, Frederick, MD, USA) containing primer pairs for 84 stem cell related genes was used to test the stem cell related genes. By using RT2 First Strand Kit (C-03, SABioscience,), total RNA was converted to first strand cDNA. PCR amplification was performed using the following cycling conditions: 95 °C for 10 min, (95 °C for 15 s, and 60 °C for 1 min) × 40 cycles in ABI Prism Sequence Detector 7900 HT (Applied Biosystems, Foster City, USA).

4.9.3 Western blot (Papers II and III)

The protein concentrations were determined using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Total cell protein was mixed with 10% SDS-polyacrylamaide gel (Bio-Rad, USA) and resolved using a PVDF membrane (Amersham, USA) using standard procedures. Membranes were blocked by incubation with 5% non-fat dry milk in TBST for one hour at room temperature. Membranes were then incubated overnight at 4 °C degree with mouse monoclonal anti-BMI1 (1:1000, Millipore, USA) or anti-GAPDH (1:10 000, Abcam, Cambridge) as control. After washing in TBST, membranes were incubated with donkey anti-mouse monoclonal antibody conjugated to horseradish peroxidase secondary antibody (1:1500, Jackson immunoresearch, USA) for one hour at room temperature. Super signal west Pico chemiluminescent substrate (Thermoscientific, USA) was used as enzyme substrate according to manufacturer`s recommendations. The membranes were visualized in SynGene scanner (VWR, USA).

4.10 Dielectrophoresis (Paper IV)

Dielectrophoresis (DEP), an electrostatic phenomenon, has been used to both characterise and separate different populations of cells for a variety of different purposes within biology and medicine, such as to study links between multidrug resistance and cancer [144]. In addition, DEP can also be applied for measurement of

electrophysiological properties in cancer, allowing it to be used as a characterisation tool where differences exist in electrical properties between different cancer cell populations. Furthermore, previous studies has demonstrated DEP as an approach to distinguish stem cells from their progeny [174] and to discriminate normal oral cells from pre-cancerous and neoplastic oral cell lines [6]. Up to now, there is no research performing DEP as a method to study the cells with stem cell properties in oral cancer. Thus, in our study, DEP was performed to determine the eletrophysiological properties of subpopulation which showed stemness properties in OSCC. The detailed procedure was described below.

Cell suspensions were washed and re-suspended twice in isotonic medium consisting of 8.5% (w/v) sucrose plus 0.3% (w/v) dextrose buffer to remove traces of highly conductive culture medium [175]. Then, cells were re-suspended in low-conductivity medium (10 mS/m) at a concentration of 1.2×10^5 cells/mL. The DEP instruments (Figure 16) were set up and cells were added in to the the DEP Well chip (DEPTech, Ringmer, UK) (Figure 16c). The cells move (Figure 16a) within the DEP well over the course of each frequency and five frequencies per decade were measured between 1 kHz and 20 MHz (Figure 16b). The system was controlled by a graphic user interface generated in MATLAB (The MathWorksInc, Nantick, MA). The change in light intensity across the well was measured (Figure 16b). By using “single shell model” approach [176], a “best fit” of the real part of the Clausius-Mossotti factor (Re[k(ω)]) versus frequency was then found for the corresponding spectrum including cytoplasmic conductivity (S/m),effective membrane conductance (S/m^2) and effective membrane capacitance (F/m^2)

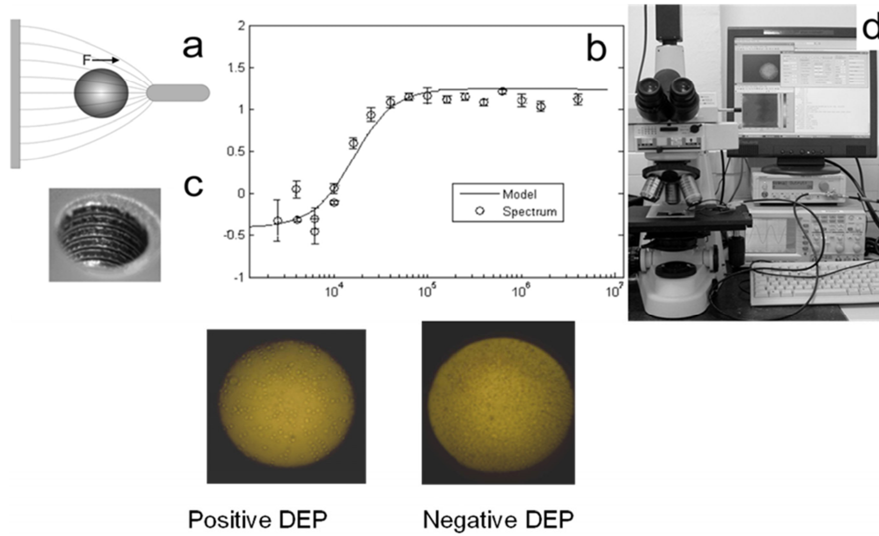

Figure 16. Instrument and example of cell movement in DEP

4.11 Statistical analysis (Papers I-IV)

Data were expressed as mean ± standard error of the mean (StEM) using. The samples were compared using a student's t-test or non-parametric tests if the normality assumption was violated. Sometimes transformation of data in relative values was performed. Values of $p < 0.05$ were considered statistically significant.

5. MAIN RESULTS

5.1 Isolation and characterization of OSCC CSCs based on FACS (Papers I and II)

5.1.1. $CD44^{high}$ cells had high clonogenicity, tumorigenicity and metastatic ability, while $CD44^{low}$ cells showed low clonogenicity, tumorigenicity and metastatic ability (Papers I and II)

Seeding OSCC cells at clonal density after FACS sorting showed that the cells of the $CD44^{high}$ subpopulation had significantly higher clonogenicity than the $CD44^{low}$cells. QPCR revealed that $CD44^{high}$ cells showed up-regulation for stem cell-related markers, such as CD44, CD133, Oct4A and Bmi1. *In vivo* tongue xenotransplantation revealed a much higher tumor burden and higher ability to infiltrate to the local lymph nodes for $CD44^{high}$ cells than for the $CD44^{low}$ cells, indicating that $CD44^{low}$ cells have lower ability to form tumors and local metastasis than the $CD44^{high}$ cells.

5.1.2. $CD44^{high}$ fraction of CSCs contained an ESA^{high} fraction which was the holoclone-forming population and had more epithelial characteristics (EPI-CSCs), and an ESA^{low} fraction which was more migratory and formed more spheres (EMT-CSCs), and these two subpopulations of CSCs were able to swift between each other *in vitro* (Paper I)

When stained in addition with the epithelial marker ESA, two subpopulations of $CD44^{high}$ were found and sorted: $CD44^{high}ESA^{low}$ and $CD44^{high}ESA^{high}$. In adherent culture, $CD44^{high}ESA^{low}$ cells had an EMT-CSC phenotype, since they displayed an elongated fibroblast-like appearance. In suspension culture, 10 times more spheres were found in $CD44^{high}ESA^{low}$ cells compared to $CD44^{high}ESA^{high}$ cells. Time-lapse videos and transwell migration assays demonstrated that $CD44^{high}ESA^{low}$ cells were more motile and migratory cells, a feature of cells undergoing EMT [177]. QPCR revealed that $CD44^{high}ESA^{low}$ cells had up-regulated many EMT markers, including

vimentin, Twist, Snail and Axl, and down-regulated epithelial-specific genes, such as E-cadherin, Calgranulin B, involucrin and keratin 15.

Single cell cloning revealed that almost all (~100%) of $CD44^{high}ESA^{high}$ clones but only a fraction (≤50%) of the $CD44^{high}ESA^{low}$ clones could re-populate mixed populations, indicating that almost all $CD44^{high}ESA^{high}$ clones were bipotent, while approximately half of the $CD44^{high}ESA^{low}$ clones were unipotent and only generated EMT cells. Further fractionation in $CD44^{high}ESA^{low/+}$ cells and $CD44^{high}ESA^{low/-}$ cells showed that 44% of the $CD44^{high}ESA^{low/+}$clones were bipotent whereas none of the $CD44^{high}ESA^{low/-}$ clones were bipotent. The $CD44^{high}ESA^{low/+}$cells were found to have considerably higher ALDH activity, considered more recently a hallmark of CSCs [178], than $CD44^{high}ESA^{low/-}$ cells. FACS analysis based on ALDH activity revealed that $CD44^{high}ESA^{low}ALDH1^{+}$ cells contained more EMT-CSCs cells than $CD44^{high}ESA^{low}ALDH^{-}$ cells, suggesting that the high ALDH1 activity marked the bipotent EMT-CSCs.

5.1.3 $CD44^{high}ESA^{high}$cells (EPI-CSCs) had high tumorigenicity while $CD44^{high}ESA^{low}$cells (EMT-CSCs) had high metastasis ability *in vivo* (Paper I)

To investigate these two CSC phenotypes, orthotopic injection into NOD/SCID mice was performed. Upon this, both EPI-CSCs $CD44^{high}ESA^{high}$ cells and EMT CSC $CD44^{high}ESA^{low}$ cells were capable to form tumors in tongues after 26 days, whereas, only EMT-CSCs $CD44^{high}ESA^{low}$ cells showed lymph node infiltration after 26 days and they showed lower tumor growth at late time points. The tumors were collected and further examined. The population of cells obtained by dissociation of mice tumors showed a mixed population by FACS, and the similarity of histology of the tumors in H&E staining indicated that these two CSC phenotypes were able to return into one heterogeneous cell population *in vivo*.

5.1.4 CAFs were capable to enhance the self-renewal and tumorigenicity of OSCC cells (Paper II)

Apart from extracellular matrix molecules, various types of stromal cells (such as fibroblasts and myofibroblasts), soluble factors and signalling molecules create the complexity of the tumor microenvironment, which is highly involved in neoplastic transformation, tumor growth and invasion, as well as in the therapeutic resistance and tumor metastasis [179]. To elucidate the role of fibroblasts in maintaining the stem cell hierarchy in OSCC, co-culturing the oral dysplastic and cancer cell lines as well as primary OSCC-derived cells together with CAFs was used in Paper II. *In vitro* adhesion culture showed that significantly more colonies were formed in co-cultured condition compared to seeding them alone in oral dysplastic cell lines and primary oral cancer-derived cells. Although not significant, the same effect was observed in oral cancer cell lines. *In vivo* tongue xenotransplantation revealed that CAFs enhanced markedly the tumorigenic ability when co-injected with oral dysplastic cells with CAFs into NOD/SCID IL2γ deficient mice. When cancer cells were co-injected with CAFs in NOD/SCID mice, tumors size increased dramatically. After co-culture with CAFS, dysplastic and cancer cells showed increased mRNA level of stem cell factors Oct4A, BMI1 and CD44, although not statistically significnat in oral cancer cell lines. In addition, we found that both normal oral fibroblasts (NOFs) and CAFs increased the expression of CD44 when co-cultured with oral dysplastic dells and primary OSCC cells, but more prominent by CAFs.

5.1.5 CAFs were able to turn the OSCC cell subpopulation depleted for CSCs ($CD44^{low}$) in a clonogenic and tumorigenic population (Paper II)

To investigate the influence of CAFs on non-stem cells in term of the stem cell abilities, $CD44^{low}$ cells derived from OSCC patients were used as a population depleted in CSCs, since they showed low-clonogenicity, low-tumorogenicity and low-metastasis abilities. When co-seeding oral dysplastic or cancer cells with fibroblasts, both NOFs and CAFs were able to induce more colonies and spheres of $CD44^{low}$ cells, but CAFs were more prominent. *In vivo* tongue xenotransplantation

showed a dramatic increase in tumor incidence, tumor size and lymph node metastasis when primary CD44^{low} OSCC-derived cells were co-injected with CAFs. All together, these finding suggested that CAFs isolated from OSCC were more competent than normal fibroblasts in enhancing tumor growth by comingled OSCC cells, which was also described in breast cancer [180]. More importantly, paper II indicated that CAFs had a critical role in promoting carcinoma tumorigenesis supported by the fact that presence of CAFs could simulate cancer cells to proliferate and induce the potential of tumorigenicity of the non-stem population. This conclusion was also supported by the study on breast [181] and pancreatic tumors [182], in which a central role of CAFs in the promotion of tumor growth have been shown.

5.1.6 TGF-β1 was identified as being the key regulator for the CAF effect on inducing cancer stem-cell like properties in OSCC cells (Paper II)

OSCC cells were treated with CM collected from CAFs (CAF-CM) or TGF-β1 (that we found it secreted at higher levels by CAFs than NOFs). When CAF-CM was added, Ca1 and H357 cells formed more spheres which were slightly smaller in size and less compact, but expressed higher level of CD44 compared to non treated cells. Regarding to TGF-β1 treatment, TGF-β1-treated cells showed significantly increased number of colonies and spheres as well as stem cell related markers CD44, BMI1, Oct4 and α6 integrin when compared with that of non-treated cells in most OSCC-derived cell lines tested, but not in dysplastic cells. In addition, using immunohistochemical double staining for vimentin and CD44, the concomitant expression of EMT and stem-cell related markers was significantly raised after TGF-β1 treatment.

5.2 Isolation and characterization of OSCC CSCs based on collagen IV adhesion (Papers III and IV)

5.2.1 The rapid adherence to collagen IV (RAC) enriched for OSCC cells with increased colony and sphere forming ability, tumor formation ability and with high expression of stem cell-related markers (Paper III)

Recent evidence demonstrated that collagen IV played a major role in the maintenance of stemness in HNSCC-derived cells [183]. To investigate this in more detail and use this ability for isolation of CSCs in OSCC, we tested various subpopulations isolated based on their adhesion to collagen IV-coated dishes at different time points: rapid adherent cells (RAC-attached in first 10 min), middle adherent cells (MAC-attached in next 30 min) and late adherent cells (LAC-attached in the sequential 4 h). Using adherent culture and bright field microscopy, different cell phenotypes were found existing among RAC, MAC and LAC in OSCC-derived cells. Measurement of the cell diameter revealed a significant smaller size in RAC compared to MAC and LAC. Scanning electron microscopy (SEM) showed a regular spherical shape with folded and ruffled cell surface in RAC, and a flat appearance with smoother cell borders in MAC and LAC.

Colony formation assay and sphere formation assay showed that RAC had significantly greater capability of colony formation and sphere formation than MAC and LAC. In line with these *in vitro* findings, *in vivo* tongue xenotransplantation demonstrated that RAC formed bigger tumors with faster tumor growth compared to MAC and LAC, although all these three subpopulations had to a certain degree tumorigenic ability. Moreover, lymph nodes metastasis was examined using immunohistochemistry staining for p53 showing that RAC had great potential of spread in the loco-regional lymph nodes than MAC and LAC. Overall, these findings indicated that RAC behaved more as stem-like cells with enhanced capability of self-renewal and migrating compared to MAC and LAC.

P75 NTR was previously identified as a stem cell marker in normal human oral epithelium [184] and epithelium of esophagus [185], as well as esophageal squamous cell carcinomas [186]. A study on OSCC has reported P75 NTR as a marker for undifferentiated cancer cells and showed that it played a role in invasion and poor prognosis [187]. In Paper III, immunohistochemistry staining showed increased expression of P75 NTR in RAC compared to MAC and LAC. Human stem cell PCR Array further revealed a distinct mRNA profile of stem cell-related genes between RAC and LAC identified by unsupervised hierarchical cluster analysis of the PCR array data. Using SAM analysis, a panel of stem cell-related genes was found to be significantly up-regulated in RAC compared to LAC, such as CCND2, FGF2, PPARG, and ABCG2. Furthermore, significant up-regulation of FGF2, PPARG, CCDN2, TERT, and RPL13A and down-regulation in BMP1 were shown in RAC compared to LAC using specific TaqMan assays.

Flow cytometry analysis for ß1 integrin, as a collagen IV receptor and a well-known stem cell marker, identified significantly higher level of β1 integrin in RAC than MAC for both OSCC cell lines and primary OSCC cells. Flow cytometry analysis after staining for DNA content identified consistently higher proportion of cells in the G2 cell cycle phase in RAC compared with MAC and LAC in both the oral dysplastic cell line, OSCC cell lines and primary OSCC-derived cells.

5.2.2 RAC cells, enriched for CSCs, possessed significantly higher effective membrane capacitance due to their particular cell membrane morphology, very rich in cellular protrusions (Paper IV)

A gradually decrease of effective membrane capacitance (C_{eff}) was observed from RAC to MAC and LAC in oral cancer cell lines. RAC displayed the highest C_{eff} among the three subpopulations. Since C_{eff} level was known to be influenced by the morphology of the cellular membrane, we further investigated morphology-related aspects. In addition to having a significantly smaller diameter than MAC and LAC, RAC displayed a regular spherical appearance but with more folded cell membrane rich in both filopodia and lamellipodia membrane protrusions, whereas MAC and

LAC had a flat appearance with smoother cell membrane surfaces and less cellular protrusions on the surface. After treating all the three subpopulations with 4-MU, a known modulator of cellular protrusions, RAC changed markedly their membrane morphology by flattening of the cell surface, with loss of filopodia and decrease of C_{eff}, thus abrogating the difference existed among three subpopulations initially. This proved that the unique high C_{eff} in RAC was due to their particular cell membrane structure.

6. DISCUSSION

6.1 CSCs in OSCC

The CSCs model postulates a cellular hierarchy in tumors, in which only a subset of tumor cells have stem-like cell properties and ability to continually sustain tumor growth [188]. Although the concept of CSCs or TICs has often been controversial, increasing number of studies have reported the existence of CSCs in various solid tumors [188]. In OSCC, it has been described previously that as few as 5,000 $CD44^{high}$ cells were able to form a tumor when xenotransplanted into immunocompromised mice, while higher number of $CD44^{low}$ cells failed to form tumors, indicating $CD44^{high}$ cells as the CSCs in OSCC. Consistently, our results presented in Paper II demonstrated that OSCC cells that highly expressed CD44 possessed greater ability of self-renewal and tumorigenicity than that of $CD44^{low}$ cells, indicating that $CD44^{high}$ cells possessed almost exclusively the tumor initiating capacity. In Paper III, we applied a method based on rapid adhesiveness to collagen IV which has been reported previously for isolating normal human epithelial stem cell [141], to identify and enrich for OSCC CSCs. We found that the subpopulation that rapidly attached to collagen IV within 10min showed the highest self-renewal ability *in vitro* and tumorigenic potential *in vivo*, suggesting that this cell population (RAC) contains the tumor-initiating cells in OSCC.

Phenotypically diverse CSCs are likely to exist in tumors [188]. Our results presented in Paper I identified two distinct phenotypes of OSCC-derived CSCs in addition to the non-CSC phenotype, using FACS sorting based on combination of CD44 expression and ESA expression: (1) the holoclone-forming $CD44^{high}ESA^{low}$ cells characterized as EPI-CSCs; (2) the sphere-forming $CD44^{high}ESA^{low}$ cells characterized as EMT-CSCs, and (3) the paraclone-forming $CD44^{low}$ cells, again demonstrated as non-CSCs. Both of these two phenotypes of CSCs had tumorigenic capacity when xenotransplatated into the tongue of NOD\SCID mice.

Taken together, the findings presented here show, through different approaches, that OSCC seem to contain a subset of cells endowed with tumor initiating capacity that is phenotypically heterogeneous.

6.2 Oral cancer stem cell niche/microenvironment

It is known that the function of normal epithelial tissue stem cells is a result of the interaction with their surrounding cells and molecules that form a niche or microenvironment. Some previous evidence indicated that also in malignancy the CSCs seem to also be highly dependent on such a niche, including tissue-associated fibroblasts, to maintain their stem cell capacity [46, 189]. As one of the main stem cell niches in tumor, CAFs could sustain tumor growth and progression, in turn, their activated phenotype being induced and maintained by tumor cells [190]. A previous study on breast cancer have shown that co-injecting tumor cells with CAFs into mice was able to increase tumor take and decrease latency, whereas co-injecting them with normal fibroblasts induced opposite results [81]. Our results presented in paper II show that CAFs enhanced self-renewal ability *in vitro* and tumorigenic ability of OSCC cells *in vivo* and could stimulate the proliferation of OSCC cells, which in turn also could stimulate CAFs to proliferate. But in contrast to the study on breast cancer, our findings showed that NOFs could also increase the self-renewal ability *in vitro,* although not as prominent as CAFs. Nevertheless, our findings are in line with previous studies which suggested CAFs as one of the most prominent and influential cell type of the tumor microenvironment in various tumors, such as breast cancer, prostate and pancreatic carcinoma [190, 191].

Previous studies reported that TGF-β1was one of the main factors released by cancer cells and mediating CAFs activation [190]. Of interest, we found in our paper II, that CAFs acquire also the ability to synthesize TGF-β1, as showed by the finding that they secreted significantly higher levels of TGF-β1 than their normal counterparts. Our results show that TGF-β1 simultaneously could increase self-renewal and migratory abilities in OSCC cells, as well as the expression of both stem cell and

epithelial-mesenchymal transition markers, suggesting that TGF-β1 might be responsible for the effect of CAFs in increasing stemness in OSCC cells. This is in line with a very recent study on breast cancer stemness [73]. Taken together, these findings indicated that targeting CAFs and its mediator TGF-β1 should be taken into consideration for more effective therapeutic strategies against oral cancer in the future.

6.3 CSCs plasticity and EMT in OSCC

A recent study suggested that CSCs are phenotypically and functionally heterogeneous and possess plasticity capacity [192]. The classic definition of cell plasticity, taken from stem cell biology, implies the ability of stem cells to differentiate into various cell lineages, and the plasticity of CSCs refers to their ability to reconstitute the cellular heterogeneity found in the original tumor [192, 193]. In addition, the plasticity potential of malignant cells has been also described in epithelial tumors as a mechanism that allows the epithelial cells to transdifferentiate into mesenchymal cells through the process of EMT and reverse MET [194]. A very early report has demonstrated EMT as an intrinsic cell plasticity ability for epithelial tumor cells in culture, by showing their ability of changing the morphological features from epithelial-like cells to scattered fibroblast-like cells that were able to invade to BM [195]. EMT cancer cells were found to either sustain their mesenchymal trait under certain stimuli, or undergo reverse MET to return to epithelial-like cells when the stimuli was removed [196] . Furthermore, findings from breast cancer cells have established a link between CSCs and EMT by showing that the $CD44^{high}CD24^{low}$ tumor initiating phenotype was also expressing EMT markers [73]. Corroborating with this study, our results presented in Paper I indicate that also CSCs in OSCC have a plasticity potential to bidirectional switch between the EMT and epithelial phenotypes, through both EMT and the reverse process of MET. This recently demonstrated phenotypic plasticity of CSCs builds more completely the early concept of migratory CSC enunciated by Brabletz based on their *in vivo* findings from colon cancer patients [65], suggesting that stationary CSCs (EPI-CSCs)

are still embed in the original epithelial tissue and remain proliferative although having differentiating capacities, while mobile CSCs (EMT-CSCs) disseminate from the original tumor and colonize at distant sites through the acquisition of EMT and then undergo MET to produce the growth of a metastatic tumor as the same epithelial trait as the parent tumor [65]. Of interest, we also showed in Paper I that for the CSCs having undergone EMT only the cells with $ESA^{low/+}ALDH1^{+}$ expression pattern CSCs could reverse this process through MET to return to the epithelial phenotype. This may indicate that one population of CSCs that have undergone EMT is fixed in the mesenchymal lineage and is responsible for the dissemination from primary tumors, while the other population that is able to return to epithelial trait might be responsible for the tumor growth at the metastatic sites.

6.4 Methods of isolation of OSCC CSCs

The ideal method to identify CSCs should be efficient, specific, sensitive, simple and rapid [81]. Several assays exist, including FACS based on specific markers or Hoechst 33342 exclusion, sphere assay and label-retention assay. FACS is the most common method for selecting CSCs, and by its use several specific surface markers have been identified as stem cell markers in tumors. CD44 was firstly used as a candidate marker for CSCs in breast tumors [36], later on in HNSCC [45]. FACS is specific and sensitive; however, it has some technical limitations. For example, we could notice that the detection of the surface markers in samples was influenced by the culture condition, the procedure of obtaining the cell suspension, and the quality of the antibodies from the different producers. In addition, it required a large number of cells for sorting, but eventually a very limited number of CSCs could be available for further analysis [81]. Moreover, several logistic factors might influence the choice of FACS sorting for CSC isolation: (a) the instrument is expensive; (b) it requires to spend a substantial time on the instrument for sorting cells enough for subsequent studies; (c) quite subjective when defining the cell population (setting the gates); (d) no “golded markers” (well established, robust markers) for CSCs; (e) quite unpredictable reproducibility [80].

Using FACS for selection of SP on the basis of Hoechst 33342 exclusion has also been claimed to isolate stem cells in various tumors including human oral cancer [98]. However, SP could give rise to just a small and highly variable number (~0.01 - 5%) of CSCs, which were not enough for the further examination [95]. Its use is also questionable in its ability for valid CSCs identification since there were tumors or cell lines that did not present an identifiable SP, although CSCs should theoretically be present in every tumor and /or cell line [197].

Recent studies have also shown that more functional methods, such as sphere formation via serum free medium with growth factor could enrich for a population with stem cell properties in OSCC, but a long time was required (~ 2-3 weeks) to grow the sphere-like bodies [164]. In papers III and IV, we applied also a functional approach, using rapid adhesiveness to collagen IV to enrich in OSCC CSCs. Compared to the other techniques mentioned above, we found that in addition to being quite sensitive and robust, this was a simple, rapid, and non-invasive method for identification and characterization of CSCs, even though it still needed large number of cells to start with and ended up with a limited number of CSCs, especially in primary cells derived from OSCC patients. By using the collagen IV rapid adhesiveness to isolate CSCs, we further suggest in paper IV that another more functional, non-invasive method, DEP, may potentially be used for reliable and label-free isolation of putative CSCs. This is a very novel approach for identification and isolation of CSCs, and thus it needs more investigation to determine and validate its use for identification of CSCs. Taken together, with all the efforts in this field, there is still no “best method” for isolation of CSCs, and continuous investigation for enrichment and characterization of CSCs is needed.

6.5 Xenotransplantation mice model for CSCs in OSCC

Although there is no animal model that could be perfectly applied to every type of human tumor [165], it is agreed that xenotransplantation tumor in immunodeficient animal models could mirror the phenotypic heterogeneity of the original tumor, and is

considered to be the most sensitive assay for identification of CSCs [126]. To date, a CSCs in many human malignancies have been identified using the xenotransplantation mice system that showed that the population of CSCs had greater tumorigenicity compared to other subtype of tumor cells [126]. By transplanting tumor cells into NOD/SCID mice, the presence of CSCs was at first demonstrated in AML, in which as few as 5000 $CD44^{++}CD38^{-}$ cells could initiate leukemic proliferation, whereas 100 times as many $CD44^{+}CD38^{+}$cellswere not able to engraft [35]. After then, CSCs have been identified in many tumors, including breast [36], brain [38], prostate [198], colon [37] and ovarian [199] cancer. Ectopic subcutaneous transplantation system has been used as one of the common mice models for CSCs studies because of its easy way for establishing and measuring the tumors, as well as tumor reproducibility [165]. However, this system cannot accurately reflect primary tumor site microenvironment, which is maybe why a lot of therapeutic compounds showed disappointing outcomes when tested clinically despite of promising activity showed in subcutaneous xenotransplantation models [200]. In addition, detection of metastatic tumors in the subcutaneous implantation model is hardly detected since its non-organ specific tumor environment. Therefore, orthotopic animal models have been more and more used instead of ectopic animal models as it provides a more natural host microenvironment that can reproduce tumor-host interactions which are lacking when ectopic transplanting the tumor cells subcutaneously [165, 201]. Xenotransplantation of various subpopulations of OSCC cells into the tongue of NOD/SCID mouse was used as an orthotropic model in our current studies and strikingly, it showed that as few as 100 RAC populations (CSCs) from human primary OSCC could initiate tumors, compared to MAC and LAC (non-CSCs) that initiated tumors at higher numbers. Furthermore, cervical lymph node metastasis could be detected in most of the mice that were injected with different dilutions of human CSC OSCC cells (100; 1,000; 5,000; 10,000). These findings are in line with previous studies where by using an orthotopic tongue xenotransplantation mouse model, the cervical lymphatic metastasis potential could be assessed in mice [165, 202]. Nevertheless, with all its advantages, the tongue xenotransplantation brings up some concerns, as we also observed. The tongue of the mice is very tiny to inject

voluminous tumor samples, and the mice were easy to lose due to post injection increase in volume of the tongue, especially when injections were made near the base of the tongue, this being able to induce difficulty in breathing for mice and ultimately asphyxia. Another very specific limitation for inducing tongue tumors in mice is that the increased volume of the tongue due to the growing tumors might lead to difficulties in eating, the mice needing to be sacrificed before the metastases were detected due to their loss in appetite, impairment in chewing and swallowing and ultimately loss in weight. Nevertheless, tongue xenotransplantation mice model still serves as a great *in vivo* experimental model, not only as a highly sensitive assay for CSCs, but also for tumorigenicity and metastasis in OSCC in general.

7. CONCLUSIONS

I. By using FACS analysis based on higher expression of CD44 combined with ESA expression, two distinct CSCs subpopulations in OSCC could be identified. They were the $CD44^{high}ESA^{high}$ subpopulation, the EPI-CSCs which was more potent in local tumour initiation and growth, and the $CD44^{high}ESA^{low}$ subpopulation, the EMT CSCs which had more potential in metastasising. These two populations seem to co-exist in OSCC by switching between them through EMT and the reverse process of MET.

II. CAFs were capable to increase the self-renewal properties of OSCC cells by inducing stem-like properties in the non-stem subpopulation of oral carcinoma cells, and TGF-β1 seemed to be the main mediator of this effect.

III. Rapid adhesion to collagen IV could be used as a sensitive enough and robust method to enrich for a population containing putative CSCs/TICs in OSCC.

IV. CSCs exhibit unique dielectrically features (high C_{eff}) that can be putatively used for their isolation, as a label-free and non-invasive method.

8. FUTURE PERSPECTIVES

I. To investigate the dynamic/kinetics of the EPI-CSCs/EMT-CSCs switch and how the equilibrium between various subpopulations of cells is maintained at the molecular level.

II. To investigate the dynamic/kinetics of the non-CSC/CSC switch and how the equilibrium between various subpopulations of cells is maintained at the molecular level.

III. To explore the role of exogenous factors such as TGF-β1 in the dynamic switch and phenotypic equilibrium.

IV. To explore the role of Axl in the dynamic switch and equilibrium of EPI-CSC/EMT-CSC.

V. Modeling and development of intracardiac mouse model system using newly developed NOD-scidIL2Rγ^{null}mice to model and compare the differential response towards niches described above in terms of tumorigenesis and metastasis in OSCC.

VI. Further developing a method for OSCC CSCs sorting based on their unique DEP characteristics.

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