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Oral epithelial stem cells – implications in normal development and cancer metastasis

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Abstract

Oral mucosa is continuously exposed to environmental forces and has to be constantly renewed. Accordingly, the oral mucosa epithelium contains a large reservoir of epithelial stem cells necessary for tissue homeostasis. Despite considerable scientific advances in stem cell behavior in a number of tissues, fewer studies have been devoted to the stem cells in the oral epithelium. Most of oral mucosa stem cells studies are focused on identifying cancer stem cells (CSC) in oral squamous cell carcinomas (OSCCs) among other head and neck cancers. OSCCs are the most prevalent epithelial tumors of the head and neck region, marked by their aggressiveness and invasiveness. Due to their highly tumorigenic properties, it has been suggested that CSC may be the critical population of cancer cells in the development of OSCC metastasis. This review presents a brief overview of epithelium stem cells with implications in oral health, and the clinical implications of the CSC concept in OSCC metastatic dissemination.

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Keywords

Oral squamous cell carcinoma; epithelial stem cells; invasion; metastasis; cancer stem cells; oral mucosa

Introduction

Oral mucosa has a remarkable regenerative potential [1]. Several stem cells markers are known to be expressed, mainly in the basal layers of oral mucosa. It has been proven that the expression of these markers is dysregulated in oral squamous cell carcinomas (OSCC), the most common cancer of the oral cavity [2]. There is a need for a better characterization of the oral stem cells in particularly of their cell behavior, tissue-specific regenerative potential and involvement in carcinogenesis. This review provides an overview of stem cells biological implications in oral mucosa with a special emphasis in OSCC.

Oral Mucosa

The epithelium on the inner surface of the lips, floor of the mouth, gingiva, cheeks and hard palate is derived from embryonic ectoderm, whereas the epithelium surrounding the tongue is derived from both endoderm and ectoderm [3–5]. The oral mucosa can be divided into: *masticatory* (hard palate and gingival), *specialized* (dorsal surface of the tongue) and *lining* (buccal mucosa, ventral surface of the tongue, soft palate, intra-oral surfaces of the lips and alveolar mucosa) [5]. Of the total surface of the oral lining, approximately 25% is keratinized resembling that of the epidermis covering the skin in regions subject to mechanical forces (masticatory mucosa of the gingiva and hard palate), 60% is the non-keratinized lining mucosa in the regions requiring flexibility to accommodate chewing, speech or swallowing (floor of the mouth, buccal regions, esophagus, etc), with the remaining 15% is the specialized mucosa (dorsum of the tongue) which can be represented as a mosaic of keratinized and non-keratinized epithelium [6]. Oral epithelium is a stratified squamous epithelium that consists in various layers: basal, spinous, granular and corneal layers for the keratinized area; basal, spinous, intermediate and superficial layers in the non-keratinized areas. The oral epithelium is in direct contact with an underlying, dense connective tissue (*lamina propria*) containing minor salivary glands, structural fibers, blood vessels, fibroblasts along with other cell types [6–11]. Its histological structure involves undulations of epithelium (*rete ridges*) protruding downwards into the lamina propria, with corresponding upward projections of lamina propria (*dermal papillae*) and thus provides increased surface contact, which prevent separation of the oral epithelium from the underlying lamina propria during mastication [12].

The squamous epithelium covering the oral mucosa relies on epithelial stem cells for tissue renewal [1]. It is unanimously accepted that normal tissue stem cells constitute a life-long reservoir of cells with active mechanisms for self-renewal. Cell division in oral mucosa epithelial cells takes place mainly in the basal layer which contains the stem cells compartment from which the oral mucosa is being regenerated [7]. After dividing, the committed cells undergo differentiation that leads to the expression of structural keratin proteins as cells move superficially, and eventually fall off the surface. In the oral

epithelium, it takes 14–24 days for a stem cell to divide and the progeny to traverse through the entire thickness of the epithelium (turnover time) [8]. Expression of several stem cells markers including, CD44, Bmi1, Sox2, Keratin 14, have been described mainly in the basal layer (Fig. 1, Table 1), suggesting that it may contain a reservoir of stem cells [5, 9]. However, the mechanism of tissue maintenance and regeneration is still largely unknown for these cells. It is interesting to note that no many studies have focused on transient amplifying (TA) progenitor cells in the oral cavity and on their potential to provide a reservoir for would healing and homeostasis [2, 5, 8, 14]. TA cells are slightly more differentiated than stem cells yet highly proliferative; they are derived from stem cells and continue to divide several times before undergoing terminal differentiation/maturation into the functional cells of the tissue; the size of the dividing transit population differs dramatically from tissue to tissue, the number of generations defining the degree of amplification that the transit population provides for each stem cells division seems to be related inversely to the frequency that stem cells will be found within the proliferating compartment [2,13].

Recent findings derived from various solid malignancies models show that cancer progenitor cells have the capacity to dedifferentiate and acquire a stem-like phenotype in response to either genetic manipulation or environmental cues, via implication of various complex molecular circuitries. These findings highlight the need for a better understanding of the dynamic, contextually regulated, equilibrium between cancer stem cells (CSCs) and cancer progenitor cells as a critical step for the development of therapeutic strategies to deplete tumors of their tumor-propagating and treatment-resistant cell subpopulations [14–15]. Better characterization of the CSCs and progenitor cells will contribute to a better understanding of normal and abnormal epithelial growth and tissues regeneration in the oral cavity.

Challenges to the integrity of the oral mucosa

The mucosal lining of the oral cavity is an environment challenged by a large variety of insults, and functions to protect the underlying tissues and organs against mechanical and chemical insults, including microorganisms and toxins, or ingested antigens and carcinogens [10]. The oral epithelium is constantly replaced with a rapid clearance of surface cells, which acts as a protective mechanism against various insults and its structure constitutes an effective barrier [10].

The turnover of the oral mucosa is faster in the lining than in the masticatory regions, thus challenges to the integrity of the oral mucosa will affect in particular the more rapidly proliferating areas and the lining regions will suffer first [16–18]. The dorsal surface of the tongue is composed of many small filiform papillae that have a very uniform shape and size, based on early detailed histological investigations and cell kinetic studies [19–20]. Each papilla is composed of four columns of cells, two dominant (anterior and posterior, also so-called *tongue proliferative units*, the functional group of proliferative basal cells derived from a single stem cell, together with the distally arranged functional differentiated cells) and two buttressing columns. The lineage characterizing this epithelium is similar to that seen in the dorsal epidermis of the mouse, self-replacing asymmetrically dividing stem cells,

occurring at a specific position in the tissue, and producing a cell lineage that has approximately three generations. The stem cells here have a particularly pronounced circadian rhythm [13, 17, 18–21] suggesting an involvement of the circadian clock in stem cell equilibrum. Of interest, disrupting this clock equilibrium in the skin, through deletion of Bmal1 (also known as Arntl) or Per1/2, resulted in a progressive accumulation or depletion of dormant stem cells, respectively [22]. Stem cell arrhythmia also led to premature epidermal ageing, and a reduction in the development of squamous tumors. Clinically, the squamous cell carcinoma of the tongue is considered one of the most aggressive tumors of the oral cavity. The potential effects of circadian disruption in tongue stem cells behavior and tongue cancer development remain largely unexplored.

Despite continuous challenges imposed on the oral mucosa, normal human oral epithelial cells undergo constant cell division leading to regeneration of tissue, provided that the cells retain their ability to limit their replicative life span through cellular senescence, induce cell cycle arrest upon DNA damaged and repair the damaged DNA before resuming the cell cycle. It is known that DNA is most vulnerable during mitosis and that mitotic activity can be affected by a number of factors, such as stress and inflammation, as well as circadian clock disruption [10, 17]. Preliminary findings from our laboratories have identified a strong expression of clock genes in areas rich in stem cells (basal layer) in oral tissues (unpublished observations). Furthermore, we also have been able to detect expression of clock genes and CD44 in the basal layer of oral mucosa surrounding the tooth root and salivary glands (Fig. 1; [23]). Of interest, expression of both stem cells markers and clock genes are found altered in oral squamous cell carcinomas [24], suggesting an implication of the circadian clock in stem cells behavior in carcinogenesis. Moreover, the expression of growth factors such as the Epithelial Growth Factor (EGF) may play a role in the oral epithelium differentiation and in CSC self-renewal in tumors originated in the head and neck area, particularly in the head and neck squamous cell carcinomas (HNSCC). EGF promotes acquisition of stem cell-like properties, increased cell proliferation and decreased sensitivity to cisplatin treatment in HNSCC [25].

CSCs and normal stem cells share similarities [8]. Normal adult stem cells have the ability to repopulate the cells that constitute the organ from which they are isolated and the capacity to propagate themselves; both processes are tightly regulated. Cancer stem cells reflect some of these same properties and are by definition able to sustain proliferation; however, the CSCs are not subject to the same genetic constraints to which normal stem cells are bound. The deregulation of pathways that control the self-renewal of normal stem cells (e.g. Wnt, Notch, Hedgehog, etc) leads to tumorigenesis in rodent models and also plays an important role in human carcinogenesis including HNSCC [2, 26–31]. Being long-lived, both CSCs and stem cells of normal tissues can be the targets of environmental carcinogens leading to the accumulation of consecutive genetic changes although several protective mechanisms have evolved to ensure the genetic integrity of the stem cell compartment in any given tissue [32]. Evolution of DNA methylation has allowed cells to respond to environmental cues in a flexible, yet stable manner, by properly regulating the response at the molecular level. However, dysregulation of DNA methylation can lead to hyper- or hypo-methylation of the promoters of critical genes, contributing to various diseases including HNSCC. Several reports have identified hypermethylation or hypomethylation in the promoters of key genes

involved in oral, head and neck cancer [33–36], and have also demonstrated unequivocally that the vast majority of human HNSCC tumors contain multiple mutations [37–38]. It has been shown that variability in DNA methylation exists within subtypes of HNSCC, and is influenced by environmental factors such as diet [39–40].

Oral squamous cell carcinoma (OSCC)

OSCC is the most prevalent and aggressive epithelial tumor of the head and neck region with the poorest outcome; in the United States alone approximately 100 new cases are daily diagnosed, while one person dies from oral cancer every hour of every day [41–44] (oralcancerfoundation.com). Worldwide, the problem is far greater with new cases annually exceeding 640,000 (oralcancerfoundation.com). Traditionally a men's illness, affecting six men for every woman, over the past 10 years that ratio has alarmingly become 2:1 also affecting younger patients [45]. Development of oral cancer proceeds through discrete molecular genetic changes that are acquired from the loss of genomic integrity after continued exposure to environmental risk factors. Predisposing risk factors such as tobacco use and alcohol consumption have a greater than multiplicative effect and account for the majority of the squamous cell carcinomas developed in the head and neck area [46]. However, these established risk factors do not account for about 40% of oral cavity cases. It has been suggested that yet unknown genetic, occupational, viral or nutritional factors could influence risk particularly in this younger patients group [47]. A growing body of evidence implicates human oral bacteria (over 700 bacterial species inhabit the oral cavity) in the etiology of oral cancers and epidemiological studies consistently report increased risks of these cancers in patients with periodontal disease or tooth loss; furthermore oral bacteria may activate alcohol and smoking-related carcinogens locally or systematically through chronic inflammation [48]. Human Papillomavirus (HPV) has recently emerged as the primary etiologic factor particularly for tumors developed in the tongue and oropharynx that are also associated with younger age at diagnosis [49–50]. Consequently, unique pathologic profiles have emerged that are consistent with the changing incidence of HNSCC [51–52]. Patients with HPV(+) head and neck cancer have a distinct risk profile, associated with a less remarkable history of tobacco and alcohol use [53–54], a more beneficial micronutrient profile [55], improved cellular immunity [56] and improved survival compared to those with HPV(−) tumors [57–58]. Notably, a significant subset (20–30%) of HPV(+) tumors fails to respond, recur locally, or spread distantly. Studies conducted at the University of Michigan have made significant contributions to the understanding of the impact of HPV infection on the pathobiology of HNSCC and response to therapy [58–60]. However, the mechanisms involved in these processes are not fully understood, and given the evolving epidemiology there is a growing controversy over the optimal strategy for oropharynx cancer treatment [59–60].

OSCC continues to be a disfiguring and deadly disease, which displays a wide range of metastatic behavior that cannot be predicted by tumor size, standard histology, or even individual gene or protein expression/activity [61, 62]. Despite advances in treatment, the survival of patients with advanced OSCC has not improved significantly over the last 30 years and remains one of the lowest among the major cancer types [42]. Metastatic tumor behavior is a critical factor in patient survival being responsible for more than 90% of cancer

associated mortality in these patients; if distant disease, the 5-year survival rate is three times less than those with nodal metastases, while survival of patients with nodal metastases is half that of similar stage patients without metastases [61, 63]. Younger (<40 years) patients with oral cancer have higher rate of distant failure [64, 65]). Therapeutic options for metastatic OSCC are limited and unsuccessful [66, 67]. Accurate prediction of metastasis in OSCC would have an immediate clinical impact through avoidance of unnecessary treatment of patients at low risk with appropriate direction of resources toward aggressive treatment of patients at high risk of having metastatic disease.

Approximately two thirds of oral cancers occur in the oral cavity (lip, tongue, floor of mouth, palate, gingival, alveolar and buccal mucosa), while the remainder occurs in the oropharynx [61]. Evidence indicates infection of oral epithelial stem cells by high-risk types of human papillomavirus (HPV); clinical observations shown early lymphatic metastasis in HPV-related HNSCC [68, 69]. The microenvironment is increasingly recognized as relevant in the process of metastasis as it is the immunity [70–72]. Studies indicated that OSCC is associated with alterations in the immune system [73–77] and that CSC may be immunologically silent or at least compromised in OSCC [79].

Clinical relevance of cancer stem cells in oral tumorigenesis

The identification of cancer stem cells (CSC) has created a new area of research with promising applications in the prognosis and therapeutics of human cancer [69, 78–91]. Accumulating evidence indicates that the CSCs also play a role in the pathogenesis and progression of carcinomas developed in the oral cavity.

To date, two models of tumor heterogeneity are unanimously accepted: the hierarchical model that assumes that CSCs represents a biologically distinct subset within the total malignant cell population in contrast with the stochastic model that assumes that every cell within a tumor has the same potential to act as a CSC [7]. Work by Prince *et al*. in 2007 was the first to identify CSC in head and neck squamous cell carcinomas (HNSCC) based on their CD44 expression; these cells possessed the qualities of self-renewal, tumorigenesis and the ability to recapitulate a heterogeneous tumor [92]. Additional studies at the University of Michigan have identified various CSC markers in HNSCC (e.g. ALDH, CD44, Bmi-1; [93–96]. Other markers also have been proposed (e.g. CD133, Oct-4, Nanog, Sox2, CD24, Snail, Twist, etc, most of them in combination with CD44 or ALDH; [2, 5, 8, 69, 90, 97–102]). Currently, CD44 and ALDH are the most common markers used to identify CSC in HNSCC.

CD44 is a cell-surface glycoprotein involved in cell-cell interactions, cell migration, and adhesion with multiple isoforms (splice variants) known to be associated with cell transformation and tumor dissemination [103–106]; Clinically, overexpression of CD44 was associated with poor prognoses and decreased 5-year survival in HNSCC patients [107–110], although its clinical relevance seems dependent on the anatomical site where the cancer originates [106, 111–114]. The aldehyde dehydrogenase (ALDH) family of cytosolic enzymes catalyses the oxidation of aliphatic and aromatic aldehydes to carboxylic acids and ALDH1 is a family member that has a role in the conversion of retinol to retinoic acid,

which is important for proliferation, differentiation and survival. ALDH1 activity seems to be responsible for the resistance of progenitor cells to chemotherapeutic agents [80, 90, 93, 115, 116]. Our group has shown that expression of ALDH and CD44 discriminates a highly tumorigenic cell subpopulation that can reconstitute the HNSCC heterogeneity; we performed *ex vivo* clonogenicity ("spheres-forming") assays to measure the frequency with which these prospectively isolated cells form colonies ("orospheres") when placed at clonal density in non-adherent conditions [117]. More recently, we reported sialyl Lewis X as a marker that associates with the metastatic abilities of CSC in OSCC [95]. We are currently investigating other putative markers to better characterize oral epithelial stem cells and their metastatic abilities in OSCC, particularly markers that we previously found associated with tumor progression and dissemination in OSCC: Aurora B [118], Survivin [119], beta-catenin [120] that are expressed in the basal layer and invasive front (Figs. 1–2). Survivin is a promising candidate for targeted anti-cancer therapy as its expression associates with poor clinical outcome, aggressive clinic-pathologic features, and resistance to radiation and chemotherapy in OSCC among other HNSCCs [83, 121–123].

Implications of oral cancer stem cells in metastasis

Better purification of the stem-like cell population in oral carcinomas is necessary to clarify what metastatic characteristics are indeed unique to these cells. Such evidence would allow clinicians to exploit this particular set of attributes to target cancer stem cells that keep a tumor growing and allow it to spread. Our group has designed *in vitro* and *in vivo* models of metastasis to study the behavior of this unique tumor cell subpopulation in HNSCC. Our data showed that CSC possesses a greater capacity for tumor growth, increased mobility and invasive characteristics [85, 117]. Our data also has confirmed the greater metastatic potential of CSC compared to non-CSC, suggesting that CSC may be responsible for the development of metastasis in HNSCC [117]. Clinically, CSC enrichment is linked to treatment failure, tumor recurrence and metastasis in head and neck carcinomas [67, 124]. There is growing evidence that CSCs behavior is orchestrated *in vivo* in tissue-specific, "niche" microenvironments. Characterization of the microenvironment surrounding CSC suggest the existence of a perivascular niche that supports stem cells maintenance and resistance to anoikis, suggesting that targeting the crosstalk between CSCs and other cells of their supportive niche may provide effective way to abrogate the tumorigenic function of these cells [72, 125].

The mechanism underlying the invasion of carcinoma cells leading to tumor dissemination involves the epithelial-mesenchymal transition (EMT) of cells with high tumorigenic potential [126, 127]. It is also known that EMT endows epithelial cells with invasive and stem cell properties [128]. Normal stem cells and CSC may share a mesenchymal phenotype that enhances their ability to preserve stemness, to regain migratory properties, and to respond to different stimuli during the expansion and differentiation [69]. Cancer stem cells seem to localize at the invasive fronts of the HNSCC in close proximity with the blood vessels [129]. Of interest, emerging evidence including our findings reveal that the CSC cell populations in carcinomas originated in the oral cavity may be heterogeneous including various CSC subpopulations with distinct phenotypic and functional states: larger-size CSC with mesenchymal features and migratory abilities *versus* proliferative CSCs that retain

epithelial characteristics (Fig. 2). Furthermore, in our spheres culture model, which is highly enriched in metastatic stem cells [95], we have observed that the majority of the cells are also highly enriched in epithelial markers, suggesting the existence of a predominant epithelial stem cells population (unpublished observations). It has been suggested that because the EMT-associated growth arrest, a re-differentiation into epithelial cells (so called Mesenchymal-Epithelial Transition, MET) may be necessary for the metastatic colonization [69, 130]. This evidence is suggestive of a new mechanism allowing metastatic colonization by uncoupling stemness from EMT and growth arrest, in favor of a parallel maintenance of stemness, proliferation phenotype and epithelial characteristics. However, it remains unclear how CSCs carry out the metastatic process in these carcinomas and how metastatic behavior of OSCC is modulated by CSC phenotypic characteristics.

Therapeutic relevance of stem cells research

Primitive stem cells capable of self-renewing proliferation and single or multiple cell lineage progeny generation have been identified in several human epithelial tissues. Although the biological characterization of various non-hematopoietic stem cells is still in its early stages in the laboratory, therapeutic experience with hematopoietic stem cells suggests that other stem cell types will likely have successful clinical applications. Better understanding of pluripotentiality, control of cellular differentiation and of epigenetic programming is critical to major future clinical applications. On the other hand, characterizing CSC subpopulations in oral, head and neck cancer will lead to a better understanding of cancer recurrence, metastasis, resistance to treatment, and should pave the way for more effective therapies for these types of cancer. In addition, the evaluation of the frequency of CSC, their molecular profiling and proliferative state, in a given tumor may be of prognostic value for the overall survival, response to therapy, risk of recurrence and metastasis. An overview of to date known anti-cancer therapies including those targeting CSC is presented in the Table 2. Current studies are conducted at the University of Michigan towards developing an autologous CSC-based therapeutic vaccine for clinical use in an adjuvant setting [131]. There are hopes that the near future will bring novel diagnostic and therapeutic approaches that will result in significant improvement of OSCC management and patient outcome.

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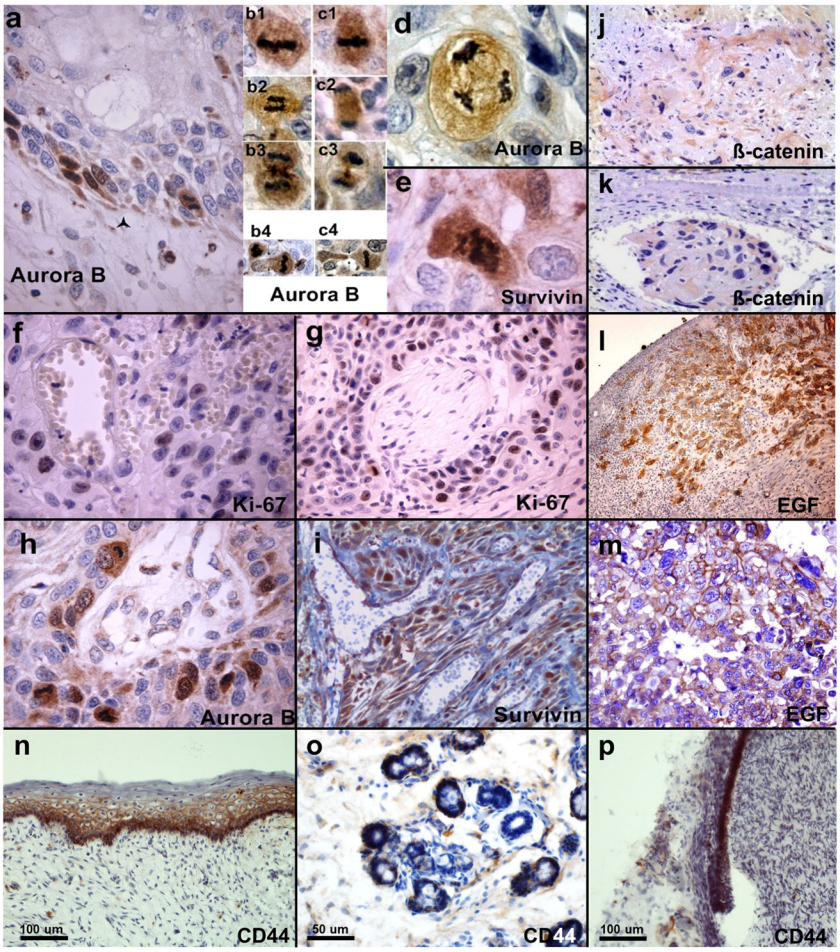

Figure 1. Markers of epithelial stem cells during normal development and in relation with poor clinical outcome in patients with OSCC

Representative photomicrographs of primary human oral carcinomas immune-stained for markers that are associated with poor clinical outcome in patients with oral carcinomas: Aurora B (a–d, h), Survivin (e, i), beta-catenin (j–k), EGFR (l–m), Ki67 (f–g) and CD44 (n–p). a, Aurora B expression in mitotically active cells in the basal layer of severe oral dysplasia; note the limits with basal membrane outlined by star. b–c, increased and aberrant mitoses overexpressing Aurora B, in aggressive OSCC at invasive front; b1–c1, metaphases; b2, early anaphase; c2, late anaphase; b3–c3, late anaphase-cytokinesis; b4–c4, anomalous cytokineses. Aurora B overexpression is coupled with survivin up-regulation in the invasion fronts of OSCC giving raise to aberrant mitoses (d, Aurora B; e, survivin). High proliferative index of aggressive and invasive cells with perivascular (f) and perineural (g) localization positive for Ki-67, is coupled with Aurora B (h) and Survivin (i) over-expression. Beta-catenin expression in the invasive front (j) and a metastatic embolus (k) of OSCC. EGFR expression in the invasive cells front (l–m). CD44 expression in the basal layer of normal human oral mucosa (n), salivary gland (o) and dental root epithelia (p). All the samples were obtained with the signed informed consent of patients under approved protocols by the Ethical Committees of the Universities of Foggia and Marseille.

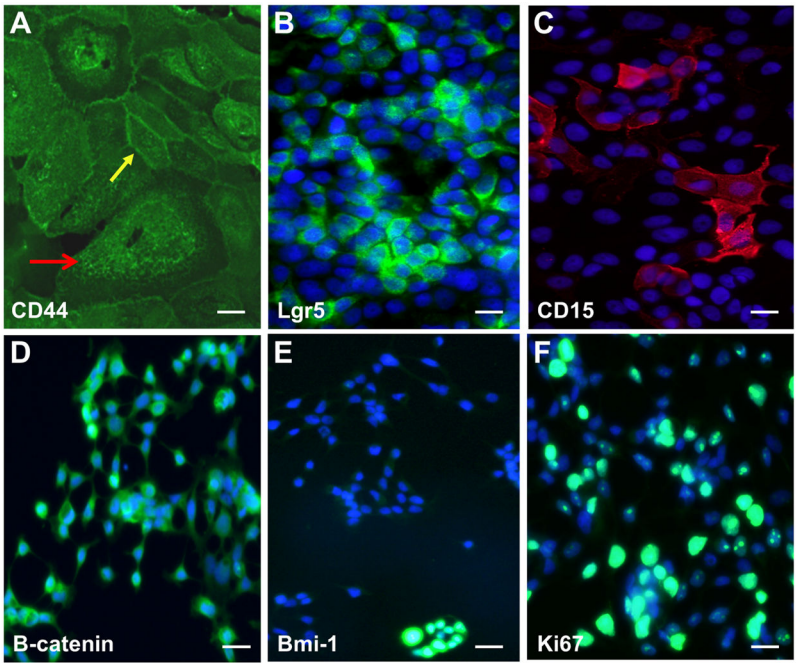

Figure 2. Putative cancer stem cells markers in oral carcinomas

Representative photomicrographs (magnification 20x) of human derived oral squamous cell carcinomas cells immune-stained for CD44 (A), Lgr5 (B), CD15 (C), beta-catenin (D), Bmi-1 (E) and Ki67 (F). Larger size mesenchymal-like CSC co-exist with normal size CSC that retain the epithelial characteristics (yellow arrow). DAPI (blue) identified nuclei.

Table 1

Markers for cancer stem cells (CSC) in head and neck squamous cell carcinomas (HNSCC).
CSC subpopulations of cells in HNCSS have been identified by the expression of specific markers (single or in combination) complemented by *in vivo* tumorigenic assay performed in immune-deficient mice and "sphere-forming" assays *in vitro* [92–117, 216–225].

CD44	Also known as phagocytic glycoprotein-1 (Pgp-1) and the receptor for hyaluronate. It is a cell surface glycoprotein involved in cell adhesion, cell proliferation, migration, and angiogenesis that exist as a large number of different isoforms resulting from alternative RNA splicing. CD44 also acts as a ligand for E-selectin. Measured by fluoresce activated cell sorting (FACS) can also be reported as $CD44^{high}$ (the 10 to 15% of cells with the highest CD44 expression) and $CD44^{low}$ (the 10 to 15% with the lowest or no expression).
Aldehyde dehydrogenase (ALDH)	ALDH gene superfamily encodes detoxifying enzymes; its activity is known to enrich cells with increased stem like properties in solid malignancies including HNSCC. Measured by FACS after staining of live cells with a non-immunologic enzymatic ALDEFLUOR kit.
EpCAM (ESA)	Epithelial cell adhesion glycosylated membrane protein involved in Wnt signaling and a cancer stem cell surface CD antigen (CD326) that can be measured by FACS. In HNSCC, two biologically distinct phenotypes of CSC have been reported based on ESA/CD44 expression: CD44(high)ESA(high) that are proliferative with epithelial characteristics (so called non–EMT CSC) versus CD44(high)ESA(low) that are migratory with mesenchymal traits (non-EMT CSC). EMT: epithelial-mesenchymal transition.
Side population (SP)	Defines cells able to efflux fluorescent DNA dye such as Hoechst 33342 and DyeCycle Violet; its phenotype depends on the concentration of ATP-binding cassette (ABC) transporters superfamily efflux pumps in the plasma membrane. In HNSCC, SP cells express high levels of Bmi-1, CD44, Oct-4 and are high in metastatic and aggressive HNSCC.
CD133 (protaminin-1)	A 120kDa glycoprotein with five transmembrane domains and two large extracellular loops with a role (through association with Src kinase) in the regulation of tumor initiating properties and the transition from an epithelial to a mesenchymal phenotype of head and neck carcinoma cells. In OSCC CD133+ stem-like cells possess higher clonogenicity, invasiveness and tumorigenesis as compared with CD133−; CD44+ cancer stem-like cells expressed higher CD133 levels than CD44− cells in HNSCC.
Bmi-1	A polycomb protein and proto-oncogenic chromatin regulator known to promote stem cells self-renewal by negatively regulating the expression of Ink4a and Arf tumor suppressors. In HNSCC Bmi-1 is highly enriched in CD133+ cells, induces the proliferation of these cells, and prevents apoptosis.
Oct-4, Nanog and Sox2	Transcription factors that play essential roles in the maintenance of pluripotency and self-renewal of embryonic stem cells. In human adult oral mucosa, stem cells were detected in the lamina propria based on their Oct4, Sox2 and Nanog expression. Triple positive Nanog/Oct-4/CD133 expression predicted worst survival in HNSCC patients. The usefulness of these factors for the sorting of CSC by FACS followed by culture and implantation in animals is hindered by the fact that they are not expressed in the cell membrane.
CD117 (c-kit, receptor of stem cell factor, SCF)	A transmembrane tyrosine-kinase receptor that is part of the platelet-derived growth-factor/colony stimulator factor 1 receptor subfamily involved in cell survival, differentiation, adhesion and chemotaxis. The co-expression of c-kit and SCF was observed in various solid tumors; this ligand/receptor system may have autocrine and paracrine effects on the regulation of tumor behavior (tumor growth and dissemination) in HNSCC.
CD24	A cell adhesion molecule commonly used as a CSC marker with CD44 in breast cancer, or with CD44/ESA in pancreatic cancer. In HNSCC, CD24+/CD44+ cells possessed stemness characteristics of self-renewal and differentiation, showed higher in vitro invasiveness and made higher number of colonies in collagen gels and were more chemo-resistant compared to CD24−/CD44+ cells. In addition, CD24+/CD44+ HNSCC cells showed a tendency to generate larger tumors in nude mice than CD24−/CD44+ cell population.

Table 2

Anti-cancer therapies targeting the cancer cells and cancer stem cells.

	Targets/Mechanisms	Drugs	Type of study	Results	References
A	**Pump targeting drugs**				
	1. ATP-binding cassette (ABC) multidrug efflux pump (transporters) inhibitors: P-glycoprotein (PGP) inhibitors	1st generation PGP inhibitors (calcium channel blockers: (e.g. verapamil), cyclosporine, tamoxifen	In vivo and in vitro	Enhanced the therapeutic effect of chemotherapeutic drugs (Vincristine, Adriamycin) circumventing tumor chemoresistance (leukemia); direct effects on cancer stem cells in head and neck cancers	[132–134]
		2nd generation: valspodar (psc-833, a cyclosporine analog)	Clinical trial	Ability to modulate multidrug resistance (MDR) caused by expression of *MDR1* gene, which encodes for the P-gp multidrug transporter, and is a determinant of both intrinsic and acquired drug resistance in many human cancers; decrease the clearance of several anticancer drugs. Usage in combination with anticancer cocktails showed limited benefits in patients with acute myeloid leukemia (AML) and metastatic cancer : non-small cell lung, gastro-intestinal, ovarian, mesothelioma, metastatic and recurrent head and neck	[133, 135–141]
		3rd generation: Zosuquidar (LY335979)	Clinical trial	Reverses P-glycoprotein-mediated multi-drug (MDR) resistance; Combined usage with Docetaxel to treat metastatic or locally recurrent carcinoma that have failed standard chemotherapy (breast cancer) or resistant malignancies (melanoma, ovary, lung, breast, sarcoma, head and neck cancer)	
		4th generation: Natural products: curcumin, flavonoids: kaempferol, genistein, silymarin, quercetin etc	In vitro	MDR reversal; blocking of multiple pathways by which cancer cells can survive (breast cancer, head and neck cancer)	
	2. Nanoparticle drug delivery	γ-secretase inhibitor- mesoporous silica nanoparticles (GSI-MSNPs)	In vivo and in vitro	Targeted inhibition of Notch signaling in cancer stem cells	[142]
B	**Targeting stemness**				
	1. Targeting CSCs dormancy/quiescence				
	1.1. stimulate the cells to enter division cycle and to become sensitive to chemotherapies	Interferon (INF) α	In vivo and Clinical trial	Stimulate dormant cells to proliferate, used in combination with imatinib to treat chronic myelogenous leukemia (CML); inhibits tumor growth and metastasis, reduce intratumoral microvessel density, increased cell apoptosis and induced prolonged survival in head and neck cancer; enhance response to standard therapies and successful immunostimulation in patients with head and neck cancer	[143–151]
		Granulocyte colony-stimulating factor (G-CSF)	Clinical trial	Stimulate dormant cells to proliferate, used in combination with tyrosine kinase inhibitor (imatinib) to treat AML, CML; dendritic cells differentiation in head and neck cancer; administration of an oncolytic herpes simplex type-1 virus encoding human G-CSF in combination with standard therapies improve loco-	

	Targets/Mechanisms	Drugs	Type of study	Results	References
				regional control and survival in patients with head and neck; however, subcutaneous administration	
				regional control and survival in patients with head and neck; however, subcutaneous administration	
				regional control and survival in patients with head and neck; however, subcutaneous administration	
				regional control and survival in patients with head and neck; however, subcutaneous administration	
		AMD3100 (plexixafor, a stromal cell- derived factor SDF-1/CXCL12- CXCR4 inhibitor)	Clinical trial; in vivo and in vitro	Mobilize stem cell in combination with G-CSF to treat non-Hodgkin's lymphoma and multiple myeloma; potent anti-metastatic effect in head and neck cancer	
	1.2. epigenetic therapy targeting chromatin acetylation and DNA replication	Histone deacetylase inhibitors (HDACi: suberoylanilide hydroxamic acid); valproic acid	Clinical trial	a/able to kill the cells in dormancy, eradicate the residual tumor by inducing apoptosis in nonproliferating cancer cell lines. Clinical trial in CML	[146]
			In Vivo and in vitro	b/able to induce replication-associated DNA damage without detectable alteration in cell cycle progression and unrelated to apoptosis; prevent aggressiveness of CSCs (colon cancer and breast cancer); induce apoptosis and cell cycle arrest, and alter the cancer stem cell phenotype in head and neck cancer; synergistic effects with standard chemotherapies in head and neck cancer; in combination with ribonuclease reductase inhibitor block efficiently tumor growth, induce tumor- cell apoptosis and induce EGFR downregulation in head and neck cancer	[147–154]
	1.3. differentiation therapy	Vitamin A derivative (retinoids and their naturally metabolized and synthetic products e.g. all-trans retinoic acid, 13- *cis*-retinoic acid, bexarotene)	Clinical trial	Successful treatment of various subtypes of leukemia harboring chromosomal translocations; limited success in the prevention and treatment of solid tumors may relate to the frequent epigenetic silencing of retinoic acid receptor beta (RARbeta); Limited effect to prevent progression and recurrence of head and neck (oral, pharyngeal) cancer	[155–156]
	2. Targeting self-renewal and proliferation signaling pathways				
	2.1. Wnt (Wingless type) pathway	anti-Wnt antibody; anti-Wnt receptors: Frizzled antibody (OMP-18R5); LRP6 antibody (anti Wnt-1 and Wnt-3)	In vitro and in vivo	The WNT receptor Frizzled (FZD)7 is essential for maintenance of the pluripotent state in human embryonic stem cells; Decrease viability and proliferation of cancer cells; decrease growth and tumorigenicity of human tumors (hepatocellular carcinoma, breast, pancreatic, lung, colon, etc); exhibits synergistic activity with standard-of-care chemotherapeutic agents; induced extended delay in the re-growth of tumors following treatment with high-dose chemotherapy	[157–161]
		Wnt protein inhibitors (VS-507)	In vivo	Decrease population of breast cancer stem cell, reduce cancer grown and metastasis	[163]
		anti-nuclear complexes; new compound (trinuclear ruthenium complex $[Ru^{III}_3(TSA\text{-}H)_2(TSA)_4][NEt_4]$ with the non-toxic 2-thiosalicylic acid ($TSA\text{-}H_2$) ligand)	In vitro	Significantly attenuates the Wnt/beta- catenin signaling at both transcriptomic and proteomic levels; Induce apoptosis, and reduces transcription and expression of nuclear components; head and neck (nasopharyngeal) cancer	[164]
		Phytochemicals: Resveratrol	In vitro and in vivo	Inhibition β-catenin/TCF- mediated transcriptional activity; effects are dose- dependent; reversal of epithelial- mesenchymal transition (EMT); inhibition of	[165–169]

	Targets/Mechanisms	Drugs	Type of study	Results	References
				cancer cell invasiveness: colorectal, melanoma, breast cancer; decrease cancer stem cells (CSC) su cancer cell invasiveness: colorectal, melanoma, breast cancer; decrease cancer stem cells (CSC) su cancer cell invasiveness: colorectal, melanoma, breast cancer; decrease cancer stem cells (CSC) su cancer cell invasiveness: colorectal, melanoma, breast cancer; decrease cancer stem cells (CSC) su cancer cell invasiveness: colorectal, melanoma, breast cancer; decrease cancer stem cells (CSC) su cancer cell invasiveness: colorectal, melanoma, breast cancer; decrease cancer stem cells (CSC) su	
		Phytochemicals: selenium, green tea (Epigallo Catechin Gallate, EGCG), vitamin D	In vitro	Various mechanisms of inhibiting various signaling pathways (including Wnt) and targeting cancer stem cells; ability to cause growth arrest and cell death selectively in cancer cells; inhibit post-initiation cancer development, including self-renewal of cancer stem cells and epithelial-mesenchymal transition (various solid tumors: ovarian, breast, colon cancer, pancreas etc)	[170–171]
		small-molecule drugs that antagonize Wnt/beta-catenin signaling pathway: ICG-001, PKF118-310	In vivo and in vitro	Alter both proliferation and differentiation; loss of self-renewal capacity, decrease chemoresistance, down-regulation of survivin expression levels; reduce tumor growth and overcome tumor relapse; chromatic remodeling; eradicate tumor-initiating cells (breast cancer; acute lymphoblastic leukemia; head and neck cancer)	[172–174]
		small-molecule drugs that inhibit ligand- induced Wnt/β-catenin signaling: Porcupine inhibitor LGK974 (secretion of Wnt protein requires Porcupine, a membrane bound O-acyltransferase dedicated to Wnt posttranslational acylation)	In vitro, in vivo, clinical trial	decrease cell and tumor growth ; decrease expression of Wnt target genes (Axin 2) (pancreatic adenocarcinoma, breast, and head and neck cancer)	[175–178]
	2.2. Shh pathway	Hedgehog pathway inhibitor: IPI-926 (saridegib); compounds and derivatives from natural products	Clinical trial	Eliminate tumors and delays regrowth (head and neck squamous carcinomas)	[179–180]
	2.3. Notch pathway	γ secretase inhibitors (GSI; the GSIs synthesized to date are divided into three classes: peptide isosteres, azepines, and sulfonamides)	Clinical trial in vitro	Effectively block Notch activity by preventing its cleavage at the cell surface; prevent metastasis and recurrence (breast cancer); prevent cell proliferation and tumor necrosis factor (TNF-α)-dependent invasion of head and neck (oral) cancer cells; inhibits cell proliferation by inducing cell cycle arrest and apoptosis, inhibits the AKT and MEK signaling and enhance radio-sensitivity in head and neck (nasopharyngeal) cancer	[181–183]
		Phytochemicals (e.g. Resveratrol); natural products (e.g. curcumin, psoralidin)	In vitro	Reduce cell proliferation and induces apoptosis (T-cell acute lymphoblastic leukaemia, breast and head and neck (esophageal) cancer; induces growth arrest and EMT inhibition in cancer stem cells (breast cancer); down-regulate Notch activating gamma secretase complex proteins (e.g. presenilin) and specific microRNAs (miRNA-21 and -34a), and upregulates tumor suppressor (let-7a miRNA) in head and neck (esophageal) cancer	[184–186]
	2.4. Aldehyde dehydrogenase (ALDH)	ALDH inhibitors: AMPAL and its analogs, Benomyl, Chloral, Chlorpropamide analogs, Citral, Coprine, Cyanamide, Daidzin, Disulfiram, diethylaminobenzaldehyde (DEAB)	In vitro	An increasing body of evidence suggests relationships among the expression of ALDH enzymes, and their cooperation with ABC transporters in the development of drug resistance in various cancers, and their underlying mechanisms are being explored. Various mechanisms of inhibiting ALDH isoforms and ALDH activity; Reduce	[187–190]

	Targets/Mechanisms	Drugs	Type of study	Results	References
				or completely reverse chemotherapy and radiation resistance of cancer stem cells; abolish cancer st	
				or completely reverse chemotherapy and radiation resistance of cancer stem cells; abolish cancer st	
				or completely reverse chemotherapy and radiation resistance of cancer stem cells; abolish cancer st	
		ALDH 1A1-targeted siRNAs	In vivo and in vitro	Sensitize taxane- and platinum-resistant cell lines to chemotherapy; significantly reduce tumor growth; targeting cancer stem cells (ovarian cancer). Knockdown of ALDH1A1 and ALDH3A1 by siRNA decreases clonogenicity and motility, and increases sensitivity to 4-hydroperoxy-cyclophosphamide in non- small cell lung cancer cell lines	[190–191]
C	**Epithelial mesenchymal transition (EMT)-targeting drugs**				
	1. Targeting signaling pathways (Wnt, Shh, Notch)	See part B2.			
	2. Snail inhibitors	Small molecule SNAIL-inhibitor GN-25	In vitro	Transcriptional reversal of the mesenchyal phenotype in cancer stem cells (breast cancer)	[192]
	3. TGFβ inhibitors	TGFβ receptor inhibitors	In Vivo	Inhibit various components of TGFβ pathway (skin and oral squamous cell Carcinoma)	[193]
	4. NF-kB inhibitors	Phytochemicals: Lupeol	In vivo	significant synergistic cytotoxic effect when combined with low-dose cisplatin (head and neck cancer)	[194]
		Phytochemicals: curcumin, resveratrol, ursolic acid, capsaicin, butein (a tetrahydroxychalcone plant polyphenol)	In vitro	Various mechanisms to inhibit activity of IKK, p65 phosphorylation, p65 translocation and DNA binding, or direct effect on cancer stem cells (by reducing ALDH and reducing their spheres-forming capacity through an inhibition of NK-kB signaling (multiple myeloma, prostate, breast, head and neck cancer)	[195, 215]
	5. miRNA therapy	miRNAs: miR200c	In vivo	Inhibit cancer stem cells by down- regulation of BMI1 and ZEB1; inhibits lung metastasis and prolongs survival rate (head and neck carcinoma)	[196]
		miRNA synergistic activators: curcumin	In vitro	Alter the expression miR-203, inhibits proliferation and increases apoptosis (bladder cancer)	[197]
D	**Survival pathways targeting drugs**				
	1. targeting growth factors	**1.1.1. receptor inhibitors**			
		anti-epithelial growth factor receptor (EGFR) monoclonal antibodies: Cetuximab	Clinical trials	In combination with chemo- or radiation therapy showed significant improvements but didn't reduce their toxicity of chemo-radiation (squamous cell carcinoma from oropharynx, hypopharynx and larynx)	[198]
		Vascular Endothelia Growth Factor Receptor (VEGFR) inhibitors: Bevaxizumab	Clinical trial	In addition to chemo-radiation showed delay in progression of the distant disease (nasopharyngeal carcinoma)	[199]
		1.1.2. kinase inhibitors:			

	Targets/Mechanisms	Drugs	Type of study	Results	References
		Tyrosine kinase inhibitors: Erlotinib; Cetiranib (VEGF tyrosine kinase inhibitor)	Clinical trial	Usage in combination with bevacizumab showed significant effect in treating metastatic and recurrent cancer (head and neck squamous cell carcinoma)	[200]
		Dual kinase inhibitors: Lapatinib	Clinical trial	Inhibition of EGFR and EGFR-2 tyrosine kinase. Lapatinib monotherapy showed non-significant difference in locally advanced squamous cell carcinoma of the head and neck	[201]
		Triple kinase [VEGFR, platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR)] inhibitors: Nintedanib	In vitro	Monotherapy and chemotherapy combination reduced proliferation and enhanced apoptosis; antiangiogenic effects (lung and pancreatic cancer)	[202]
		1.1.3. targeting MEK/ERK (EGFR downstream signaling pathway)			
		Raf inhibitors: sorafenib	Clinical trial	Single agent trials showed poor response, but well tolerated and favorable progression-free survival in recurred and metastatic squamous cell carcinoma of the head and neck	[203]
		MEK inhibitors: Trametinib (GSK1120212)	In vitro, in vivo	Showed therapeutic potential for tumors with activating mutations in BRAF. Ongoing clinical trials in head and neck cancer	[204]
	1.2. Targeting PIK3 (Phosphatidylinositol- 3-kinase)/Akt (Protein Kinase B)/ mTOR (MammalianTarget Of Rapamycin) pathways and promote PTEN (Phosphatase and tensin homolog)	AKT inhibitor: Perifosine	Clinical trial	Monotherapy showed poor response in incurable, recurrent or metastatic head and neck cancer	[205]
		PIK3 inhibitors LY294002 (reversible inhibitor), wortmannin (irreversible inhibitor)	In vivo	Inhibit tumor-induced angiogenesis and tumor growth (glioma, prostate cancer); preclinical evaluation in head and neck cancer	[206–207]
		mTOR inhibitors: rapamycin, mTOR kinase inhibitors: Torin1, Torin 2	Clinical trials and preclinical	Studies targeting various solid tumors and cancer stem cells (Lymphoma, glioma); decrease the capacity of sphere formation as well as ALDH activity, suppress the stimulation of stem-like cells by chemotherapy in colorectal cancer; preclinical evaluation in head and neck cancer	[208–209]
		Dual PIK3/mTOR inhibitors: NVP- BEZ235	In vivo and in vitro	Usage in combination with sorafenib inhibits tumor cell proliferation and increases tumor cell apoptosis, to overcome drug resistance in renal cell carcinoma; Phosphatidylinositol-3- phosphate kinase, AKT and dual PI3K- mTOR inhibitors caused marked in vitro enhancement of cytotoxicity induced by HDACIs in head and neck cancer	[209–211]
	1.3. Calcium influx inhibitors	Econazole, Ketotifen, Carboxyamidotriazole	In Vitro	loss of viability and clonogenicity of cancer stem cells (breast cancer); decrease neural stem cell differentiation; inhibition of cell proliferation, migration and chemoinvasion (head and neck cancer)	[212–214]