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# Identity Matters: Tumor cell plasticity in head and neck squamous cell carcinoma

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## Abstract

**Keywords:**

## Introduction

Cellular plasticity is a phenomenon describes the ability of certain cells to adopt different phenotypes and functions, which is considered a characteristic feature of embryonic stem cells. However, such trait has also been observed in adult differentiated cells when challenged by chronic physiological or pathological conditions such as wound repair and tumorigenesis (Yuan et al., 2019). In the context of cancer, cell plasticity endows tumors with enhanced self-renewal and pro-invasiveness capacities. Further, by attaining different identities (phenotypes), tumor cells can bypass cell cycle arrest, apoptosis and circumvent the therapeutic insults (Boumahdi and de Sauvage 2020). Indeed, tumor microenvironment (TME) plays a crucial role in fueling tumor plasticity by exposing tumor cells to a wide variety of stimuli from substantially heterogeneous niche, thereby imposing a significant obstacle in cancer management (Yuan et al., 2019; da Silva-Diz et al., 2018).

Head and neck squamous cell carcinoma (HNSCC) encompasses a group of common and aggressive epithelial tumors with strong associations to smoking, alcohol dependence and infection with human papillomavirus (HPV) types 16 and 18 (Johnson et al., 2020). Recently, oral dysbiosis has been linked to HNSCC plus to certain oral potentially malignant lesions (Metsäniitty et al., 2021). These tumors arise more frequently in the oral cavity as oral SCC (OSCC), but also develop in oropharynx and larynx (Mehanna et al., 2010). Owing to their invasiveness and “metastability”, HNSCCs accounted for nearly one million new cases and over half a million deaths in 2018 alone (Ferlay et al., 2019; Bray et al., 2018). Currently, treatment options include surgery, chemo-radiation, targeted therapy, immunotherapy, or a combination of these modalities. Despite the remarkable improvement in HNSCC management, metastasis and drug resistance remain the main cause of mortality. Although survival can be prolonged with a multimodal approach, this can however induce drug toxicity and deteriorate the patients’ quality of life. Thus, the 5-year survival of these patients remains stagnant at approximately 50% (Johnson et al., 2020; Xing et al., 2021; Muzaffar et al., 2021).

Recent technical advances in single-cell genomics such as single-cell RNA sequencing (scRNA-seq) have revealed the complexity and heterogeneity of HNSCCs, which influence tumor plasticity and clinical response alike. Within such diverse ecosystem, tumor cells exhibit diverse expression of signatures related to cell cycle, stress, epithelial-to-mesenchymal transition (EMT), hypoxia, and epithelial differentiation (Puram et al., 2017). Importantly, TME also comprise a distinct subpopulation of cancer stem (or stem-like) cells (CSCs) with enhanced self-renewal, phenotypic plasticity (differentiation), tumorigenesis and metastability in multiple cancers including HNSCCs (**Figure 1**; Prince et al., 2007; Clay et al., 2010; Biddle et al., 2016). To develop more effective anticancer therapies, it will be critical to understand CSCs and how they can promote carcinogenesis

and multidrug resistance. Herein we review our current understanding of CSCs in HNSCC, highlighting some recently identified mechanisms that mediate their phenotypic plasticity and immune evasion. We also discuss critical factors governing their dynamic interplay within TME, including our evolving appreciation of the contribution of oral microbiota.

## Intratumor heterogeneity in HNSCC

Given the limited success of the traditional anticancer approaches, attention has been focused on identifying the genotype variations between patients to predict their response to targeted drugs. However, TME harbors, within the same patient, subpopulations of tumor cells with different phenotypes and mutations, referred to as intra-tumoral heterogeneity (Bedard et al., 2013). Intratumor heterogeneity drives the clinical resistance and poses a major challenge for designing effective therapies in solid tumors including HNSCC (Canning et al., 2019; Bedard et al., 2013). Initially, two models were proposed to explain the intra-tumoral heterogeneity. On the one hand, the stochastic model of clonal evolution postulates that every tumor cell with an appropriate set of somatic mutations can initiate and sustain a "metastable" tumor growth. However, this model speculates tumors as a homogeneous mass, hence falls short of explaining the variations in tumorigenic potential and multidrug resistance. On the other hand, according to the hierarchical cell model, cancer initiation and progression are driven mainly by a smaller subpopulation of cells—CSCs—that are intrinsically different from the majority, more differentiated, tumor cells (Shackleton et al., 2009; Fanelli et al., 2020).

In essence, CSCs were termed as such to highlight their stem cell-like properties including their self-renewal, transdifferentiation and migration abilities. In this regard, there has been an overwhelming evidence supporting this "stemness" model. CSCs were first characterized as a minority of $CD34^{+ve}/CD38^{-ve}$ cells in acute myeloid leukemia (Lapidot et al., 1994). Thenceforward, CSCs with other surface markers have been identified in different cancers. For instance, $CD44^{+ve}$ and $CD133^{+ve}$ CSCs sustained the capacity to initiate new tumors in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse models of breast and colorectal cancers, respectively (Al-Hajj et al., 2003; O'Brien et al., 2007). The first identification of CSCs in HNSCC was reported by Prince and colleagues, who showed that only $CD44^{+ve}$ cancer cells—comprising <10% of the tumors—initiated new malignant growths in NOD/SCID mice (Prince et al., 2007). Interestingly, as few as $5 \times 10^3$ cells of early-passaged $CD44^{+ve}$ CSCs were able to produce new tumors *in vivo*, whereas $CD44^{-ve}$ cells failed to give rise tumors event at a 100-fold higher density. Of note, $CD44^{+ve}$ cell-derived tumors comprised phenotypically diverse cells of both $CD44^{+ve}/CD44^{-ve}$ clones, suggesting that CSCs may also drive the intra-tumoral heterogeneity in HNSCC patients. These findings signify the role of

CD44 in identifying the CSCs in other tumors of epithelial origin (Prince et al., 2007). Thereupon, several cell surface receptors and intracellular proteins were reported as applicable CSC markers in HNSCC, as summarized in **Table 1**.

## CSC markers in HNSCC

To date, Fluorescence Activated Cell Sorting (FACS) and the magnetic bead sorting are the most commonly applied approaches to detect and isolate CSCs from different tumor types. With these methods, various cell surface markers have been used either individually or in combination to detect HNSCC CSCs. Beside their importance to understand the complex behavior of CSCs, these markers start to emerge as valuable biomarkers and therapeutic targets in HNSCC. Here, we briefly outline recent findings of certain markers that are commonly applied in HNSCC. A list of other potential markers is found in **Table 1**.

**CD44:** The cluster differentiation CD44 is the key stem cell marker in solid tumors and the first used to study HNSCC-derived CSCs. CD44 is a multistructural and multifunctional transmembrane adhesion receptor that binds to several extracellular matrix (ECM) ligands, particularly to hyaluronic acid (HA; aka hyaluronan)—a glycosaminoglycan involved in pivotal tumorigenic events. CD44 mediated cancer cell proliferation, migration, angiogenesis and stemness properties, which ultimately led to tumor progression and metastasis (Ludwig et al., 2019; Hujanen et al., 2021; Boxberg et al., 2018; Wang and Bourguignon 2011). Recently, Ludwig et al. analyzed the association of CD44 with the pro-angiogenic genotype in HNSCC using the Cancer Genome Atlas. Interestingly, they found that HNSCC has the second highest CD44 expression among all cancer types included in the Pan-Cancer Atlas. Moreover, using an orthotopic carcinogen-induced mouse model, $CD44^{+ve}$ expression was consistently upregulated at different stages of oral carcinogenesis, from dysplastic lesions to advanced carcinomas (Ludwig et al., 2019). Using primary human HNSCC samples and patient-derived xenografts, Lee et al. examined the immunogenicity of CSCs. Strikingly, $CD44^{+ve}$ CSCs in HNSCC revealed EMT features and were less immunogenic than other $CD44^{-ve}$ tumor cells when cultured with autologous $CD8^{+ve}$ tumor-infiltrating lymphocytes (TILs). Further, programmed death-ligand 1 (PD-L1) was selectively expressed on $CD44^{+ve}$ CSCs compared with the $CD44^{-ve}$ cells (Lee et al., 2016). In addition, HNSCC-derived spheroids, exhibited increased expression of CD44 whereas the levels of other putative CSC markers, such as CD24 and CD133 were not notably increased (Byun et al., 2022).

Human CD44 is encoded by the highly conserved *CD44* gene on chromosome 11. Following extensive alternative splicing, it generates multiple variant isoforms including the standard (CD44s)

and variant (CD44v) forms, in which the latter represented a promising prognostic and therapeutic target in different cancers. In this regard, Wang and colleagues showed that HNSCC cells (HSC-3) expressed at least four CD44 isoforms (v3, v6, v10) and CD44s. Of note, these CD44 isoforms mediated cancer cell migration, proliferation, and cisplatin sensitivity. Importantly, the variant isoforms (v3, v6, v10), alone or in combination, showed a greater proportion of metastatic lymph nodes and tumor progression compared with the standard form (Wang et al., 2009). A meta-analysis study revealed a significant association between CD44 and worsening T stage, N status, higher tumor grades and 5-year overall survival (OS) rates in patients with HNSCC (Chen et al., 2014). However, in spite of its wide use in CSC studies, the increased levels of $CD44^{+ve}$ cells in some cohorts suggest that such cell subpopulation may not represent a pure mass of CSCs. Thus, combining multiple surface markers such as CD44 and aldehyde dehydrogenase (ALDH; $CD44^{high}/ALDH^{high}$) has been increasingly used for studying CSCs in HNSCCs (Kamarajan et al., 2017).

**ALDH:** This family comprises a group of intracellular detoxifying enzymes that oxidize exogenous and endogenous aldehydes, hence mediating drug resistance in cancer patients (Januchowski et al., 2013). ALDH has been considered as a functional marker for human HNSCC-derived CSCs (Kamarajan et al., 2017; Chen et al., 2009; Clay et al., 2010). Among the first reports on this molecule in HNSCC, Chen et al. showed that $ALDH1^{+ve}$ tumor cells displayed EMT features and radioresistance and represented a reservoir for tumor initiation (Chen et al., 2009). Unlike the copious expression of CD44, most HNSCC cells had low ALDH activity; nevertheless, $ALDH^{high}$ cells (1.0% to 7.8%) clearly co-expressed CD44 and sustained high tumorigenic potential in NOD/SCID mice (Clay et al., 2010). In agreement with these studies, HNSCC $ALDH1^{+ve}$ CSCs exhibited higher cancer stemness properties including tumor spheres-forming capability than that of $ALDH1^{-ve}$ cells (Yu et al., 2011).

Indeed, these findings support the role of ALDH as a selective marker for HNSCC CSCs and hold a therapeutic and prognostic promise. In this regard, targeting ALDH with Aldi-6 (ALDH3A1 inhibitor) sensitized HNSCC cells to cisplatin and reduced tumor growth burden *in vivo* (Kim et al., 2017). Prince et al. presented a feasible approach to prepare $ALDH^{high}$ CSC-based vaccines that can induce anti-HNSCC immunity, implying a clinical utility to treat cancer patients (Prince et al., 2016). Recently, a multifactorial analysis revealed that HNSCC patients with a negative immunoexpression of ALDH1A1 had 5.25 times higher OS compared with the $ALDH1A1^{+ve}$ group ($P = 0.01$). Furthermore, using univariate and multivariate analysis, only ALDH1A1 staining positivity showed a significant effect on OS in HNSCC patients compared with other CSC markers such as CD44 (Szafarowski et al., 2020). Of interest, HNSCC-$ALDH1^{+ve}$ CSCs were found to possess high levels

of the transcriptional repressor Bmi-1, another putative marker of CSCs, which also regulated their stemness and drug resistance (Chen et al., 2009).

**Bmi1:** The B-cell-specific moloney murine leukemia virus insertion site 1 (Bmi1) is a key factor responsible for self-renewal and enrichment of stem cells. In their seminal work on characterizing CSCs in HNSCC, Prince et al. reported a differential expression of Bmi1 in the tumorigenic $CD44^{+ve}$ population, indicating a potential role for this molecule in tumor plasticity (Prince et al., 2007). Interestingly, Bmi1 levels were abnormally upregulated in patients with HNSCC, which correlated positively with chemo- and radioresistance (Vormittag et al., 2009). Thus, these findings have made it an attractive target for CSC examination in HNSCC studies. Recently, a comparative study found that CSC markers Bmi1 and BCL11B were able to discriminate between healthy and HNSCC tissues, whereas ALDH1A1 and CD44 were both expressed to a comparable extent in healthy mucosa and cancerous tissues (Sharaf et al., 2021). Interestingly, $Bmi1^{+ve}$ cells were convincingly found as slow-cycled tumor-initiating CSCs that did not only initiate the tumor, but also mediated cervical lymph node metastasis in mouse model of chemically-induced HNSCC (4-nitroquinoline-1-oxide: 4-NQO). As congruous with CSC features, such $Bmi1^{+ve}$ tumor cells were highly tumorigenic and chemo-resistant, whereas a combination of Bmi1 inhibitor and cisplatin treatment potently inhibited HNSCC (Chen et al., 2017).

Using a multicolor lineage tracing method in 4-NQO-induced mouse model of OSCC, Tanaka and colleagues reported that $Bmi1^{+ve}$ cells could serve as oral CSCs. They showed that $Bmi1^{+ve}$ cells were scattered in the developing tumors, which then proliferated to produce new patches, fueling the tumor growth and maintenance. However, some $Bmi1^{+ve}$ cells remained single and gradually disappeared from the malignant tissue, implying that Bmi1 was also expressed by differentiated cells, which have limited capacity to self-renew and maintain tumor growth (Tanaka et al., 2016). This also signifies the importance of using multiple surface markers for better characterization of CSCs. Importantly, a recent study showed that Bmi1 inhibition not only helped to abolish $Bmi1^{+ve}$ CSCs in HNSCC, but also augmented PD1 blockade by activating tumor cell-intrinsic immunity, which hindered the metastasis and prevented tumor relapse (Jia et al., 2020).

**CSC plasticity—a partial phenotypic transition?**

In addition to the aforementioned patterns of intra-tumoral heterogeneity, a newer more nuanced model was recently proposed as “CSC plasticity”, whereby CSCs reversibly switch between stemness and differentiated cell states. Such transition is mediated by genetic and epigenetic instabilities

together with cues from key processes, particularly EMT and mesenchymal-to-epithelial transition (MET; Ghuwalewala et al., 2016; La Fleur et al., 2012; Biddle et al., 2016; Fanelli et al., 2020).

During embryogenesis, cells undergo a highly dynamic and reversible shift between epithelial and mesenchymal states. When the shift is toward the mesenchymal phenotype, cells undergo EMT and obtain potent migratory and invasive characteristics. The main EMT transcription factors include the Snail family (Snail 1 and 2), the Twist family (Twist 1 and 2), and the ZEB family (ZEB1 and 2) of transcription factors (Yeung and Yang 2017). Opposite to EMT, cells may however start losing these migratory features and shift towards an epithelial state by acquiring junctional attachments and apico-basal polarization—a process referred to as MET (Thiery et al., 2009). Although the role of MET/EMT in cancer plasticity is still under investigation, it is nevertheless widely accepted that much of the intra-tumoral heterogeneity, invasion, metastasis and drug resistance are driven by these processes. CSCs can employ EMT to dissociate from the primary tumors, intravasate into the circulation and initiate new locoregional/distant metastatic colonies. Upon reaching a preferable metastatic niche, cells therein revert to an epithelial state (MET) to terminate migration, promote proliferation and seed new heterogeneous tumor colonies (Nieto et al., 2016).

In HNSCC, CSCs were mainly localized to the tumor invasive front, wherein both EMT and metastasis are executed, in contrast to the upper layers of the tumor bulk which remains largely epithelial (Sterz et al., 2010). Consistently, Chowdhury et al. showed that the tumor leading edges in a subset of OSCC were enriched by $CD44^{high}$/$ALDH^{high}$ CSCs that demonstrated greater proliferative and invasive activities. Notably, $CD44^{high}$ CSCs from HNSCC tumors revealed clear EMT-related features including migration and invasiveness. Further, $CD44^{high}$ cells formed bilateral lung metastases in NOD-SCID mice, in contrast to $CD44^{low}$ which failed to generate similar metastatic growths (Davis et al., 2010). When analyzed the expression of EMT-associated genes in twenty-five HNSCC-derived cell lines, Johansson et al. found that EMT program-expressing cells were $CD44^{high}$ with an enhanced motility. Moreover, the expression of Twist1, a key inducer of EMT, was correlated with radioresistance in HNSCC (Johansson et al., 2016). Of interest, such $CD44^{high}$, EMT-expressing cells had low levels of the epidermal growth factor receptor—a pattern associated with stemness (Johansson et al., 2016; La Fleur et al., 2012). In support of these reports, Twist1 directly regulated the expression of the potential CSC marker Bmi1. Furthermore, Twist1 and Bmi1 were mutually essential to promote EMT and tumour-initiating capability and associated with unfavorable clinical outcomes in HNSCC (Yang et al., 2010). In OSCC, EMT characteristics such as ZEB1 overexpression and the loss of E-cadherin were remarkably higher in $CD44^{high}$/$CD24^{low}$ CSCs, signifying the fact that tumor cell stemness concur with EMT in HNSCC (Ghuwalewala et al. 2016). Recently, 16 canonical EMT markers were surveyed in pan-cancer cohort collected from various

tumors, which confirmed the presence of EMT features in HNSCC patients (Gibbons and Creighton 2018).

Recent emerging evidence has shown that tumor cells do not necessarily undergo a complete phenotypic transition. Intriguingly, studies on various cancer types indicated that tumor cells tend to execute partial (p-EMT) or hybrid EMT by concurrently bearing epithelial and mesenchymal phenotypes (Bakir et al., 2020). Supporting the "CSC plasticity" model, transcriptional profiles of ~6000 single cells from HNSCC patients revealed that pEMT-expressing cancer cells were localized to the leading edge of the primary tumors (Puram et al., 2017). Interestingly, these cells were in proximity with cancer-associated fibroblasts and their pEMT program was concluded as an independent predictor of nodal metastasis, grade, and adverse pathologic features in patients with HNSCC (Puram et al., 2017). In this regard, tumor-initiating cells can enhance their mesenchymal gene repertoire at the tumoral front to invade and disseminate distally; while clearly maintaining certain epithelial characteristics to reseed and establish new metastatic growths. Such plasticity is influenced by many TME-related factors including hypoxia (Mimeault et al., 2013).

**Role of hypoxic TME**

Hypoxia, either persistent or temporary, is a hallmark of most solid tumors. Accumulating experimental data suggests hypoxia as a crucial inducer of EMT and CSC plasticity, wherein hypoxia-inducible factors (HIFs) associate with tumor cell stemness, metastasis, angiogenesis and drug resistance (Mimeault et al., 2013). Of note, recent studies indicated that CSCs favor hypoxic niches for their growth and maintenance of stemness (Lee and Simon 2012). Like other solid tumors, hypoxia ensues in HNSCC when the blood supply becomes insufficient due to tumor growth, vascular disturbances or metabolic stress. Importantly, hypoxia had anti-apoptotic effects, predicted poor therapeutic response and OS, correlated with tumor aggressiveness and promoted the formation of functional tumor cell invadopodia in HNSCC (Sasabe et al., 2005; Moreno Roig et al., 2018; Beasley et al., 2002; Díaz et al., 2013; Wiechec et al., 2022).

The tumorigenic and pro-stemness effects of hypoxia could in part be mediated through EMT. In this context, co-expression of hypoxic and EMT markers (Twist 2/Snip1 and HIF-1α, respectively) served as an independent prognosticator for both OS and disease-free survival in patients with tongue SCC (TSCC; Liang et al., 2011). Furthermore, hypoxia induced the expression of key transcriptional factors regulating EMT and promoted pulmonary metastasis in OSCC (Huang et al., 2009; Wiechec et al., 2022). A recent study found that hypoxia-related genes were enriched in $CD44^{+ve}$ CSCs from patients with HNSCC. Interestingly, functional assays indicated that HIF-1α promoted stemness, drug resistance and EMT in these $CD44^{+ve}$ CSCs. Noteworthy, inhibition of HIF1α-driven pathways

reversed the CD44-mediated chemoresistance *in vivo*, implying new therapeutic opportunities in HNSCC (Byun et al., 2022).

Collectively, these reports denote that hypoxia is not only a promoter of tumor stemness and EMT but can also regulate the therapeutic response to anticancer agents. Additional evidence on the impact of hypoxia on tumorigenesis and stemness can be found in these detailed reviews (Yeo et al., 2016; Mimeault and Batra 2013; Lee and Simon 2012). Another important aspect of hypoxia-induced stemness is the induction of a new pattern of tumor vasculature, termed vascular mimicry (VM), which will be discussed in the next section.

**Plasticity underlies tumor cell mimicry**

*i. Endothelial-like cell mimicry*

Angiogenesis is a key hallmark of cancer. However, the limited success of antiangiogenic strategies remains a daunting challenge in treating many solid tumors including HNSCCs (Ribatti et al., 2019). In fact, despite the growing number of clinical trials, targeting the classical angiogenic pathways (e.g. vascular endothelial growth factor, VEGF) showed a modest improvement in the OS of cancer patients. In addition, inhibiting the VEGF pathway has been associated with persistent tumor invasiveness, increased distant metastasis and worsening treatment outcome (Ebos et al., 2009; Pàez-Ribes et al., 2009). Questions have therefore emerged, not only how tumor cells can survive such harsh hypoxic conditions but also how they become more “metastable” and resistant following the treatment with angiogenic inhibitors.

Tumor vasculature has long been assumed to arise from preexisting endothelial cells (ECs). However, a significant number of studies suggest that CSCs utilize their plasticity to acquire differentiation programs of distinct cell types, including EC-like phenotype (Seftor et al., 2012). Indeed, the CSC marker CD44 has been implicated in promoting several tumor proangiogenic events (Wang and Bourguignon 2011). In this regard, VM represents an alternative pattern of tumor microcirculation, in which aggressive tumor cells mimic ECs by initiating perfusable networks of vessel-like structures *in vitro* (Salem and Salo 2021). Notably, tumor cells capable of VM share considerable molecular similarity with that of CSCs (Seftor et al., 2012; Fan et al., 2013). For instance, up to 90% of CSCs were able to transdifferentiate into functional ECs in glioblastoma, the most common and aggressive brain tumor, implying that a significant part of tumor vasculature has a neoplastic origin (Ricci-Vitiani et al., 2010). Recently, we showed that metastatic OSCC cells co-express the endothelial marker CD31 *in vitro*. Furthermore, tissue samples from OSCC patients revealed distinct $CD31^{+ve}$ mosaic VM lumens that also contained red blood cells (Almahmoudi et al., 2021; Hujanen et al., 2021). Interestingly, and unlike VM-free regions, such VM-competent cells

display $CD44^{high}$/E-$cadherin^{low}$ phenotype, denoting a common stemness-related state (Hujanen et al., 2021).

Beside angiogenesis, tumor lymphatics play a pivotal role in cancer progression. In a recent study on basal-like breast cancer, CSCs were capable to undergo lymphatic cell differentiation via the activation of VEGF-C pathway, by which lymphatic vessel-like channels were initiated. Further *in vivo* and *in vitro* experiments revealed that lymphangiogenic mimicry (LM) served as conduits for CSCs to the lymphatic vessels and accelerated lymphatic metastases (Wang et al., 2018). Our group has recently demonstrated that the lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), a homologue of the CSC-marker CD44, is expressed by OSCC cells and promoted their dissemination and vessel-like formation (Karinen et al., 2021). Notably, patients with tumors expressing high levels of LYVE-1 tend to suffer more lymph node metastasis and worse survival (Karinen et al., 2021; Karinen et al., 2022). Of interest, LYVE-1 is a receptor for HA—a key component of ECM that influences, directly and indirectly, the self-renewal and maintenance of CSCs (Chanmee et al., 2015). Thus, targeting LYVE-1 or VEGF-C pathways (or both) may represent a novel therapeutic approach to conquer CSC-driven lymphatic metastasis in HNSCC. Notwithstanding, the potential clinical applications of LM remain unclear and warrant further in-depth investigations.

*ii. Immune and neuronal cell mimicry*

In addition to the vascular cell phenotype, several recent studies uncovered more intriguing differentiation programs of CSCs including the acquisition of immune and neuronal cell states to facilitate tumor progression (Gangoso et al., 2021; Gao et al., 2021; Zeng et al., 2019). However, a conventional bulk transcriptomic profiling can be ineffective in distinguishing such tumor-intrinsic programs from other TME-infiltrating cells.

Based on scRNA-seq datasets and experimental models, an elevated immune-like transcriptional program was recently seen in cells of multiple cancer types, which was progressively acquired during the course of malignant transformation. Specifically, an enrichment of B cell, T cell and myeloid cell signatures was revealed in tumor cells from lung, breast, kidney, and pancreatic cancers. The score was, however, lower in tumor cells than in immune cells, implying a partial acquisition of this immune mimicry during progression from normalcy to neoplasm (Gao et al., 2021). Of note, such mimetic profile might adversely influence the prognostic value of immune cell signatures in cancer studies. Hence, through the exclusion of tumor cell-related immune genes, a new optimized immune response signature was proposed, which offered more reliable prognostic estimates (Gao et al., 2021). These findings are consistent with a recent work showing a striking gain in the immune evasion capabilities of CSCs following the acquisition of a transcriptional module that

hampered the immune response in glioblastoma multiforme (Gangoso et al., 2021). In primary HNSCC, Lee at al. provided a mechanism by which long-lived $CD44^{+ve}$ CSCs can selectively evade host immune responses. Interestingly, $CD44^{+ve}$ cells expressed higher levels of PD-L1 compared with the $CD44^{-ve}$ cells, which was associated with constitutive phosphorylation of STAT3 and decreased immunogenicity. Importantly, PD-1/PD-L1 blockade partially reversed the weakened immunogenicity and activated the TILs response against $CD44^{+ve}$ CSCs (Lee et al., 2016). Indeed, these data highlight the complex genetic and phenotypic changes of CSCs and their infinite ability to hijack the immune response and remodel TME. Although this is still a new area of research, it will be paramount to investigate whether the immune mimicry is implicated in HNSCC, wherein mechanisms underpinning its immunosuppressive TME remain an enigma.

In order to survive and initiate metastatic colonization in the brain, tumor cells must render their new neural niche to an amenable microenvironment. In this context, Neman et al. showed that $HER2^{+ve}$, breast-to-brain metastatic (BBM), CSCs can attain neuronal phenotype by expressing and metabolizing Gamma-aminobutyric acid (GABA)—the chief inhibitory neurotransmitter in mammalian brains. GABA was utilized by tumor cells to promote their proliferation and GABAergic neuronal properties. Further, BBM cells expressed Reelin, a critical neuronal glycoprotein for brain development, which was directly associated with HER2 (Neman et al., 2014). Supporting these observations, a recent preprint (not peer-reviewed) study showed that small-cell lung cancer (SCLC) cells undergo a neuronal mimicry—a transition towards neuronal phenotype during tumor progression and metastasis. More importantly, this neuronal mimicry was critical for establishing SCLC growth in the brain, whereby tumor cells secrete neuronal pro-survival factors (e.g. Reelin) to recruit astrocytes and promote brain metastases (Fangfei et al., 2021). In essence, although brain metastasis in HNSCC is a rare event comprising <1% of all cases (Barrett et al., 2018), these reports further assert the concept of CSCs and urge the need for more studies in this exciting area that has much more to reveal.

## Oral microbiota and CSCs: Emerging evidence

The “polymorphic microbiome” has recently been proposed as a new hallmark of cancer (Hanahan 2022). Indeed, dysbiosis of the commensal microbiota can contribute, both positively and negatively, to the initiation and progression of cancer and, through immunomodulation, to anticancer drug response. Recent research has now advanced our understanding of the interactions between microbiota and tumor cells and there is a growing appreciation of their potential to transform treatment strategies for cancer patients. This review briefly outlines a few examples of microbiota commonly involved with HNSCC, to then focus on their possible role in tumor plasticity.

Oral cavity hosts diverse microbial communities inhabiting different sites such as teeth, saliva, tongue and other mucosal surfaces. Using germ-free animal models, particular microorganisms, chiefly but not exclusively bacteria, were shown to impact key features associated with tumor cell stemness such as EMT, proliferation, migration and invasion. Among these, two prominent oral bacteria, *Porphyromonas gingivalis* (*P. gingivalis*) and *Fusobacterium nucleatum* (*F. nucleatum*) influenced oral carcinogenesis and/or cancer progression. For example, infection with *P. gingivalis*, a major anaerobic Gram-negative periodontopathogen, induced EMT-driven morphological changes in OSCC cells and augmented their migratory and invasive capacities. Importantly, *P. gingivalis*-infected tumor cells exhibited evident plasticity features including the upregulation of CSC markers (CD44 and CD133) and chemotherapeutic resistance (Ha et al., 2015). Using a murine model of periodontitis-associated oral TSCC (OTSCC), infection with both *P. gingivalis* and *F. nucleatum* markedly enhanced the tumors' size and invasiveness. Furthermore, administration of these periodontopathogens significantly induced the activation of Signal Transducer and Activator of Transcription 3 (STAT3)—an important regulator of CSCs in HNSCC (Binder Gallimidi et al., 2015). In agreement, exposure to *P. gingivalis*, *F. nucleatum* or *E. coli*-derived lipopolysaccharide resulted in a strong upregulation of EMT transcripts (vimentin, Snail, and Twist) in OSCC cells. In contrast, the epithelial adhesion molecule, E-cadherin, was downregulated, implying shifting towards a mesenchymal state. Of interest, infected tumor cells showed morphological changes resembling that of fibroblasts, while unstimulated cells maintained a classical epithelial cobble-stone morphology (Abdulkareem et al., 2018).

Epstein–Barr virus (EBV) is a human herpesvirus that is associated with several malignancies including the nasopharyngeal carcinoma (NPC). Lun et al. (2012) found that CSC markers (CD44 and SOX2) were overexpressed in a minor population of $EBV^{+ve}$ NPC cells. Notably, these CSCs were resistant to chemotherapy and showed high spheroid formation efficiency. Consistent with CSC properties, authors revealed that the sorted $CD44^{+ve}$ cells generated heterogeneous population of both $CD44^{+ve}$ and $CD44^{-ve}$ cells (Lun et al., 2012). The exact mechanisms by which EBV, and other pathogenic microbiota, induce tumor cell stemness are still being elucidated. For instance, the latent membrane protein 1 (LMP1) constitutes a key oncoprotein of EBV. Interestingly, Kondo et al. (2011) revealed that EBV instigates the development of $CD44^{high}/CD24^{low}$ phenotype of CSCs and cancer progenitor cells in NPC via LMP1. Moreover, LMP1 induction in nasopharyngeal epithelial cells resulted in high tumorigenicity, rapid proliferation and enhanced self-renewal abilities. Morphologically, LMP1-expressing cells changed into fibroblast-like, spindle-shaped cells.

HPV-induced oropharyngeal HNSCC represents one of the most rapidly increasing cancers in high-income countries which also affects younger individuals (Lechner et al., 2022). Despite the

evident prognostic value of HPV status in HNSCC patients, its impact on CSCs and tumor cell stemness remains poorly understood and requires more research. Tang et al. (2013) showed that HPV status in HNSCC does not correlate with the proportion of $ALDH^{high}$ CSCs since their proportion was not significantly different between the $HPV^{+ve}$ and $HPV^{-ve}$ cell lines. On contrary, another study showed that $HPV16^{+ve}$ HNSCC had a 62.5-fold greater intrinsic CSC pool than $HPV^{-ve}$ cells (Zhang et al., 2014). Further, transfecting $HPV^{-ve}$ tumor cells with HPV16 genome enhanced CSC features including ALDH activity, tumor growth, migration/invasion and self-renewal capacity (Lee et al., 2015). In fact, it is difficult to explain how $HPV^{+ve}$ tumors—characterized by substantially better prognosis and outcome—harbor higher levels of CSCs, which render tumors more aggressive and drug resistant. Nevertheless, more recent studies suggested that HPV status in HNSCC could impact CSC populations after initiating the therapeutic regimen. In this sense, Reid et al. (2020) demonstrated that $HPV^{-ve}$ tumors had significant elevations in $CD44^{+ve}/ALDH^{+ve}$ CSCs following irradiation, with more increasing escalation, compared to the $HPV^{+ve}$ cell lines.

Collectively, although these illustrative examples are rather limited and comprise small sample sizes, they encourage further research to uncover the relationship between oral microbiota and CSCs and how it can influence carcinogenesis in the head and neck tissues.

**A need for new models?**

Besides the *in vivo* models, the characteristics of CSCs are traditionally assessed using plentiful three-dimensional (3D) techniques (comprehensively reviewed in: Langhans 2018; Zhang et al., 2020). Among these, the sphere (or spheroid-like) formation assay is commonly used to assess the ability of CSCs to grow in extremely low attachment/serum conditions and form tumorspheres. These tumorspheres are also termed “orospheres” when formed by HNSCC CSCs, indicating their origin from oral cavity and head and neck tumors (Krishnamurthy and Nör 2013). Typically, tumorspheres are generated using scaffold-free (e.g., Hanging drop cultures and low adhesion plates) and scaffold-based models. The latter scaffold-based 3D cultures provide more physiologically relevant conditions, particularly when incorporating biologically-derived hydrogels such as collagen, fibrin or Matrigel. These matrices facilitate cell-to-cell and cell-to-ECM interactions, thereby allowing more reliable assessment of CSC tumorigenicity, stemness and drug response including possible chemo-/radioresistance. In particular, Matrigel, a matrix secreted by Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells, has been broadly used to assess CSC features (e.g., invasion) due to its high content of ECM proteins, growth factors and proteoglycans (Zhang et al., 2020; Langhans 2018; Aramini et al., 2022). However, animal-derived matrices such as Matrigel exhibit several limitations, making them suboptimal for human CSC research, including non-human origin,

inter-patch variations, and the presence of several undefined factors (Pamarthy and Sabaawy 2021). Therefore, the safety and efficacy of 85% of the drugs identified in such models are not translated to early clinical trials (Ledford 2011).

In order to provide a reliable alternative to animal-derived matrices, our group has developed "Myogel", a matrix isolated from human leiomyoma that provides a physiologically relevant 3D milieu for *ex vivo* cancer modelling. Although its proteome is substantially different from other EHS-based matrices, yet, Myogel comprises key ECM proteins such as laminin, collagen (types IV, XII and XIV), tenascin-C, heparan sulfate proteoglycans, nidogen and epidermal growth factor (Salo et al., 2015). Indeed, balance in the physiological pH environment is important to the regulation and maintenance of stem cell activities including proliferation, viability and differentiation (Teo et al., 2014). In this context, Myogel pH is neutral and more stable during cell culture than that of Matrigel, hence providing a good control of CSC experiments. Importantly, key CSC-related properties were more efficiently represented in Myogel, including tumor cell migration, invasion and response to targeted anti-HNSCC therapy (Salo et al., 2015; Tuomainen et al., 2019). Furthermore, primary HNSCC cell lines showed a greater tendency to form VM in Myogel, whereas human endothelial cells formed consistent and dense tubes throughout the matrix, suggesting that ECM is an important modulator of the tumor cell-derived tubulogenesis (Hujanen et al., 2021). Thus, when selecting a 3D scaffold for CSC studies, it is important to consider matrices that sustain stemness traits and faithfully recapitulate the structural and molecular features of human TME.

Tumor-initiating capacity is considered to be the gold standard for identifying and confirming CSC-like cells. Currently, most *in vivo* studies of CSCs rely upon tumor engraftment into an immunocompromised mouse model—typically the NOD/SCID mice. These mice are B cell- and T cell-deficient model and exhibit defective activity of dendritic cells, macrophages and natural killer cells, thus enhancing the chances of successful xenotransplantation (Skidan and Steiniger 2014). However, in addition to cost and labor challenges, these traditional models do not reliably represent the complex TME interactions in the patients including the lack of specific cytokines necessary for the activities of CSCs. To overcome such constraints, Morton et al. (2016) developed a humanized xenochimeric mouse model of HNSCC (XactMice) comprising human hematopoietic stem and progenitor cells (HSPCs) that can home into the transplanted tumor and replicate its natural TME. Following the harvesting of HSPCs from either donated cord blood or adult peripheral blood, cells were expanded *ex vivo* and injected into sub-lethally irradiated NOD/SCID gamma mice to generate XactMice, wherein HNSCC cells were later engrafted. Interestingly, XactMice tumors showed epithelial, stromal and immune gene signature that aligns more closely to the original TME observed clinically—an effect partly mediated by HSPC-/tumor cell-driven cytokines (Morton et al., 2016).

Additionally, the same group has developed a dual infusion protocol of HSPCs and mesenchymal stem cells, resulting in higher degrees of “humanization” of the HNSCC mouse model. This includes incremental human bone marrow engraftment, excessive increase in human immune cells and intratumor homing with better lineage reconstitution. This optimized model is more closely resembling that of the originating patient's tumor, suggesting an enhanced capability to accurately recapitulate a human TME (Morton et al., 2018).

Another potential *in vivo* model for CSC research is Zebrafish, which has been increasingly used as an anticancer screening platform. Recently, our group has utilized zebrafish larvae as xenograft model of human HNSCC to evaluate tumor cell metastasis, VM formation and personalized drug response (Karinen et al., 2021; Hujanen et al., 2021; Wahab et al., 2021; Al-Samadi et al., 2019). Indeed, zebrafish has several advantages over murine models considering its efficiency, feasibility plus to the associated cost- and labor-effectiveness. However, similar to mice, zebrafish model is devoid of critical cytokines, chemokines and other TME factors released in human patients. To alleviate this limitation, Rajan and colleagues (2020) pioneered the first “humanized” zebrafish that expresses multiple human hematopoietic-specific cytokines. By transplanting primary HSPCs and leukemia cells, they showed that their model exhibits hematopoietic niche homing that more precisely represent the behavior of human leukemia. Further, such humanized zebrafish promoted survival, self-renewal and multi-lineage differentiation of HSPCs (Rajan et al., 2020). Although this platform has not yet been harnessed for HNSCC CSCs, the findings pave the way for a new modelling paradigm in humanized animal-based research.

Taking together, developing physiologically relevant *ex vivo* and *in vivo* models that closely recapitulate CSCs’ niche and enable their characterization and therapeutic targeting will definitely facilitate the translation of basic discoveries to the clinical practice in a timely manner. Such approaches are paramount for the development of novel drugs that can selectively target and destroy CSCs.

## Conclusions and future perspectives

HNSCCs encompass highly heterogeneous tumors, wherein CSCs seem to play pivotal and multifaceted roles in their initiation, progression, metastasis, clinical resistance and possible relapse. Recent technological advances, such as scRNA-seq and tumor modelling, have led to unprecedented understanding of CSCs and their intricate interplay with other TME factors. However, much work remains to be undertaken before this understanding can be translated into successful anticancer therapies, since the prognosis for HNSCC patients remains generally dismal. To this end, several areas need to be further elucidated including, inter alia, pathways involved in CSC signaling including

with local microbiota; the molecular underpinning of CSC-driven drug resistance; and immune cell mimicry. Another challenge is to developing better cancer models for CSC research. Despite the tremendous progress brought by humanized animal platforms, yet they remain relatively immature with several shortcomings. For instance, besides cost and labor intensiveness, the possibility to develop xenogenic graft versus host disease (GVHD) mediated by engrafted human T-cells is a major obstacle in such models. GVHD not only affects the number of viable experimental animals but also complicates data interpretation by overlaying a second set of diseases (Greenblatt et al., 2012). Nevertheless, the humanized animal models remain a promising approach with a high potential for improvement to catalogue the diverse range of cellular activities in head and neck carcinogenesis.

Lastly, but importantly, we need to identify specific (i.e. exclusive) markers for CSCs in HNSCC. To date, there are no available universal markers that can label CSCs alone. On contrary, many of the current markers are also expressed by other cell types such as normal stem cells. Thus, combining different putative labels of CSCs is recommended. Importantly, novel therapies should be carefully designed to exclusively targeting this subpopulation of tumor cells and to minimize possible elimination of tissue stem cells or disrupting vital functions (Skidan and Steiniger 2014; Prince et al., 2016). Additionally, anti-cancer vaccines targeting CSCs through dendritic cells and other immune cell types showed promising results in HNSCC and could be further elaborated in future (Prince et al., 2016). In sum, tumor cell plasticity and CSCs represent an exciting and expanding battleground with great implications for cancer therapy that are only beginning to be appreciated in head and neck oncology.

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**Conflict of interest**

The authors declare no conflict of interest.

**Figure legend**

**Figure 1. Cancer Stem Cells (CSCs) in head and neck squamous cell carcinomas**. CSCs are a small subpopulation of tumor cells which exhibit the following main capabilities: **a)** self-renewal; **b)** differentiation into multiple types of cancer cells; and **c)** tumor-initiation (tumorigenesis) when transplanted into an animal host.

**Figure 2. Examples of models used to assess cancer stem cells (CSCs) features. a)** Ex vivo models are broadly categorized to scaffold-free cultures (e.g. low attachment plates and hanging drops) and scaffold-based models (e.g. Matrigel and Myogel) that used to assess CSC features such as tumorspheres formation and invasion. **b)** In vivo models are mainly used to assess the capacity of CSC-like cells to initiate new tumors (tumorigenicity and metastability) using different models, particularly non-obese diabetic/severe combined immunodeficient mice and humanized models.

## References


Abdulkareem AA, Shelton RM, Landini G, Cooper PR, Milward MR. Periodontal pathogens promote epithelial-mesenchymal transition in oral squamous carcinoma cells in vitro. Cell Adh Migr. 2018 Mar 4;12(2):127-137. doi: 10.1080/19336918.2017.1322253.

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. 2003 Apr 1;100(7):3983-8. doi: 10.1073/pnas.0530291100. Epub 2003 Mar 10. Erratum in: Proc Natl Acad Sci U S A. 2003 May 27;100(11):6890.

Almahmoudi R, Salem A, Hadler-Olsen E, Svineng G, Salo T, Al-Samadi A. The effect of interleukin-17F on vasculogenic mimicry in oral tongue squamous cell carcinoma. Cancer Sci. 2021 Jun;112(6):2223-2232. doi: 10.1111/cas.14894.

Al-Samadi A, Tuomainen K, Kivimäki A, Salem A, Al-Kubati S, Hyytiäinen A, Parikka M, Mesimäki K, Wilkman T, Mäkitie A, Grenman R, Salo T. PCR-based zebrafish model for personalised medicine in head and neck cancer. J Transl Med. 2019 Jul 22;17(1):235. doi: 10.1186/s12967-019-1985-1.

Aramini B, Masciale V, Grisendi G, Bertolini F, Maur M, Guaitoli G, Chrystel I, Morandi U, Stella F, Dominici M, Haider KH. Dissecting Tumor Growth: The Role of Cancer Stem Cells in Drug Resistance and Recurrence. Cancers (Basel). 2022 Feb 15;14(4):976. doi: 10.3390/cancers14040976.

Bakir B, Chiarella AM, Pitarresi JR, Rustgi AK. EMT, MET, Plasticity, and Tumor Metastasis. Trends Cell Biol. 2020 Oct;30(10):764-776. doi: 10.1016/j.tcb.2020.07.003.

Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Biol. 1999 Feb 22;144(4):789-801. doi: 10.1083/jcb.144.4.789.

Barrett TF, Gill CM, Miles BA, Iloreta AMC, Bakst RL, Fowkes M, Brastianos PK, Bederson JB, Shrivastava RK. Brain metastasis from squamous cell carcinoma of the head and neck: a review of the literature in the genomic era. Neurosurg Focus. 2018 Jun;44(6):E11. doi: 10.3171/2018.2.FOCUS17761.

Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K, Millard P, Fuggle S, Harris AL. Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. Cancer Res. 2002 May 1;62(9):2493-7.

Bedard PL, Hansen AR, Ratain MJ, Siu LL. Tumour heterogeneity in the clinic. Nature. 2013 Sep 19;501(7467):355-64. doi: 10.1038/nature12627.

Biddle A, Gammon L, Liang X, Costea DE, Mackenzie IC. Phenotypic Plasticity Determines Cancer Stem Cell Therapeutic Resistance in Oral Squamous Cell Carcinoma. EBioMedicine. 2016 Jan 9;4:138-45. doi: 10.1016/j.ebiom.2016.01.007.

Binder Gallimidi A, Fischman S, Revach B, Bulvik R, Maliutina A, Rubinstein AM, Nussbaum G, Elkin M. Periodontal pathogens Porphyromonas gingivalis and Fusobacterium nucleatum promote tumor progression in an oral-specific chemical carcinogenesis model. Oncotarget. 2015 Sep 8;6(26):22613-23. doi: 10.18632/oncotarget.4209.

Boumahdi S, de Sauvage FJ. The great escape: tumour cell plasticity in resistance to targeted therapy. Nat Rev Drug Discov. 2020 Jan;19(1):39-56. doi: 10.1038/s41573-019-0044-1.

Boxberg M, Götz C, Haidari S, Dorfner C, Jesinghaus M, Drecoll E, Boskov M, Wolff KD, Weichert W, Haller B, Kolk A. Immunohistochemical expression of CD44 in oral squamous cell carcinoma in relation to histomorphological parameters and clinicopathological factors. Histopathology. 2018 Oct;73(4):559-572. doi: 10.1111/his.13496.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018 Nov;68(6):394-424. doi: 10.3322/caac.21492.

Byun JY, Huang K, Lee JS, Huang W, Hu L, Zheng X, Tang X, Li F, Jo DG, Song X, Huang C. Targeting HIF-1α/NOTCH1 pathway eliminates CD44+ cancer stem-like cell phenotypes, malignancy, and resistance to therapy in head and neck squamous cell carcinoma. Oncogene. 2022 Feb;41(9):1352-1363. doi: 10.1038/s41388-021-02166-w.

Canning M, Guo G, Yu M, Myint C, Groves MW, Byrd JK, Cui Y. Heterogeneity of the Head and Neck Squamous Cell Carcinoma Immune Landscape and Its Impact on Immunotherapy. Front Cell Dev Biol. 2019 Apr 9;7:52. doi: 10.3389/fcell.2019.00052.

583 Cao G, Savani RC, Fehrenbach M, Lyons C, Zhang L, Coukos G, Delisser HM. Involvement of endothelial
584 CD44 during in vivo angiogenesis. Am J Pathol. 2006 Jul;169(1):325-36. doi:
585 10.2353/ajpath.2006.060206. Erratum in: Am J Pathol. 2006 Nov;169(5):1899.
586 Chanmee T, Ontong P, Kimata K, Itano N. Key Roles of Hyaluronan and Its CD44 Receptor in the Stemness
587 and Survival of Cancer Stem Cells. Front Oncol. 2015 Aug 10;5:180. doi: 10.3389/fonc.2015.00180.
588 Chen D, Wu M, Li Y, Chang I, Yuan Q, Ekimyan-Salvo M, Deng P, Yu B, Yu Y, Dong J, Szymanski JM,
589 Ramadoss S, Li J, Wang CY. Targeting BMI1+ Cancer Stem Cells Overcomes Chemoresistance and
590 Inhibits Metastases in Squamous Cell Carcinoma. Cell Stem Cell. 2017 May 4;20(5):621-634.e6. doi:
591 10.1016/j.stem.2017.02.003.
592 Chen D, Wu M, Li Y, Chang I, Yuan Q, Ekimyan-Salvo M, Deng P, Yu B, Yu Y, Dong J, Szymanski JM,
593 Ramadoss S, Li J, Wang CY. Targeting BMI1+ Cancer Stem Cells Overcomes Chemoresistance and
594 Inhibits Metastases in Squamous Cell Carcinoma. Cell Stem Cell. 2017 May 4;20(5):621-634.e6. doi:
595 10.1016/j.stem.2017.02.003.
596 Chen J, Zhou J, Lu J, Xiong H, Shi X, Gong L. Significance of CD44 expression in head and neck cancer: a
597 systemic review and meta-analysis. BMC Cancer. 2014 Jan 13;14:15. doi: 10.1186/1471-2407-14-15.
598 Chen YC, Chen YW, Hsu HS, Tseng LM, Huang PI, Lu KH, Chen DT, Tai LK, Yung MC, Chang SC, Ku
599 HH, Chiou SH, Lo WL. Aldehyde dehydrogenase 1 is a putative marker for cancer stem cells in head
600 and neck squamous cancer. Biochem Biophys Res Commun. 2009 Jul 31;385(3):307-13. doi:
601 10.1016/j.bbrc.2009.05.048.
602 Chowdhury FN, Reisinger J, Gomez KE, Chimed TS, Thomas CM, Le PN, Miller B, Morton JJ, Nieto CM,
603 Somerset HL, Wang XJ, Keysar SB, Jimeno A. Leading edge or tumor core: Intratumor cancer stem
604 cell niches in oral cavity squamous cell carcinoma and their association with stem cell function. Oral
605 Oncol. 2019 Nov;98:118-124. doi: 10.1016/j.oraloncology.2019.09.011.
606 Chung MK, Jung YH, Lee JK, Cho SY, Murillo-Sauca O, Uppaluri R, Shin JH, Sunwoo JB. CD271 Confers
607 an Invasive and Metastatic Phenotype of Head and Neck Squamous Cell Carcinoma through the
608 Upregulation of Slug. Clin Cancer Res. 2018 Feb 1;24(3):674-683. doi: 10.1158/1078-0432.CCR-17-
609 0866.
610 Clay MR, Tabor M, Owen JH, Carey TE, Bradford CR, Wolf GT, Wicha MS, Prince ME. Single-marker
611 identification of head and neck squamous cell carcinoma cancer stem cells with aldehyde
612 dehydrogenase. Head Neck. 2010 Sep;32(9):1195-201. doi: 10.1002/hed.21315.
613 Coletta RD, Yeudall WA, Salo T. Grand Challenges in Oral Cancers. Front Oral Health. 2020 Jun 9;1:3. doi:
614 10.3389/froh.2020.00003.
615 da Silva-Diz V, Lorenzo-Sanz L, Bernat-Peguera A, Lopez-Cerda M, Muñoz P. Cancer cell plasticity: Impact
616 on tumor progression and therapy response. Semin Cancer Biol. 2018 Dec;53:48-58. doi:
617 10.1016/j.semcancer.2018.08.009.
618 Davis SJ, Divi V, Owen JH, Bradford CR, Carey TE, Papagerakis S, Prince ME. Metastatic potential of cancer
619 stem cells in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg. 2010
620 Dec;136(12):1260-6. doi: 10.1001/archoto.2010.219.
621 Díaz B, Yuen A, Iizuka S, Higashiyama S, Courtneidge SA. Notch increases the shedding of HB-EGF by
622 ADAM12 to potentiate invadopodia formation in hypoxia. J Cell Biol. 2013 Apr 15;201(2):279-92. doi:
623 10.1083/jcb.201209151.
624 Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after
625 short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell. 2009 Mar 3;15(3):232-
626 9. doi: 10.1016/j.ccr.2009.01.021.
627 Elkashty OA, Abu Elghanam G, Su X, Liu Y, Chauvin PJ, Tran SD. Cancer stem cells enrichment with surface
628 markers CD271 and CD44 in human head and neck squamous cell carcinomas. Carcinogenesis. 2020
629 Jun 17;41(4):458-466. doi: 10.1093/carcin/bgz182.
630 Fan YL, Zheng M, Tang YL, Liang XH. A new perspective of vasculogenic mimicry: EMT and cancer stem
631 cells (Review). Oncol Lett. 2013 Nov;6(5):1174-1180. doi: 10.3892/ol.2013.1555.
632 Fanelli GN, Naccarato AG, Scatena C. Recent Advances in Cancer Plasticity: Cellular Mechanisms,
633 Surveillance Strategies, and Therapeutic Optimization. Front Oncol. 2020 Apr 22;10:569. doi:
634 10.3389/fonc.2020.00569.
635 Fangfei Qu, Siqi Cao, Wojciech Michno, Chioma J. Madubata, Alyssa Puno, Alexandros Drainas, Myung
636 Chang Lee, Dian Yang, Angus Toland, Christina Kong, Millie Das, Monte Winslow, Anca Paşca, Julien

637 Sage. Neuronal mimicry generates an ecosystem critical for brain metastatic growth of SCLC. Preprint
638 at bioRxiv (2021). doi: https://doi.org/10.1101/2021.08.10.455426
639 Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Piñeros M, Znaor A, Bray F. Estimating the
640 global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. Int J Cancer. 2019
641 Apr 15;144(8):1941-1953. doi: 10.1002/ijc.31937.
642 Gangoso E, Southgate B, Bradley L, Rus S, Galvez-Cancino F, McGivern N, Güç E, Kapourani CA, Byron
643 A, Ferguson KM, Alfazema N, Morrison G, Grant V, Blin C, Sou I, Marques-Torrejon MA, Conde L,
644 Parrinello S, Herrero J, Beck S, Brandner S, Brennan PM, Bertone P, Pollard JW, Quezada SA, Sproul
645 D, Frame MC, Serrels A, Pollard SM. Glioblastomas acquire myeloid-affiliated transcriptional
646 programs via epigenetic immunoediting to elicit immune evasion. Cell. 2021 Apr 29;184(9):2454-
647 2470.e26. doi: 10.1016/j.cell.2021.03.023.
648 Gao R, He B, Huang Q, Wang Z, Yan M, Lam EW, Lin S, Wang B, Liu Q. Cancer cell immune mimicry
649 delineates onco-immunologic modulation. iScience. 2021 Sep 15;24(10):103133. doi:
650 10.1016/j.isci.2021.103133.
651 Ghuwalewala S, Ghatak D, Das P, Dey S, Sarkar S, Alam N, Panda CK, Roychoudhury S.
652 CD44(high)CD24(low) molecular signature determines the Cancer Stem Cell and EMT phenotype in
653 Oral Squamous Cell Carcinoma. Stem Cell Res. 2016 Mar;16(2):405-17. doi:
654 10.1016/j.scr.2016.02.028.
655 Gibbons DL, Creighton CJ. Pan-cancer survey of epithelial-mesenchymal transition markers across the Cancer
656 Genome Atlas. Dev Dyn. 2018 Mar;247(3):555-564. doi: 10.1002/dvdy.24485. Epub 2017 May 4.
657 Greenblatt MB, Vrbanac V, Tivey T, Tsang K, Tager AM, Aliprantis AO. Graft versus host disease in the
658 bone marrow, liver and thymus humanized mouse model. PLoS One. 2012;7(9):e44664. doi:
659 10.1371/journal.pone.0044664.
660 Ha NH, Woo BH, Kim DJ, Ha ES, Choi JI, Kim SJ, Park BS, Lee JH, Park HR. Prolonged and repetitive
661 exposure to Porphyromonas gingivalis increases aggressiveness of oral cancer cells by promoting
662 acquisition of cancer stem cell properties. Tumour Biol. 2015 Dec;36(12):9947-60. doi:
663 10.1007/s13277-015-3764-9.
664 Han J, Fujisawa T, Husain SR, Puri RK. Identification and characterization of cancer stem cells in human head
665 and neck squamous cell carcinoma. BMC Cancer. 2014 Mar 11;14:173. doi: 10.1186/1471-2407-14-
666 173.
667 Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer Discov. 2022 Jan;12(1):31-46. doi: 10.1158/2159-
668 8290.CD-21-1059.
669 Huang CH, Yang WH, Chang SY, Tai SK, Tzeng CH, Kao JY, Wu KJ, Yang MH. Regulation of membrane-
670 type 4 matrix metalloproteinase by SLUG contributes to hypoxia-mediated metastasis. Neoplasia. 2009
671 Dec;11(12):1371-82. doi: 10.1593/neo.91326.
672 Hujanen R, Almahmoudi R, Salo T, Salem A. Comparative Analysis of Vascular Mimicry in Head and Neck
673 Squamous Cell Carcinoma: In Vitro and In Vivo Approaches. Cancers (Basel). 2021 Sep
674 23;13(19):4747. doi: 10.3390/cancers13194747.
675 Irani S, Dehghan A. The Expression and Functional Significance of Vascular Endothelial-Cadherin, CD44,
676 and Vimentin in Oral Squamous Cell Carcinoma. J Int Soc Prev Community Dent. 2018 Mar-
677 Apr;8(2):110-117. doi: 10.4103/jispcd.JISPCD_408_17.
678 Januchowski R, Wojtowicz K, Zabel M. The role of aldehyde dehydrogenase (ALDH) in cancer drug
679 resistance. Biomed Pharmacother. 2013 Sep;67(7):669-80. doi: 10.1016/j.biopha.2013.04.005.
680 Jia L, Zhang W, Wang CY. BMI1 Inhibition Eliminates Residual Cancer Stem Cells after PD1 Blockade and
681 Activates Antitumor Immunity to Prevent Metastasis and Relapse. Cell Stem Cell. 2020 Aug
682 6;27(2):238-253.e6. doi: 10.1016/j.stem.2020.06.022.
683 Johnson DE, Burtness B, Leemans CR, Lui VWY, Bauman JE, Grandis JR. Head and neck squamous cell
684 carcinoma. Nat Rev Dis Primers. 2020;6(1):92. doi: 10.1038/s41572-020-00224-3.
685 Joshua B, Kaplan MJ, Doweck I, Pai R, Weissman IL, Prince ME, Ailles LE. Frequency of cells expressing
686 CD44, a head and neck cancer stem cell marker: correlation with tumor aggressiveness. Head Neck.
687 2012 Jan;34(1):42-9. doi: 10.1002/hed.21699.
688 Kamarajan P, Rajendiran TM, Kinchen J, Bermúdez M, Danciu T, Kapila YL. Head and Neck Squamous Cell
689 Carcinoma Metabolism Draws on Glutaminolysis, and Stemness Is Specifically Regulated by
690 Glutaminolysis via Aldehyde Dehydrogenase. J Proteome Res. 2017 Mar 3;16(3):1315-1326. doi:
691 10.1021/acs.jproteome.6b00936.

692 Karinen S, Hujanen R, Salo T, Salem A. The prognostic influence of lymphatic endothelium-specific
693 hyaluronan receptor 1 in cancer: A systematic review. Cancer Sci. 2022 Jan;113(1):17-27. doi:
694 10.1111/cas.15199.
695 Karinen S, Juurikka K, Hujanen R, Wahbi W, Hadler-Olsen E, Svineng G, Eklund KK, Salo T, Åström P,
696 Salem A. Tumour cells express functional lymphatic endothelium-specific hyaluronan receptor in vitro
697 and in vivo: Lymphatic mimicry promotes oral oncogenesis? Oncogenesis. 2021 Mar 5;10(3):23. doi:
698 10.1038/s41389-021-00312-3.
699 Kim J, Shin JH, Chen CH, Cruz L, Farnebo L, Yang J, Borges P, Kang G, Mochly-Rosen D, Sunwoo JB.
700 Targeting aldehyde dehydrogenase activity in head and neck squamous cell carcinoma with a novel
701 small molecule inhibitor. Oncotarget. 2017 Apr 10;8(32):52345-52356. doi:
702 10.18632/oncotarget.17017.
703 Kondo S, Wakisaka N, Muramatsu M, Zen Y, Endo K, Murono S, Sugimoto H, Yamaoka S, Pagano JS,
704 Yoshizaki T. Epstein-Barr virus latent membrane protein 1 induces cancer stem/progenitor-like cells in
705 nasopharyngeal epithelial cell lines. J Virol. 2011 Nov;85(21):11255-64. doi: 10.1128/JVI.00188-11.
706 Koo BS, Lee SH, Kim JM, Huang S, Kim SH, Rho YS, Bae WJ, Kang HJ, Kim YS, Moon JH, Lim YC. Oct4
707 is a critical regulator of stemness in head and neck squamous carcinoma cells. Oncogene. 2015 Apr
708 30;34(18):2317-24. doi: 10.1038/onc.2014.174.
709 Krishnamurthy S, Nör JE. Orosphere assay: a method for propagation of head and neck cancer stem cells.
710 Head Neck. 2013 Jul;35(7):1015-21. doi: 10.1002/hed.23076.
711 La Fleur L, Johansson AC, Roberg K. A CD44high/EGFRlow subpopulation within head and neck cancer cell
712 lines shows an epithelial-mesenchymal transition phenotype and resistance to treatment. PLoS One.
713 2012;7(9):e44071. doi: 10.1371/journal.pone.0044071.
714 Langhans SA. Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning.
715 Front Pharmacol. 2018 Jan 23;9:6. doi: 10.3389/fphar.2018.00006.
716 Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri
717 MA, Dick JE. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice.
718 Nature. 1994 Feb 17;367(6464):645-8. doi: 10.1038/367645a0.
719 Lechner M, Liu J, Masterson L, Fenton TR. HPV-associated oropharyngeal cancer: epidemiology, molecular
720 biology and clinical management. Nat Rev Clin Oncol. 2022 May;19(5):306-327. doi: 10.1038/s41571-
721 022-00603-7.
722 Ledford H. Translational research: 4 ways to fix the clinical trial. Nature. 2011 Sep 28;477(7366):526-8. doi:
723 10.1038/477526a.
724 Lee KE, Simon MC. From stem cells to cancer stem cells: HIF takes the stage. Curr Opin Cell Biol. 2012
725 Apr;24(2):232-5. doi: 10.1016/j.ceb.2012.01.005.
726 Lee SH, Lee CR, Rigas NK, Kim RH, Kang MK, Park NH, Shin KH. Human papillomavirus 16 (HPV16)
727 enhances tumor growth and cancer stemness of HPV-negative oral/oropharyngeal squamous cell
728 carcinoma cells via miR-181 regulation. Papillomavirus Res. 2015 Dec 1;1:116-125. doi:
729 10.1016/j.pvr.2015.08.001.
730 Lee Y, Shin JH, Longmire M, Wang H, Kohrt HE, Chang HY, Sunwoo JB. CD44+ Cells in Head and Neck
731 Squamous Cell Carcinoma Suppress T-Cell-Mediated Immunity by Selective Constitutive and Inducible
732 Expression of PD-L1. Clin Cancer Res. 2016 Jul 15;22(14):3571-81. doi: 10.1158/1078-0432.CCR-15-
733 2665.
734 Liang X, Zheng M, Jiang J, Zhu G, Yang J, Tang Y. Hypoxia-inducible factor-1 alpha, in association with
735 TWIST2 and SNIP1, is a critical prognostic factor in patients with tongue squamous cell carcinoma.
736 Oral Oncol. 2011 Feb;47(2):92-7. doi: 10.1016/j.oraloncology.2010.11.014
737 Lim YC, Han JH, Kang HJ, Kim YS, Lee BH, Choi EC, Kim CH. Overexpression of c-Met promotes invasion
738 and metastasis of small oral tongue carcinoma. Oral Oncol. 2012 Nov;48(11):1114-9. doi:
739 10.1016/j.oraloncology.2012.05.013.
740 Ludwig N, Szczepanski MJ, Gluszko A, Szafarowski T, Azambuja JH, Dolg L, Gellrich NC, Kampmann A,
741 Whiteside TL, Zimmerer RM. CD44(+) tumor cells promote early angiogenesis in head and neck
742 squamous cell carcinoma. Cancer Lett. 2019 Dec 28;467:85-95. doi: 10.1016/j.canlet.2019.10.010.
743 Lun SW, Cheung ST, Cheung PF, To KF, Woo JK, Choy KW, Chow C, Cheung CC, Chung GT, Cheng AS,
744 Ko CW, Tsao SW, Busson P, Ng MH, Lo KW. CD44+ cancer stem-like cells in EBV-associated
745 nasopharyngeal carcinoma. PLoS One. 2012;7(12):e52426. doi: 10.1371/journal.pone.0052426.

Lüttich L, Besso MJ, Heiden S, Koi L, Baumann M, Krause M, Dubrovska A, Linge A, Kurth I, Peitzsch C. Tyrosine Kinase c-MET as Therapeutic Target for Radiosensitization of Head and Neck Squamous Cell Carcinomas. Cancers (Basel). 2021 Apr 14;13(8):1865. doi: 10.3390/cancers13081865.

Mehanna H, Paleri V, West CM, Nutting C. Head and neck cancer--Part 1: Epidemiology, presentation, and prevention. BMJ. 2010 Sep 20;341:c4684. doi: 10.1136/bmj.c4684.

Metsäniitty M, Hasnat S, Salo T, Salem A. Oral Microbiota-A New Frontier in the Pathogenesis and Management of Head and Neck Cancers. Cancers (Basel). 2021 Dec 23;14(1):46. doi: 10.3390/cancers14010046.

Mimeault M, Batra SK. Hypoxia-inducing factors as master regulators of stemness properties and altered metabolism of cancer- and metastasis-initiating cells. J Cell Mol Med. 2013 Jan;17(1):30-54. doi: 10.1111/jcmm.12004.

Modur V, Joshi P, Nie D, Robbins KT, Khan AU, Rao K. CD24 Expression May Play a Role as a Predictive Indicator and a Modulator of Cisplatin Treatment Response in Head and Neck Squamous Cellular Carcinoma. PLoS One. 2016 Jun 8;11(6):e0156651. doi: 10.1371/journal.pone.0156651.

Moreno Roig E, Yaromina A, Houben R, Groot AJ, Dubois L, Vooijs M. Prognostic Role of Hypoxia-Inducible Factor-2α Tumor Cell Expression in Cancer Patients: A Meta-Analysis. Front Oncol. 2018 Jun 11;8:224. doi: 10.3389/fonc.2018.00224.

Morton JJ, Bird G, Keysar SB, Astling DP, Lyons TR, Anderson RT, Glogowska MJ, Estes P, Eagles JR, Le PN, Gan G, McGettigan B, Fernandez P, Padilla-Just N, Varella-Garcia M, Song JI, Bowles DW, Schedin P, Tan AC, Roop DR, Wang XJ, Refaeli Y, Jimeno A. XactMice: humanizing mouse bone marrow enables microenvironment reconstitution in a patient-derived xenograft model of head and neck cancer. Oncogene. 2016 Jan 21;35(3):290-300. doi: 10.1038/onc.2015.94. Epub 2015 Apr 20.

Morton JJ, Keysar SB, Perrenoud L, Chimed TS, Reisinger J, Jackson B, Le PN, Nieto C, Gomez K, Miller B, Gao D, Somerset H, Wang XJ, Jimeno A. Dual use of hematopoietic and mesenchymal stem cells enhances engraftment and immune cell trafficking in an allogeneic humanized mouse model of head and neck cancer. Mol Carcinog. 2018 Nov;57(11):1651-1663. doi: 10.1002/mc.22887.

Murillo-Sauca O, Chung MK, Shin JH, Karamboulas C, Kwok S, Jung YH, Oakley R, Tysome JR, Farnebo LO, Kaplan MJ, Sirjani D, Divi V, Holsinger FC, Tomeh C, Nichols A, Le QT, Colevas AD, Kong CS, Uppaluri R, Lewis JS Jr, Ailles LE, Sunwoo JB. CD271 is a functional and targetable marker of tumor-initiating cells in head and neck squamous cell carcinoma. Oncotarget. 2014 Aug 30;5(16):6854-66. doi: 10.18632/oncotarget.2269.

Muzaffar J, Bari S, Kirtane K, Chung CH. Recent Advances and Future Directions in Clinical Management of Head and Neck Squamous Cell Carcinoma. Cancers (Basel). 2021 Jan 18;13(2):338. doi: 10.3390/cancers13020338.

Nathansen J, Lukiyanchuk V, Hein L, Stolte MI, Borgmann K, Löck S, Kurth I, Baumann M, Krause M, Linge A, Dubrovska A. Oct4 confers stemness and radioresistance to head and neck squamous cell carcinoma by regulating the homologous recombination factors PSMC3IP and RAD54L. Oncogene. 2021 Jun;40(24):4214-4228. doi: 10.1038/s41388-021-01842-1.

Neman J, Termini J, Wilczynski S, Vaidehi N, Choy C, Kowolik CM, Li H, Hambrecht AC, Roberts E, Jandial R. Human breast cancer metastases to the brain display GABAergic properties in the neural niche. Proc Natl Acad Sci U S A. 2014 Jan 21;111(3):984-9. doi: 10.1073/pnas.1322098111.

Nieto MA, Huang RY, Jackson RA, Thiery JP. EMT: 2016. Cell. 2016 Jun 30;166(1):21-45. doi: 10.1016/j.cell.2016.06.028.

O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature. 2007 Jan 4;445(7123):106-10. doi: 10.1038/nature05372.

Oliveira LR, Castilho-Fernandes A, Oliveira-Costa JP, Soares FA, Zucoloto S, Ribeiro-Silva A. CD44+/CD133+ immunophenotype and matrix metalloproteinase-9: Influence on prognosis in early-stage oral squamous cell carcinoma. Head Neck. 2014 Dec;36(12):1718-26. doi: 10.1002/hed.23527.

Pàez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Viñals F, Inoue M, Bergers G, Hanahan D, Casanovas O. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell. 2009 Mar 3;15(3):220-31. doi: 10.1016/j.ccr.2009.01.027.

Pamarthy S, Sabaawy HE. Patient derived organoids in prostate cancer: improving therapeutic efficacy in precision medicine. Mol Cancer. 2021 Sep 29;20(1):125. doi: 10.1186/s12943-021-01426-3.

Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF, Ailles LE. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci U S A. 2007 Jan 16;104(3):973-8. doi: 10.1073/pnas.0610117104.

Prince MEP, Zhou L, Moyer JS, Tao H, Lu L, Owen J, Etigen M, Zheng F, Chang AE, Xia J, Wolf G, Wicha MS, Huang S, Ren X, Li Q. Evaluation of the immunogenicity of ALDH(high) human head and neck squamous cell carcinoma cancer stem cells in vitro. Oral Oncol. 2016 Aug;59:30-42. doi: 10.1016/j.oraloncology.2016.05.013.

Puram SV, Tirosh I, Parikh AS, Patel AP, Yizhak K, Gillespie S, Rodman C, Luo CL, Mroz EA, Emerick KS, Deschler DG, Varvares MA, Mylvaganam R, Rozenblatt-Rosen O, Rocco JW, Faquin WC, Lin DT, Regev A, Bernstein BE. Single-Cell Transcriptomic Analysis of Primary and Metastatic Tumor Ecosystems in Head and Neck Cancer. Cell. 2017 Dec 14;171(7):1611-1624.e24. doi: 10.1016/j.cell.2017.10.044.

Rajan V, Melong N, Hing Wong W, King B, Tong SR, Mahajan N, Gaston D, Lund T, Rittenberg D, Dellaire G, Campbell CJV, Druley T, Berman JN. Humanized zebrafish enhance human hematopoietic stem cell survival and promote acute myeloid leukemia clonal diversity. Haematologica. 2020 Oct 1;105(10):2391-2399. doi: 10.3324/haematol.2019.223040.

Reid P, Staudacher AH, Marcu LG, Olver I, Moghaddasi L, Brown MP, Bezak E. Influence of the human papillomavirus on the radio-responsiveness of cancer stem cells in head and neck cancers. Sci Rep. 2020 Feb 17;10(1):2716. doi: 10.1038/s41598-020-59654-4.

Ribatti D, Annese T, Ruggieri S, Tamma R, Crivellato E. Limitations of Anti-Angiogenic Treatment of Tumors. Transl Oncol. 2019 Jul;12(7):981-986. doi: 10.1016/j.tranon.2019.04.022.

Ricci-Vitiani L, Pallini R, Biffoni M, Todaro M, Invernici G, Cenci T, Maira G, Parati EA, Stassi G, Larocca LM, De Maria R. Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells. Nature. 2010 Dec 9;468(7325):824-8. doi: 10.1038/nature09557.

Routila J, Qiao X, Weltner J, Rantala JK, Carpén T, Hagström J, Mäkitie A, Leivo I, Ruuskanen M, Söderlund J, Rintala M, Hietanen S, Irjala H, Minn H, Westermarck J, Ventelä S. Cisplatin overcomes radiotherapy resistance in OCT4-expressing head and neck squamous cell carcinoma. Oral Oncol. 2022 Apr;127:105772. doi: 10.1016/j.oraloncology.2022.105772.

Salem A, Salo T. Vasculogenic Mimicry in Head and Neck Squamous Cell Carcinoma-Time to Take Notice. Front Oral Health. 2021 Mar 31;2:666895. doi: 10.3389/froh.2021.666895.

Salo T, Sutinen M, Hoque Apu E, Sundquist E, Cervigne NK, de Oliveira CE, Akram SU, Ohlmeier S, Suomi F, Eklund L, Juusela P, Åström P, Bitu CC, Santala M, Savolainen K, Korvala J, Paes Leme AF, Coletta RD. A novel human leiomyoma tissue derived matrix for cell culture studies. BMC Cancer. 2015 Dec 16;15:981. doi: 10.1186/s12885-015-1944-z.

Sasabe E, Tatemoto Y, Li D, Yamamoto T, Osaki T. Mechanism of HIF-1alpha-dependent suppression of hypoxia-induced apoptosis in squamous cell carcinoma cells. Cancer Sci. 2005 Jul;96(7):394-402. doi: 10.1111/j.1349-7006.2005.00065.x

Scully S, Francescone R, Faibish M, Bentley B, Taylor SL, Oh D, Schapiro R, Moral L, Yan W, Shao R. Transdifferentiation of glioblastoma stem-like cells into mural cells drives vasculogenic mimicry in glioblastomas. J Neurosci. 2012 Sep 12;32(37):12950-60. doi: 10.1523/JNEUROSCI.2017-12.2012.

Seftor RE, Hess AR, Seftor EA, Kirschmann DA, Hardy KM, Margaryan NV, Hendrix MJ. Tumor cell vasculogenic mimicry: from controversy to therapeutic promise. Am J Pathol. 2012 Oct;181(4):1115-25. doi: 10.1016/j.ajpath.2012.07.013.

Shackleton M, Quintana E, Fearon ER, Morrison SJ. Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell. 2009 Sep 4;138(5):822-9. doi: 10.1016/j.cell.2009.08.017.

Sharaf K, Lechner A, Haider SP, Wiebringhaus R, Walz C, Kranz G, Canis M, Haubner F, Gires O, Baumeister P. Discrimination of Cancer Stem Cell Markers ALDH1A1, BCL11B, BMI-1, and CD44 in Different Tissues of HNSCC Patients. Curr Oncol. 2021 Jul 19;28(4):2763-2774. doi: 10.3390/curroncol28040241.

Skidan I, Steiniger SC. In vivo models for cancer stem cell research: a practical guide for frequently used animal models and available biomarkers. J Physiol Pharmacol. 2014 Apr;65(2):157-69.

Sterz CM, Kulle C, Dakic B, Makarova G, Böttcher MC, Bette M, Werner JA, Mandic R. A basal-cell-like compartment in head and neck squamous cell carcinomas represents the invasive front of the tumor and is expressing MMP-9. Oral Oncol. 2010 Feb;46(2):116-22. doi: 10.1016/j.oraloncology.2009.11.011.

853 Sun S, Wang Z. Head neck squamous cell carcinoma c-Met$^{+}$ cells display cancer stem cell properties and are
854 responsible for cisplatin-resistance and metastasis. Int J Cancer. 2011 Nov 15;129(10):2337-48. doi:
855 10.1002/ijc.25927.
856 Szafarowski T, Sierdziński J, Ludwig N, Głuszko A, Filipowska A, Szczepański MJ. Assessment of cancer
857 stem cell marker expression in primary head and neck squamous cell carcinoma shows prognostic value
858 for aldehyde dehydrogenase (ALDH1A1). Eur J Pharmacol. 2020 Jan 15;867:172837. doi:
859 10.1016/j.ejphar.2019.172837.
860 Tanaka T, Atsumi N, Nakamura N, Yanai H, Komai Y, Omachi T, Tanaka K, Ishigaki K, Saiga K, Ohsugi H,
861 Tokuyama Y, Imahashi Y, Hisha H, Yoshida N, Kumano K, Okazaki K, Ueno H. Bmi1-positive cells
862 in the lingual epithelium could serve as cancer stem cells in tongue cancer. Sci Rep. 2016 Dec
863 22;6:39386. doi: 10.1038/srep39386.
864 Tang AL, Owen JH, Hauff SJ, Park JJ, Papagerakis S, Bradford CR, Carey TE, Prince ME. Head and neck
865 cancer stem cells: the effect of HPV--an in vitro and mouse study. Otolaryngol Head Neck Surg. 2013
866 Aug;149(2):252-60. doi: 10.1177/0194599813486599.
867 Teo AL, Mantalaris A, Lim M. Influence of culture pH on proliferation and cardiac differentiation of murine
868 embryonic stem cells Biochem. Eng. J. 2014; 8-15.
869 Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease.
870 Cell. 2009 Nov 25;139(5):871-90. doi: 10.1016/j.cell.2009.11.007.
871 Tuomainen K, Al-Samadi A, Potdar S, Turunen L, Turunen M, Karhemo PR, Bergman P, Risteli M, Åström
872 P, Tiikkaja R, Grenman R, Wennerberg K, Monni O, Salo T. Human Tumor-Derived Matrix Improves
873 the Predictability of Head and Neck Cancer Drug Testing. Cancers (Basel). 2019 Dec 30;12(1):92. doi:
874 10.3390/cancers12010092.
875 Vormittag L, Thurnher D, Geleff S, Pammer J, Heiduschka G, Brunner M, Grasl MCh, Erovic BM. Co-
876 expression of Bmi-1 and podoplanin predicts overall survival in patients with squamous cell carcinoma
877 of the head and neck treated with radio(chemo)therapy. Int J Radiat Oncol Biol Phys. 2009 Mar
878 1;73(3):913-8. doi: 10.1016/j.ijrobp.2008.10.040.
879 Vsiansky V, Gumulec J, Raudenska M, Masarik M. Prognostic role of c-Met in head and neck squamous cell
880 cancer tissues: a meta-analysis. Sci Rep. 2018 Jul 10;8(1):10370. doi: 10.1038/s41598-018-28672-8.
881 Wahab A, Hyytiäinen A, Wahbi W, Tuomainen K, Tervo S, Conesa-Zamora P, Jauhiainen L, Mäkinen LK,
882 Paavonen T, Toppila-Salmi S, Salem A, Almangush A, Salo T, Al-Samadi A. The effect of fascin 1
883 inhibition on head and neck squamous cell carcinoma cells. Eur J Oral Sci. 2021 Dec;129(6):e12819.
884 doi: 10.1111/eos.12819.
885 Wang QS, He R, Yang F, Kang LJ, Li XQ, Fu L, Sun B, Feng YM. FOXF2 deficiency permits basal-like
886 breast cancer cells to form lymphangiogenic mimicry by enhancing the response of VEGF-C/VEGFR3
887 signaling pathway. Cancer Lett. 2018 Apr 28;420:116-126. doi: 10.1016/j.canlet.2018.01.069.
888 Wang SJ, Bourguignon LY. Role of hyaluronan-mediated CD44 signaling in head and neck squamous cell
889 carcinoma progression and chemoresistance. Am J Pathol. 2011 Mar;178(3):956-63. doi:
890 10.1016/j.ajpath.2010.11.077.
891 Wang SJ, Wong G, de Heer AM, Xia W, Bourguignon LY. CD44 variant isoforms in head and neck squamous
892 cell carcinoma progression. Laryngoscope. 2009 Aug;119(8):1518-30. doi: 10.1002/lary.20506.
893 Wiechec E, Matic N, Ali A, Roberg K. Hypoxia induces radioresistance, epithelial-mesenchymal transition,
894 cancer stem cell-like phenotype and changes in genes possessing multiple biological functions in head
895 and neck squamous cell carcinoma. Oncol Rep. 2022 Mar;47(3):58. doi: 10.3892/or.2022.8269.
896 Xing DT, Khor R, Gan H, Wada M, Ermongkonchai T, Ng SP. Recent Research on Combination of
897 Radiotherapy with Targeted Therapy or Immunotherapy in Head and Neck Squamous Cell Carcinoma:
898 A Review for Radiation Oncologists. Cancers (Basel). 2021 Nov 15;13(22):5716. doi:
899 10.3390/cancers13225716.
900 Yang MH, Hsu DS, Wang HW, Wang HJ, Lan HY, Yang WH, Huang CH, Kao SY, Tzeng CH, Tai SK, Chang
901 SY, Lee OK, Wu KJ. Bmi1 is essential in Twist1-induced epithelial-mesenchymal transition. Nat Cell
902 Biol. 2010 Oct;12(10):982-92. doi: 10.1038/ncb2099.
903 Yeo CD, Kang N, Choi SY, Kim BN, Park CK, Kim JW, Kim YK, Kim SJ. The role of hypoxia on the
904 acquisition of epithelial-mesenchymal transition and cancer stemness: a possible link to epigenetic
905 regulation. Korean J Intern Med. 2017 Jul;32(4):589-599. doi: 10.3904/kjim.2016.302.
906 Yeung KT, Yang J. Epithelial-mesenchymal transition in tumor metastasis. Mol Oncol. 2017 Jan;11(1):28-39.
907 doi: 10.1002/1878-0261.12017.

908 Yu CC, Hu FW, Yu CH, Chou MY. Targeting CD133 in the enhancement of chemosensitivity in oral
909 squamous cell carcinoma-derived side population cancer stem cells. Head Neck. 2016 Apr;38 Suppl
910 1:E231-8. doi: 10.1002/hed.23975.
911 Yu CC, Lo WL, Chen YW, Huang PI, Hsu HS, Tseng LM, Hung SC, Kao SY, Chang CJ, Chiou SH. Bmi-1
912 Regulates Snail Expression and Promotes Metastasis Ability in Head and Neck Squamous Cancer-
913 Derived ALDH1 Positive Cells. J Oncol. 2011; 2011:609259. doi: 10.1155/2011/609259.
914 Yuan S, Norgard RJ, Stanger BZ. Cellular Plasticity in Cancer. Cancer Discov. 2019 Jul;9(7):837-851. doi:
915 10.1158/2159-8290.CD-19-0015.
916 Zeng Q, Michael IP, Zhang P, Saghafinia S, Knott G, Jiao W, McCabe BD, Galván JA, Robinson HPC, Zlobec
917 I, Ciriello G, Hanahan D. Synaptic proximity enables NMDAR signalling to promote brain metastasis.
918 Nature. 2019 Sep;573(7775):526-531. doi: 10.1038/s41586-019-1576-6.
919 Zhang C, Yang Z, Dong DL, Jang TS, Knowles JC, Kim HW, Jin GZ, Xuan Y. 3D culture technologies of
920 cancer stem cells: promising ex vivo tumor models. J Tissue Eng. 2020 Jun 24;11:2041731420933407.
921 doi: 10.1177/2041731420933407.
922 Zhang M, Kumar B, Piao L, Xie X, Schmitt A, Arradaza N, Cippola M, Old M, Agrawal A, Ozer E, Schuller
923 DE, Teknos TN, Pan Q. Elevated intrinsic cancer stem cell population in human papillomavirus-
924 associated head and neck squamous cell carcinoma. Cancer. 2014 Apr 1;120(7):992-1001. doi:
925 10.1002/cncr.28538.
926 Zhang Q, Shi S, Yen Y, Brown J, Ta JQ, Le AD. A subpopulation of CD133(+) cancer stem-like cells
927 characterized in human oral squamous cell carcinoma confer resistance to chemotherapy. Cancer Lett.
928 2010 Mar 28;289(2):151-60. doi: 10.1016/j.canlet.2009.08.010.
929 Zhao D, Wang SH, Feng Y, Hua CG, Zhao J, Tang XF. Intratumoral c-Met expression is associated with
930 vascular endothelial growth factor C expression, lymphangiogenesis, and lymph node metastasis in oral
931 squamous cell carcinoma: implications for use as a prognostic marker. Hum Pathol. 2011
932 Oct;42(10):1514-23. doi: 10.1016/j.humpath.2010.03.012.
933 Zhou L, Wei X, Cheng L, Tian J, Jiang JJ. CD133, one of the markers of cancer stem cells in Hep-2 cell line.
934 Laryngoscope. 2007 Mar;117(3):455-60. doi: 10.1097/01.mlg.0000251586.15299.35.
935 Zimmerer RM, Ludwig N, Kampmann A, Bittermann G, Spalthoff S, Jungheim M, Gellrich NC, Tavassol F.
936 CD24+ tumor-initiating cells from oral squamous cell carcinoma induce initial angiogenesis in vivo.
937 Microvasc Res. 2017 Jul;112:101-108. doi: 10.1016/j.mvr.2017.03.006.
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940 **Table 1. Cancer stem cell markers in head and neck squamous cell carcinoma**

| CSC Marker | Protein type and localization | Main tumorigenic potential in HNSCC | References |
|---|---|---|---|
| ALDH | Enzyme; cytoplasmic | ▪ $ALDH^{+ve}$ cells (as few as $5\times10^2$) initiated visible malignant growths *in vivo* that resembled the original tumors.<br>▪ ALDH activity in HNSCC involved its isoforms ALDH1-3; and it was associated with increased levels of several tumorigenic properties, e.g., sphere formation, enhanced migration and drug resistance.<br>▪ ALDH had therapeutic and prognostic potential; and $ALDH^{high}$ CSC-based vaccines induced anti-HNSCC immunity. | Clay et al., 2010; Prince et al., 2016; Chen et al., 2009; Yu et al.; 2011; Chen et al., 2010; Kim et al., 2017; Szafarowski et al., 2020 |
| Bmi1 (PCGF4) | Binding protein; nuclear | ▪ Bmi1 mediated metastasis in mouse model of chemically-induced HNSCC, while $Bmi1^{+ve}$ cells showed features of tumor-initiating CSCs<br>▪ $Bmi1^{+ve}$ cells were chemoresistant and fueled tumor growth and maintenance. ▪ Bmi1 targeting augmented immunotherapeutic drugs and reduced metastasis. | Prince et al., 2007; Chen et al., 2017; Vormittag et al., 2009; Sharaf et al., 2021; Tanaka et al., 2016; Jia et al., 2020 |
| CD133 (PROM1) | Transmembrane protein; cell surface protrusions | ▪ $CD133^{+ve}$ cells represented > 5% of OSCC cells and showed properties of CSCs including chemoresistance, self-renewal, clonogenicity, proliferation and differentiation *in vitro* and *in vivo* compared with the $CD133^{-ve}$ cells.<br>▪ $CD133^{+ve}$ cells had higher levels of pluripotency-associated genes<br>▪ Targeting CD133 ameliorated the drug resistance while combining anti-CD133 and cisplatin led to the maximal inhibition of tumor initiating properties<br>▪ $CD133^{+ve}/CD44^{+ve}$ immunophenotype predicted poor prognosis of early-stage OSCC patients. | Yu et al., 2016; Oliveira et al., 2014; Zhang et al., 2010 |

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|---|---|---|---|
| CD24 | Membrane receptor; cell surface | ▪ CD24 expression level directly affects cisplatin sensitivity as well as the expression of key apoptotic, stem and drug resistance genes in LSCC cells<br>▪ $CD24^{high}$ LSCC tumors had unfavorable response to cisplatin treatment<br>▪ $CD24^{+ve}$ OSCC cells showed a significantly higher functional angiogenic capillary density in NOD/SCID mice compared with $CD24^{-ve}$ cells<br>▪ $CD24^{+ve}/CD44^{+ve}$ cells possessed stemness characteristics of self-renewal and differentiation, higher cell invasion and clonogenicity *in vitro* and generated larger tumors in nude mice<br>▪ $CD24^{+ve}/CD44^{+ve}$ cells were chemoresistant to gemcitabine and cisplatin.<br>▪ In contrast, Ghuwalewala et al. showed that OSCC-derived CSCs are $CD44^{high}/CD24^{low}$ cells, which had EMT characteristics, increased clonogenicity, sphere forming ability, invasion and elevated chemoresistance. | Modur et al., 2016; Zimmerer et al., 2017; Han et al., 2014; Ghuwalewala et al. 2016 |
| CD271<br>(NGFR) | Transmembrane protein; cell surface | ▪ $CD271^{+ve}/CD44^{+ve}$ subpopulation was highly tumorigenic cells and showed higher cell proliferation, sphere/colony formation, chemo- and radio-resistance<br>▪ Targeting CD271 inhibits tumor cell proliferation and tumorigenicity<br>▪ CD271-overexpressing cells resulted in a more invasive and metastatic phenotype including the upregulation of EMT-related transcription factors<br>▪ CD271 expression correlated with greater nodal metastasis and shorter disease-free survival *in vivo*. | Murillo-Sauca et al., 2014; Elkashty et al., 2020; Chung et al.,2018 |
| CD44<br>(HCAM) | Transmembrane protein; cell surface | ▪ It is the most frequently studied CSC marker in cancer including in HNSCC.<br>▪ $CD44^{+ve}$ cells comprised <10% of the cells in HNSCC and gave rise to new tumors *in vivo*, which reproduced the original tumor heterogeneity and could be serially passaged | Prince et al., 2007; Wang and Bourguignon 2011; Boxberg et al., 2018; Joshua et al., 2012; Chen et al., 2014; Ludwig et al., 2019; Hujanen et al., 2021; |

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|---|---|---|---|
| | | ▪ CD44$^{+ve}$ cells were less immunogenic; had a higher EMT features and elevated potential for 3D sphere-forming ability, migration and drug resistance<br>▪ CD44 signaling pathway promoted tumor growth, metastasis, cell survival, drug resistance, tumor-related angiogenesis and VM.<br>▪ CD44 expression was correlated with poorer clinicopathological parameters of HNSCC patients<br>▪ CD44 isoforms enhance migration, proliferation, and cisplatin sensitivity and hold prognostic potential in HNSCC | Irani and Dehghan 2018; Lee et al., 2016; Byun et al., 2022; Wang et al., 2009 |
| c-Met (HGFR) | Transmembrane receptor tyrosine kinase; cell surface | ▪ c-Met$^{+ve}$ HNSCC cells showed CSC properties *in vivo*, which was suggested superior to CD44 and only slightly inferior to ALDH.<br>▪ c-Met$^{+ve}$ HNSCC cells have higher self-renewal, chemoresistance and colony-forming ability *in vitro*; while enhanced metastatic ability *in vivo*<br>▪ c-Met$^{+ve}$ staining strongly correlated with neck metastasis and increased depth of tumor invasion in OTSCC<br>▪ c-Met activation enhanced migration and invasion of OTSCC cells *in vitro*<br>▪ c-Met overexpression in OSCC cells was significantly associated with lymphangiogenesis including higher peritumoral lymphatic vessel, higher incidence of peritumoral lymphatic invasion, and positive lymph node status<br>▪ c-Met targeting may have therapeutic effect in HNSCC via radiosensitization<br>▪ c-Met overexpression in HNSCC was significantly correlated with poor overall survival and unfavorable clinicopathological features. | Sun and Wang 2011; Lim et al., 2012; Zhao et al., 2011; Lüttich et al., 2021; Vsiansky et al., 2018; Zhou et al., 2007 |
| Oct4 (POU5F1) | Transcription factor; nuclear | ▪ Oct4 promoted conversion of differentiated HNSCC cells into CSCs | Koo et al., 2015; Routila et al., 2022; Nathansen et al., 2021 |

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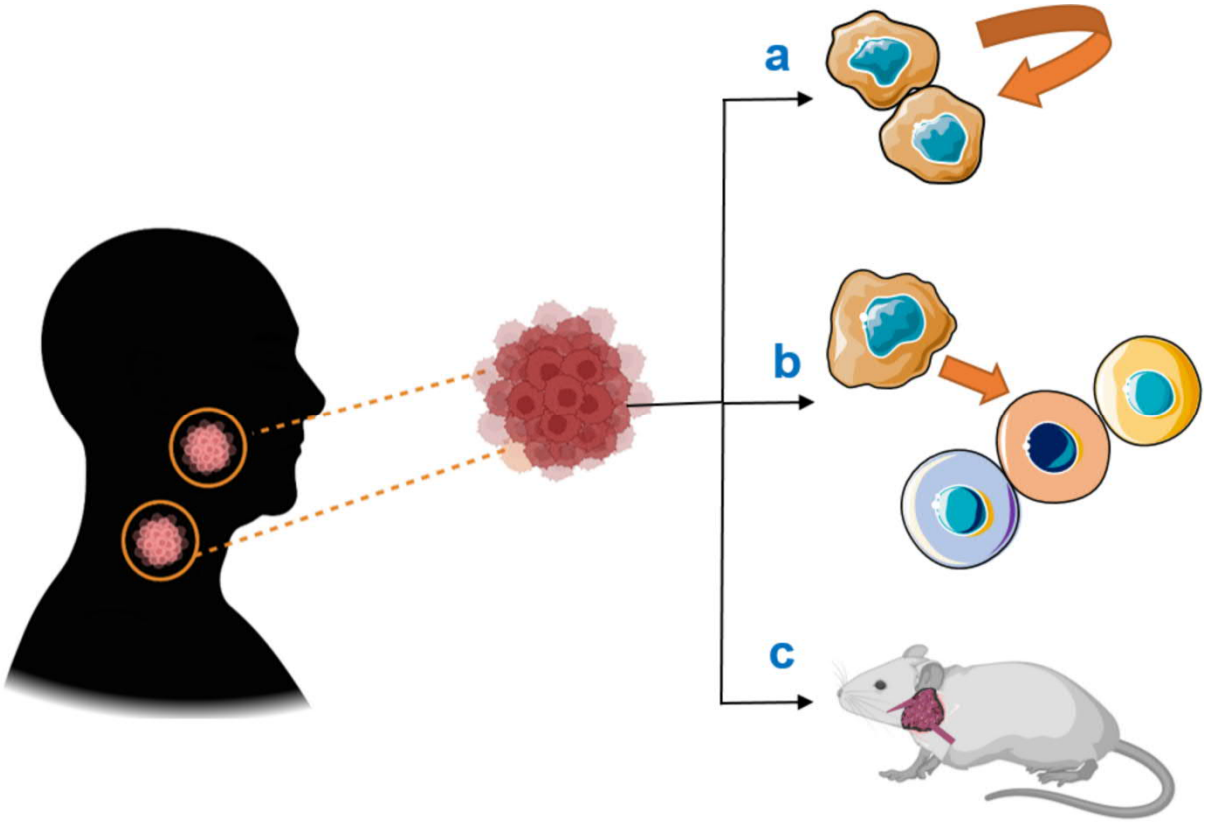
|---|---|
| | ▪ Oct4$^{high}$ CSCs have more stem cell-like traits including self-renewal, chemoresistance, invasion capacity and tumorigenicity *in vitro* and *in vivo*. |
| | ▪ Oct4 expression served as a potent prognostic marker in HNSCC patients. |
| | ▪ Using several independent patient cohorts, Oct4 expression predicted impaired survival in the radiotherapy-only HNSCC patients |
| | ▪ Oct4 positivity served as a biomarker of benefit from DNA damaging chemotherapies; it was implicated in the irradiation-induced DNA damage response in HNSCC and contributes to the regulation of the radioresistant CSCs |
| | ▪ Oct4 overexpression in the HNSCC cell line resulted in apoptosis resistance |

3D, three-dimensional; ALDH, aldehyde dehydrogenase; Bmi1, B-cell-specific moloney murine leukemia virus insertion site 1; CD, cluster of differentiation; c-Met, c-mesenchymal–epithelial transition factor; CSC, cancer stem cell; EMT, epithelial-mesenchymal transition; GPR49, G-protein coupled receptor 49; HCAM, homing cell adhesion molecule; HGFR, hepatocyte growth factor receptor; HNSCC, head and neck squamous cell carcinoma; Lgr5, Leucine-rich repeat-containing G-protein coupled receptor 5; LSCC, laryngeal squamous cell carcinoma; NGFR, nerve growth factor receptor; NOD/SCID, non-obese diabetic/severe combined immunodeficient; Oct4, octamer-binding transcription factor 4; OSCC, oral squamous cell carcinoma; OTSCC, oral tongue squamous cell carcinoma; PCGF4; polycomb group RING finger protein 4; POU5F1; Pic-1, Oct1,2, Unc-86 transcription factor 1; PROM1, prominin-1; VM, vascular mimicry

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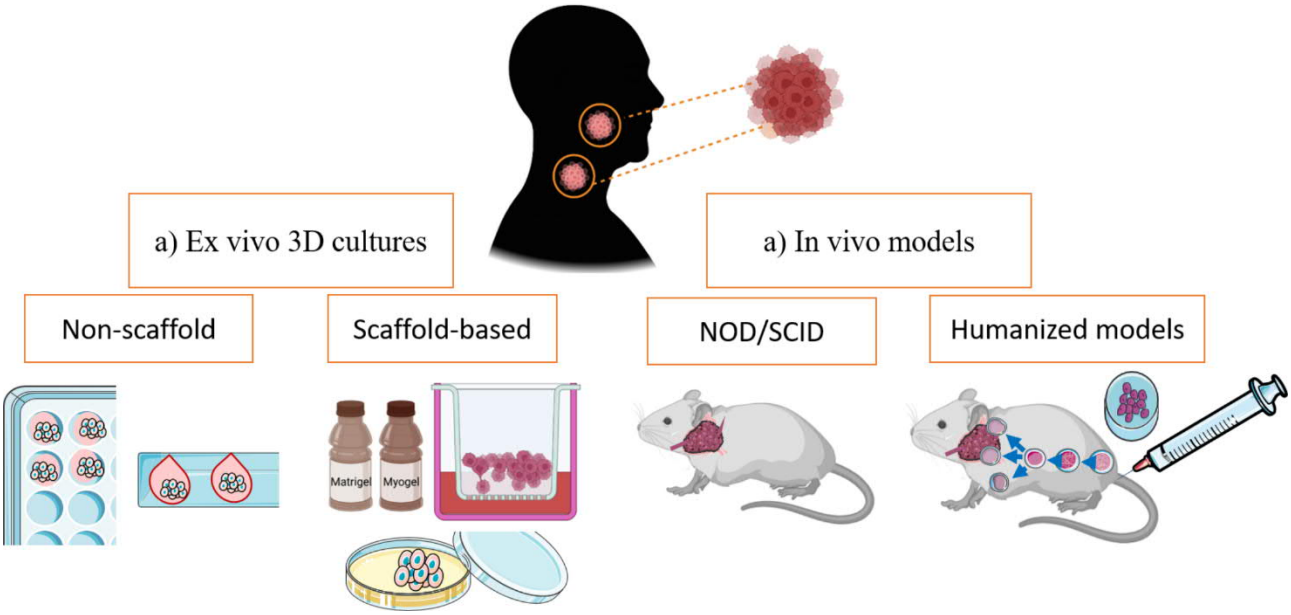
a) Ex vivo 3D cultures
a) In vivo models
Non-scaffold
Scaffold-based
NOD/SCID
Humanized models
Matrigel
Myogel

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