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Stem-like cells and therapy resistance in squamous cell carcinomas

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Abstract

Cancer stem cells (CSCs) within squamous cell carcinomas (SCCs) are hypothesized to contribute to chemotherapy and radiation resistance and represent potentially useful pharmacologic targets. Hallmarks of the stem cell phenotype that may contribute to therapy resistance of CSCs include quiescence, evasion of apoptosis, resistance to DNA damage, and expression of drug transporter pumps. A variety of CSC populations within SCCs of the head and neck and esophagus have been defined tentatively, based on diverse surface markers and functional assays. Stem-like self-renewal and differentiation capacities of these SCC subpopulations are supported by sphere formation and clonogenicity assays *in vitro* as well as limiting dilution studies in xenograft models. Early evidence supports a role for SCC CSCs in intrinsic therapy resistance, while detailed mechanisms by which these subpopulations evade treatment remain to be defined. Development of novel SCC therapies will be aided by pursuing such mechanisms as well as refining current definitions for CSCs and clarifying their relevance to hierarchical versus dynamic models of stemness.

Keywords

cancer stem cells; drug resistance; squamous cell carcinoma

I. Introduction

Squamous cell carcinomas (SCCs) of the digestive tract share a distinct biology and arise almost exclusively within the mucosa of the head and neck and proximal third of the esophagus. Head and neck squamous cell carcinoma (HNSCC) is the 6th leading cause of cancer worldwide (Argiris et al., 2008). In the United States, smoking is the major risk factor for SCC of the head and neck or esophagus, with heavy alcohol use serving as a potent cofactor. Oncogenic human papilloma viruses were also recently recognized as an important and growing etiology for HNSCCs of the tonsil and base of the tongue, with the HPV-16 subtype predominating in the United States (Ang et al., 2010).

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Conflict of Interest

All authors declare no conflict of interest.

Currently, advanced stage HNSCCs require multimodality therapies that may combine surgery, radiation, cytotoxic chemotherapy and/or targeted therapy against the epidermal growth factor receptor (EGFR). Yet these aggressive treatments continue to produce high rates of recurrence as well as severe treatment-related disabilities for long-term survivors. Over the past decade, the cancer stem cell (CSC) hypothesis has emerged as a new paradigm for many solid tumors, with CSCs proposed to play a broad role in intrinsic resistance to existing drugs and radiation therapy. Pursuing strategies that pharmacologically target HNSCC CSCs therefore holds potential for benefit in the form of improved survival and decreased treatment-related morbidity.

Conceptually akin to normal stem cells, CSCs were originally conceived as a minority subset of malignant cells with capacity for both unlimited self-renewal and hierarchical differentiation. They are predicted to show additional hallmarks of normal stem cells including resistance to DNA damage and apoptosis, allowing them to evade both drugs and radiation and subsequently drive tumor repopulation post-therapy (Figure 1). In addition, CSCs have been attributed with enhanced migratory and invasive capacity, which may occur in association with an epithelial to mesenchymal transition (EMT)-related gene signature. Here we briefly delineate the evolving conceptual framework of the CSC hypothesis. In this context, we review multiple working definitions of CSC subpopulations within SCCs. We subsequently appraise the early evidence regarding the significance of these subsets in intrinsic therapy resistance and the mechanisms underlying this resistance.

II. CSCs

A. Hierarchical CSC model

A hierarchical cancer stem cell model posits that ongoing tumor propagation requires a minority subset of tumor cells with phenotypic traits shared with normal adult stem cells. These cells are deemed necessary to sustain the bulk of a tumor comprised of rapidly proliferating and terminally differentiated cells. By dividing asymmetrically, CSCs simultaneously renew themselves and generate a hierarchy of more differentiated lineages that lack independent tumor propagating ability.

The hierarchical CSC model was first supported experimentally by Dick et al., who identified a subpopulation of acute myeloid leukemia (AML) cells that were CD34^{high}CD38^{low} and could generate xenograft tumors fully recapitulating the cell surface marker heterogeneity of the original tumor (Lapidot et al., 1994; Bonnet & Dick, 1997). In contrast, more differentiated CD34^{low} and CD34^{high}CD38^{high} cells were not tumorigenic. Corroborating findings in AML and chronic myelogenous leukemia have since been described across multiple model systems, including genetically engineered mice, and in patients (Lane & Gilliland, 2010). Over the past decade, solid tumors have also been dissected to identify subpopulations showing enhanced tumorigenicity in xenograft models in conjunction with stem cell-like phenotypes in *in vitro* assays. The CD44^{high}CD24^{low} subset in breast cancer was the first such example and has become perhaps the most extensively characterized population in this regard (Al-Hajj et al., 2003; Visvader & Lindeman, 2008). Since then, stem-like subpopulations have been similarly defined across numerous solid tumor types including brain, prostate, and colon (Singh et al., 2004; Collins et al., 2005; Dalerba et al., 2007; O'Brien et al., 2007; Ricci-Vitiani et al., 2007).

B. Dynamic CSC model

There is growing evidence that some cells in solid tumors meet the experimental criteria used for CSCs but do not adhere to a strict hierarchical model of stemness. Specifically, putative non-CSC populations may revert to the CSC state when provided a permissive microenvironment and thus also contribute to tumor propagation. For instance, more

differentiated, luminal breast cancer cell phenotypes transition to the CD44^{high}CD24^{low} CSC state and allow tumor propagation when co-inoculated with irradiated carrier cells (Gupta et al., 2011). In malignant melanoma, multiple markers of subpopulations with CSC properties have been defined (Boiko et al., 2010, Schatton et al., 2008). However, engraftment of even single human melanoma cells has been shown feasible with simple xenograft assay modifications (Quintana et al., 2008). Furthermore, the lack of CSC marker enrichment among engrafted cells in such modified xenograft experiments supports an absence of hierarchical organization based on currently used melanoma markers (Quintana et al., 2010). Accordingly, expression of the H3K4 histone demethylase JARID1B induces a stem-like state in melanoma cells and is required for long-term tumor propagation, and yet JARID1B^{low} and JARID1B^{high} phenotypes are highly plastic and undergo rapid inter-conversion (Roesch et al., 2010). A comparable epigenetic transition regulated by another JARID1 family member, JARID1A, was shown to be rapidly and reversibly induced by exposure to cytotoxic and EGFR-targeted therapy (Sharma et al., 2010). Such dynamic reversibility between CSC and non-CSC populations has implications for any pharmacologic approach, which must then simultaneously target multiple epigenetic cell states to achieve tumor eradication. At present, the degree to which the current definitions of SCC CSCs conform to hierarchical or dynamic models of stemness remains largely untested.

III. CSCs in SCCs

A. Defining SCC CSCs

CSCs in SCC have been defined by diverse methodologies using cell lines, primary tumor specimens, and patient-derived xenografts (PDXs). A number of assays (sphere-formation, Hoechst dye exclusion, Aldefluor®) and markers (e.g., CD44, CD133) have been used to identify, isolate, and subsequently characterize CSC populations in SCCs (Table 1). Expression of these markers is associated with a variety of other proteins associated with stemness, differentiation, apoptosis regulation and/or drug resistance (Table 2). In distinguishing SCC CSCs, investigators have relied upon two cardinal features of stem cells: self-renewal and differentiation. Though controversial in its interpretation, serial xenotransplantation in animal models remains a key functional assay for self-renewal and lineage capacity and thus for evaluating the stemness of a tumor subpopulation (Clarke et al., 2006). Such studies in SCCs have largely been performed using xenotransplantation of human cells to immune deficient mice rather than in syngeneic mouse models. High tumor formation ability at low cell numbers in limiting dilution assays is used as a correlate of stemness. Self-renewal and differentiation are confirmed based on the subpopulation forming tumors of comparable heterogeneity upon secondary passage. A central caveat of such studies is that cells with innate CSC properties in a human tumor may not necessarily coincide with those that engraft most efficiently in the mouse microenvironment. Also, modifications in assay conditions have been shown to dramatically affect the frequency of human cancer cells determined to be tumor-forming (Quintana et al., 2008). In this regard, changes in tumor disaggregation methods, Matrigel use, and co-injection of non-malignant carrier cells are all known to alter xenotransplantation assay results.

B. Sphere-forming SCC cells

First used to define neural stem cells (Reynolds and Weiss, 1992), sphere-formation assays select stem cells by growing a bulk population at low density on a non-adherent substrate, in absence of serum and presence of defined growth factors. Outgrowth of individual stem cell clones is represented by floating sphere formation. Spheres can be subsequently disaggregated and passaged under distinct culture conditions promoting self-renewal versus differentiation. Variations of this assay are now widely used both for defining stem-like subpopulations *in vitro* and assessing the self-renewal and differentiation potential of

populations selected by other criteria. Accordingly, some studies designate SCC CSCs based solely on the sphere-formation assay, while others use it as a measure of self-renewal and differentiation. Pastrana et al. provide critical review of this assay, highlighting the strengths and limitations to its interpretation (Pastrana et al., 2011).

In contrast to some other solid tumor types, most SCC cell lines and primary tumor cells are relatively inefficient in sphere forming capacity under currently used assay conditions. Sphere-forming cells were identified in only 3 of 47 primary HNSCC specimens dissociated to single cells and grown in tumor sphere medium (Lim et al., 2011). These spheres were passaged as secondary and tertiary spheres, demonstrating self-renewal. HNSCC sphere-derived cells possessed increased colony formation in soft agar relative to their counterparts passaged under differentiating conditions. Chiou et al. enriched for CSCs by culturing two HNSCC cell lines under sphere-forming conditions, producing populations with increased activity in invasion and colony forming assays *in vitro* (Chiou et al., 2008). Additionally, xenografting these sphere-forming cells in limiting dilution assays demonstrated increased tumorigenicity as well as enhanced invasion and neovascularization. Similarly, spheres generated from certain HNSCC cell lines can be serial passaged, and cells derived from the spheres have been shown to be highly invasive *in vitro* (Chen et al., 2011b). These sphere-derived HNSCC cells also showed increased aldehyde dehydrogenase activity, another common CSC marker (section IIID).

C. Side populations in SCC

Goodell et al. originally identified a small subset of bone marrow cells with increased efflux of the vital DNA binding dye, Hoechst 33342. Termed side population (SP) cells based on their location in 2D flow cytometry plots, this subset was demonstrated to contain hematopoietic stem cells (Goodell et al., 1996). Since that time, SP cells have since been associated with stemness in other tissue types and used to isolate CSC candidates in various cancers, including SCC.

The frequency of SP cells in primary SCCs and cell lines reportedly varies from 0.2 to 3%. HNSCC SP cells have shown increased sphere formation, self-renewal over serial passage, and differentiation to restore normal tumor heterogeneity (Tabor et al., 2011; Yanamoto et al., 2011; Sun et al., 2010; Yajima et al., 2009; Loebinger et al., 2009). HNSCC SP cells can also have increased *in vitro* proliferative and colony-forming capacities (Tabor et al., 2011; Loebinger et al., 2008) as well as enhanced tumorigenicity *in vivo* (Yanamoto et al., 2011; Loebinger et al., 2008). SPs have also been defined in primary HNSCCs, accounting for about 0.5% of the tumor (Yanamoto et al., 2011). SP cells isolated from primary esophageal squamous cell carcinomas (ESCCs) show comparable behavior, with increased colony formation *in vitro* and xenograft tumor formation at a limiting dilution of only 100 cells (Li et al., 2011).

D. Aldehyde dehydrogenase activity and SCC CSCs

The aldehyde oxidative function of the aldehyde dehydrogenase family of enzymes participates in retinoic acid biosynthesis and is thus innately linked to regulation of differentiation in squamous epithelia (Douville, et al, 2009). High aldehyde dehydrogenase isoform 1 (ALDH1) activity has been detected in some normal stem cell populations, particularly hematopoietic progenitor cells (Kastan et al., 1990), and subsequently used to isolate CSC candidates in different cancers, including SCCs. A widely used assay for ALDH1 activity is based on the fluorochrome Aldefluor® (BODIPY-conjugated aminoacetaldehyde, Storms et al., 1999), which passively diffuses into the cell and is converted by ALDH1 to BODIPY-aminoacetate. This product is retained within the cell, resulting in a green fluorescence of ALDH^{high} cells.

ALDH^{high} cells isolated from primary HNSCCs were shown to be more tumorigenic as xenografts than ALDH^{low} cells in two studies, but with relatively modest differences in limiting dilution. Specifically, 3,000 ALDH^{high} cells formed tumors in all mice injected, whereas ALDH^{low} cells were not tumorigenic until more than 10,000 cells were used (Chen et al., 2009; Chen et al., 2010). ALDH^{high} cells from primary HNSCC specimens form tumors in mice from as few as 500 cells and recapitulate original tumor histology and heterogeneity with respect to ALDH1 activity (Clay et al., 2010). ALDH^{high} HNSCC cells also appear to have more proliferative and invasive potential as well as higher sphere-forming capacity than ALDH^{low} or parental populations (Chen et al, 2009; Chen et al., 2010; Chen et al., 2011b).

One study further fractionates ALDH^{high} HNSCC cells based on high expression of the cell surface marker CD44 and low expression of CD24 (Chen et al., 2009). CD44 is a cell surface marker used to define CSCs in multiple tumor types including SCCs (section IIIE1) and CD24 is a negative CSC marker in breast cancer that has failed consistent validation in SCCs. ALDH^{high}/CD44⁺/CD24⁻ cells possessed higher tumorigenicity than the ALDH^{high} or CD44⁺/CD24⁻ cells subsets alone and showed the highest *in vitro* proliferation, colony formation, invasion, and sphere formation of all the subsets (Chen et al., 2009). Similarly, addition of CD49f, a normal stem cell marker, to selection of ALDH^{high} cells identified a subpopulation with enhanced stemness features in the HNSCC HEP3 cell line. Notably, these CD49f⁺/ALDH^{high} cells demonstrated a non-hierarchical plasticity with the non-CSC phenotypes defined based on these two markers (Bragado et al., 2012).

E. CSC makers in SCC

1. CD44—The cell surface glycoprotein CD44 is a receptor for matrix hyaluronic acid. The functions of multiple splice variants of this molecule remain poorly understood but may hold significance in the progression of several malignancies (Naor et al., 2002). CD44 has become the most commonly used cell surface marker for CSCs across multiple tumor types and is perhaps the most universally validated CSC marker in HNSCC at present. Prince et al. identified a subpopulation (<10%) of CD44-expressing cells in primary HNSCC specimens with CSC properties (Prince et al., 2007). These CD44⁺ HNSCC cells were highly tumorigenic compared with CD44⁻ cells and successfully propagated in serial xenotransplantation assays. Tumors formed from sorted CD44⁺ cells reproduced the original tumor morphology and heterogeneity with respect to CD44 expression. In a subsequent study, ALDH1 activity was combined with CD44 to select CSCs from primary HNSCCs (Krishnamurthy et al., 2010). CD44⁺/ALDH^{high} cells showed enhanced xenograft tumorigenicity and formed tumors that recapitulated the heterogeneity of the original. This study also described a “gradient of stemness” with respect to colony forming efficiency: CD44⁺/ALDH^{high} > CD44⁺/ALDH^{low} > CD44⁻/ALDH^{low}.

It is important to note that CD44⁺ cells are not consistently a minority subset in HNSCCs, forming up to 80% of cells in many tumors (Joshua et al., 2012). Resembling these tumors, most HNSCC cell lines are nearly 100% CD44⁺, and yet a few cell lines have been further fractionated by some investigators based on distinctions in CD44 cell surface level. Such a CD44^{high} subpopulation (2.1%) isolated from a HNSCC cell line displayed increased sphere-formation, proliferation, migration, and invasion (Okamoto et al., 2009) as well as high CD133 and low CD24 expression, surface signatures of CSCs in other cancers. Furthermore, HNSCC cells grown in tumor sphere media are enriched for CD44^{high} cells (Chikamatsu et al., 2012). In select ESCC cell lines, higher cell surface CD44 levels correlate with tumorigenicity and induced differentiation of these cells decreases CD44 expression (Zhao et al., 2011).

2. CD133—CD133 was initially described as a cell surface marker specific for hematopoietic stem cells (Miraglia et al., 1997; Yin et al., 1997) and subsequently has been pursued extensively as a CSC marker across multiple tumor types (Keysar & Jimeno, 2010). While not a broadly validated marker in SCCs, the existence of a subpopulation of CD133⁺ cells has been reported in certain HNSCC cell lines (1–2% cells) as well as in primary tumor tissues (1–3%) (Zhang et al., 2011). These CD133⁺ cells isolated from HNSCC lines showed increased sphere formation compared with CD133[−] cells, and HNSCC-derived spheres were enriched for CD133⁺ cells (Zhang et al., 2011). This subpopulation also exhibited higher xenograft tumorigenicity than CD133[−] cells and gave rise to both CD133⁺ and CD133[−] cells. Silencing CD133 expression abrogated sphere formation in two HNSCC cell lines and simultaneously decreased colony formation, migration, and invasion while promoting differentiation (Chen et al., 2011a).

3. Other markers

a. c-Met: Signaling by the receptor tyrosine kinase c-Met has been implicated in the progression of a variety of cancers including HNSCC (Gentile et al., 2008; Di Renzo et al., 2000; De Herdt & Baatenburg, 2008). Sun and Wang report a subpopulation of c-Met⁺ cells in three PDXs of HNSCCs that display CSC properties (Sun and Wang, 2011). The c-Met⁺ subset was shown to have enhanced tumorigenicity, with as few as 100 cells forming xenograft tumors that were similarly heterogeneous and could be serially passaged. c-Met⁺ HNSCC cells were metastatic by intracardiac injection whereas c-Met[−] cells were not. As expected, cells positive for both c-Met and CD44 were more tumorigenic than single marker positive cells.

b. GRP78: Expression of membrane-bound 78 kDa glucose-regulated protein (GRP78^{mem}) is another potential regulator of stemness and tumorigenicity in HNSCC cells (Wu et al., 2010). GRP78 (also known as binding immunoglobulin protein BiP) is an endoplasmic reticulum chaperone protein relevant to embryonic stem cell survival (Gonzalez-Gronow et al., 2009; Luo et al., 2006). GRP78 has also been shown to play a role in HNSCC growth and metastatic potential (Chiu et al., 2008). Wu et al. observed increased expression of GRP78^{mem} in six HNSCC cell lines grown in tumor sphere media. Characteristic of CSCs, isolated GRP78^{mem+} cells were sphere-forming, tumorigenic *in vivo*, and generated both GRP78^{mem+} and GRP78^{mem−} cells. SiRNA-mediated silencing of GRP78 diminished sphere formation and drove cells toward a differentiated phenotype (involcrin⁺/CK18⁺).

c. p75^{NTR}: The low-affinity neurotrophin receptor p75^{NTR} regulates neuron survival, differentiation and apoptosis and has been used as a marker of various stem and progenitor cell populations (Okumura et al., 2003; Campagnolo et al., 2001; Yamamoto et al., 2007; Qi et al., 2008; Boiko et al., 2010). Cells positive for p75^{NTR} from four ESCC cell lines showed increased capacity to be passaged as spheres as well as higher tumorigenicity than p75^{NTR−} cells *in vivo* (Huang et al., 2009).

4. Stemness markers in SCC CSCs

a. Oct4, Sox2, Nanog: The transcription factors Oct4, Sox2, and Nanog are required to maintain pluripotency and self-renewal in embryonic stem cells (ESCs) (Loh et al., 2006; Boyer et al., 2005). Enhanced expression of these factors is often observed in SCC CSCs, supporting the innate stemness of subpopulations currently defined based on other current markers. Sphere-forming SCC CSCs have been shown to express increased levels of Oct4, Nanog, and Sox2 mRNA and protein (Lim et al., 2011; Chiou et al., 2008; Chen et al., 2011b). Nestin, an intermediate filament protein widely employed as a marker of neural stem cells (Park et al., 2010), is also increased in HNSCC tumor spheres (Lim et al., 2011; Chiou et al., 2008). ALDH^{high} HNSCC subpopulations show expression patterns similar to

ESCs including high expression of Oct4, Nanog, and Sox2, as well as nestin and the transcription factor Klf4 (Chen et al., 2010; Chen et al., 2009). Elevated Oct4 and Nanog mRNA levels are also present in SP cells (Tabor et al., 2011; Sun et al., 2010). Similarly, CSC subpopulations expressing the cell surface proteins CD44 and CD133 also display heightened levels one or more of these factors (Chikamatsu et al., 2012; Zhang et al., 2010; Chen et al., 2011a).

b. Bmi1: Bmi1 is a member of the Polycomb family of transcription repressors, which have been implicated in processes that regulate stem cell fate (Park et al., 2004). Bmi1 is necessary for efficient self-renewal of adult hematopoietic and neuronal stem cells (Park et al., 2003; Molofsky et al., 2003). SCC CSCs defined by various methods show high expression of Bmi1 (Chen et al., 2010; Chen et al., 2010; Yamamoto et al., 2011; Prince et al., 2007; Chikamatsu et al., 2012; Huang et al., 2009). Knockdown of Bmi1 in ALDH^{high} CSCs significantly inhibited the colony-forming and invasion capacities of these cells, supporting a role for Bmi1 in regulating a CSC state in HNSCC (Chen et al., 2010). Furthermore, microarray analysis revealed a shift away from an ESC-like gene profile upon Bmi1 downregulation.

c. Other stemness markers: Various other factors known to play roles in stem cell regulation are also found to be differentially expressed in SCC CSCs. For example, p63, a marker of tissue-specific stem cells in squamous epithelia (Pellegrini et al., 2001), is upregulated in p57^{NTR+} ESCC cells. The Wnt/ β -catenin and Notch pathways play key regulatory roles in adult stem cells in various tissues (Conboy and Rando, 2002; Korkaya et al., 2009; Brabletz et al., 2009; Fre et al., 2005; Blanpain et al., 2006). Notch1 signaling normally drives keratinocyte differentiation in squamous epithelia but appears to have alternate, stemness-promoting functions upon malignant transformation (Ohashi et al., 2010; Ohashi et al., 2011). Expression of Notch1 and β -catenin is increased in CD44⁺ and CD133⁺ HNSCC cells, respectively (Chikamatsu et al., 2012; Zhang et al., 2010). CD133⁺ CSCs also upregulate expression of the stem cell-associated gene hTERT (Zhang et al., 2010). Concurrently, markers of squamous epithelial differentiation like involucrin and CK18 are typically decreased in CSC populations (Lim et al., 2011; Zhao et al., 2010; Chen et al., 2011a; Huang et al., 2009).

IV. Epithelial to mesenchymal transition and stemness in SCCs

Epithelial to mesenchymal transition (EMT) diversifies cell types during embryogenesis and also allows epithelial cells to acquire a migratory, mesenchymal-like phenotype during wound healing. There is accumulating evidence that a similar EMT contributes to invasion and metastasis of carcinoma cells (Singh and Settleman, 2010; Yang & Weinberg, 2008). The relevance of EMT to CSCs was first defined in CD44⁺C24⁻ breast cancer cells, which exhibit a prominent mesenchymal-like gene expression profile (Mani et al., 2008; Morel et al., 2008).

Currently defined SCC CSCs also possess mesenchymal-like traits, and inducing EMT in HNSCC cells correlates with the emergence of CSCs and vice versa. Gene expression profiling of ALDH^{high} HNSCC cells demonstrated an EMT-associated expression signature (Chen et al., 2009). Overexpression of stem cell surface protein CD133 in HNSCCs similarly induces expression of mesenchymal markers vimentin and fibronectin while down-regulating epithelial specific antigen (ESA) (Chen et al., 2011a). Sphere-forming HNSCC cells express increased levels of Snail, Twist, α -SMA, and vimentin and possess a more invasive phenotype (Chen et al., 2011b). Similarly, CD44^{high}ESA^{low} cells within HNSCC cell lines possess fibroblast-like morphology, and express high mesenchymal markers

vimentin, Snail, Twist, and Axl, versus low E-cadherin (Biddle et al., 2011). Accordingly, inducing EMT by adding TGF β enriches for these CD44^{high}ESA^{low} CSCs.

Modulation of EMT-related genes can affect CSC populations in HNSCC. Lo et al. found overexpression of the metastasis-promoting gene S100A4 to drive EMT and stemness in HNSCC cell lines (Lo et al., 2011). Likewise, silencing of S100A4 simultaneously inhibited sphere-formation, Oct4 and Nanog expression, and xenograft tumor formation. Inhibition of Snail in ALDH^{high} HNSCC cells also suppresses the CSC phenotype, evidenced by decreased sphere formation and tumorigenicity (Chen et al., 2009).

V. Therapy-resistance in CSCs

The critical function of adult stem cells in normal tissue homeostasis necessitates their resistance to diverse stressors, including hypoxia, nutrient deprivation, radiation, and chemical toxins. The cancer stem cell hypothesis predicts that CSCs possess comparable resistance to chemotherapy and radiation and thus serve as a reservoir for tumor repopulation post-therapy. Limited studies of CSCs in SCCs show evidence of such enhanced therapy-resistance (Table 3), while mechanistic understanding in this area remains to be fully developed.

A. Resistance to chemotherapy

Some studies have used cell viability assays (MTT, MTS) to assess the sensitivity of SCC CSCs defined by functional readouts such as sphere-forming capacity, SP status, and ALDH activity to a variety of chemotherapeutic drugs. Cells dissociated from primary HNSCC-derived spheres showed greater resistance to multiple cytotoxic drugs relative to HNSCC cells grown under differentiating conditions (Lim et al., 2011). SP cells isolated from HNSCC cell lines display increased survival compared with non-SP cells after treatment with the cytotoxic drug 5-fluorouracil (5-FU) (Tabor et al., 2011; Yamamoto et al., 2011, Yajima et al., 2009). SPs from HNSCC and ESCC lines have also shown enhanced viability relative to non-SPs upon treatment with conventional and targeted drugs including platinum compounds (Li et al., 2011; Yajima et al., 2009) and bortezomib (Li., et al., 2011). Sensitivity to the drug taxol can be increased in primary HNSCC-derived ALDH^{high} cells through siRNA-mediated silencing of the stemness gene Bmi1 (Chen et al., 2010). SPs from HNSCC lines were shown to maintain increased colony formation compared with parental cells when cultured in the presence of the topoisomerase I inhibitor mitoxantrone (Loebinger et al., 2008).

Other studies have tested drug resistance in CSCs defined by cell surface markers. For example, a CD44^{high} subpopulation from the HNSCC Gun-1 line showed modestly increased viability, measured by MTS assay, compared with CD44^{low} cells after treatment with a panel of cytotoxic drugs (5-FU, docetaxel, paclitaxel, cisplatin, carboplatin) (Okamoto et al., 2009).

Drug treatment has also been shown to enrich for populations of SCC CSCs. Treatment of HNSCC cells with 5-FU and paclitaxel enriches for SP and CD133⁺ cells, respectively (Yajima et al., 2009; Zhang et al., 2010). A subpopulation of p75^{NTR+} cells is increased in ESCC when exposed to cisplatin (Huang et al., 2009). c-Met⁺ CSCs are markedly enriched in HNSCC-xenografted mice treated with cisplatin (Sun & Wang, 2011). These cisplatin resistant tumor cells also have enhanced secondary tumor growth, supporting a role for c-Met⁺ CSCs in disease relapse. Interestingly, HNSCC cells selected for cisplatin resistance were found to possess several CSC properties including increased proliferation, sphere-forming capacity, colony formation, and invasion compared to the drug sensitive parent cells

(Tsai et al., 2011). Cisplatin resistant HNSCC cells also express high levels stem cell surface markers (CD133 and c-Kit) as well as stemness markers Oct4, Nanog, Nestin, and Bmi1.

B. Resistance to radiation

Radiation resistance has been shown to increase within CSC subpopulations in SCC cell lines and primary tumors. Sphere-forming cells derived from an HNSCC cell line were less sensitive to up to 10 Gray of ionizing radiation than parental cells (Chiou et al., 2008). ALDH^{high} and ALDH^{high}/CD44⁺/CD24⁻ populations sorted from primary HNSCC tumors displayed a comparably decreased radiation dose response relative to parental or ALDH^{low} cells (Chen et al., 2009). Two cell lines containing small subsets of CD44^{hi} cells were also used to show a modest increase in survival of this subpopulation following exposure to 10 Gray (Chikamatsu et al., 2012). Sensitivity to irradiation may be restored through silencing of genes involved with CSC maintenance; knockdown of Bmi1 or GPR78 restored sensitivity to ionizing radiation in primary ALDH^{high} HNSCC cells and GPR78^{mem+} cells from HNSCC lines, respectively (Wu et al., 2010; Chen et al., 2010). Importantly, how CSCs respond to the radiation dosing and fractionation regimens used clinically for HNSCC remains unknown.

C. Mechanisms of drug resistance in CSCs

Acquired drug resistance in clonal populations of tumor cells can arise by both induction of epigenetic changes and selection of spontaneous genetic variants that confer survival advantage during treatment. Based on the cancer stem cell hypothesis, drug therapy may selectively enrich intrinsically resistant CSCs and/or promote acquired resistance by inducing epigenetic shifts that drive differentiation to a stem-like state. Resistance in CSCs likely derives from multiple factors including quiescence, resistance to DNA damage/capacity for DNA repair, and expression of ABC-transporter pumps and anti-apoptotic proteins (Figure 2).

1. Multidrug efflux proteins—Alteration of effectors that regulate the accumulation of drugs within cells is one of the most studied mechanisms of multidrug resistance. Adenosine triphosphate-binding cassette (ABC) transporters, a class of multidrug efflux pumps, are known to be associated with cancer drug resistance. Normal stem cells express high levels of specific ABC-transporters, which function to protect them from certain damaging agents (Scharenberg, et al., 2002; Moitra, et al., 2011). Similarly, CSCs can express higher levels of these efflux proteins that afford protection to some chemotherapeutic drugs.

a. ABCG2: ABCG2 is an ABC-transporter that homodimerizes at the plasma membrane and actively effluxes a range of substrates, including both cytotoxic compounds and fluorescent DNA binding Hoechst dyes (Sarkadi et al., 2004). It is therefore not surprising that CSCs selected based on their enhanced ability to exclude Hoechst (SP cells) often show increased resistance to chemotherapeutic drugs. SCC SP cells have been reported to display increased ABCG2 expression and/or activity (Li et al., 2011; Tabor et al., 2011; Yamamoto et al., 2011; Geng et al., 2011; Sun et al., 2010; Yajima et al., 2009), which may mediate resistance to diverse cancer drugs including platinum compounds, bortezomib, and 5-FU (Li et al., 2011; Tabor et al., 2011; Yamamoto et al., 2011; Sun et al., 2010; Yajima et al., 2009). In support of a role for ABC family proteins in CSC drug resistance, SCC SP cells can be sensitized to chemotherapy upon general inhibition of ABC transporters by the calcium channel blocker verapamil (Loebinger et al., 2008).

Tumor spheres generated from primary HNSCC specimens showed increased ABCG2 expression (Chiou et al., 2008; Lim et al., 2011) as well as a higher fraction of SP cells compared with the same cells maintained in differentiating media (Lim et al., 2011).

Furthermore, these spheres displayed less sensitivity to paclitaxel, cisplatin, 5-FU, and docetaxel than their differentiated counterparts. CSCs defined by expression of the cell surface markers CD44 and CD133 or by ALDH1 activity also express elevated levels of ABCG2 and can be more resistant to various therapies (Okamoto et al., 2009; Zhang et al., 2010; Zhao et al., 2011; Chen et al., 2009; Chen et al., 2010). Finally, SCC cells with a CSC-like phenotype selected for cisplatin resistance show enhanced ABCG2 expression (Tsai et al., 2011).

b. Other ABC- transporters: ABCB1, also known as MDR1 or P-glycoprotein, can bind a variety of hydrophobic compounds including the anticancer drugs doxorubicin, vinblastine, and taxol (Gottesman, 2002). High expression of ABCB1 is found in SCC CSC populations selected by multiple methods, including SP analysis and ALDH1 activity (Li et al., 2011; Yajima et al., 2009; Chen et al., 2009). CD44⁺ cells from ESCC specimens, which are enriched upon treatment with 5-FU or cisplatin, show increased expression of ABCA5 in addition to ABCG2 (Zhao et al., 2011). ALDH^{high} and ALDH1^{high}/CD44⁺/CD24⁻ populations of HNSCC cells were shown to express multiple efflux pumps including ABCG2, ABCB1, and ABCC1 (or MRP1) (Chen et al., 2009). SP cells from ESCC tumors were shown to have high expression of a number of different ABC-transporters (ABCB1, ABCG2, ABCA3, ABCC1), as well (Li et al., 2011).

2. Resistance to apoptosis—Evasion of apoptosis as a pro-survival strategy is a hallmark of both cancer and stem cells (Kruyt & Schuringa, 2010; Hanahan & Weinberg, 2011); thus activation of anti-apoptotic pathways likely plays a role in resistance of CSCs to therapy. Differential expression of apoptosis-related genes, most commonly B cell lymphoma/leukemia-2 (Bcl-2) family genes, is described in SCC CSCs. The BCL2 oncogene product suppresses apoptosis through inhibition of caspase activation (Ola et al., 2011). SP cells from an HNSCC line showed increased levels of BCL2 and BCL2A1 gene expression (Yajima et al., 2009) along with high expression of another pro-survival gene, CFLAR. CD44^{high} CSCs within certain HNSCC lines have decreased basal levels of apoptosis and display an increased resistance to the apoptotic inducing stimuli TNF-alpha, anti-Fas, and TRAIL (Chikamatsu et al., 2012). This CD44^{high} subpopulation also up-regulated Bcl-2, Bcl-2 family genes BCL2A1 and BCL2L1, and inhibitor of apoptosis protein (IAP) family genes BNIP1 and NAIP. Manipulation of mediators that regulate SCC CSC dynamics can restore sensitivity to apoptosis. Knockdown of Bmi1 in ALDH^{high} primary HNSCC cells increased apoptosis along with sensitivity to taxol (Chen et al., 2010). Similarly, silencing of GRP78 induced the expression of pro-apoptotic molecules Bax and Caspase 3 in CSCs in HNSCC cell lines (Wu et al., 2010).

3. EMT—EMT has received considerable attention for its emerging role in intrinsic and acquired drug resistance (Singh and Settleman, 2010). We have previously demonstrated that a low-turnover, mesenchymal-like subpopulation within HNSCC cell lines and PDXs resists both cytotoxic and EGFR-targeted therapy (Basu et al., 2010; Basu et al. 2011). Although mechanisms underlying this EMT-based resistance are incompletely defined, they likely overlap with those attributed to CSC populations, which can share a mesenchymal-like gene signature (section IV).

4. Other potential mechanisms of drug resistance—Diverse additional mechanisms likely underlie intrinsic therapy resistance in CSCs. For example, Akt activity is enhanced in SP cells from primary ESCC tumors (Li et al., 2011). Activation of the pro-survival PI3K/Akt pathway is associated with chemoresistance, and inhibition of this pathway induces apoptosis and decreases growth of drug-resistant tumor cells (Lee et al., 2004; Abdul-Ghani et al., 2006; Cordo Russo et al., 2008; Garcia et al., 2009). Inhibition of Akt in these SP cells

decreased ABCG2 activity, thus linking this pathway to drug efflux mechanisms (Li et al., 2011). CD133⁺ HNSCC cells display increased activation of the tyrosine kinase Src, which continues to hold interest as a potential target for overcoming cytotoxic drug resistance (Grant and Dent, 2004; Chen et al., 2011a). p75^{NTR}⁺ ESCC CSCs express low levels of the major copper influx transporter CTR1, which has been shown to mediate cisplatin uptake, potentially contributing to the resistance of this population to the drug (Huang et al., 2009). Future analyses of SCC CSCs will likely identify other targetable signaling components as regulators of their drug resistance.

VI. CSCs in SCC clinical samples and prognosis

Identifying CSCs in human tissues is limited by the inability of any single current marker to accurately select all the cells of interest while excluding other phenotypes. Still, some markers associated with SCC CSCs in preclinical investigations have been validated in clinical specimens, and, in some cases, correlated with disease grade and/or prognosis.

Normal stem cells reside in the basal layer of mucosa in the upper aerodigestive tract (Janes and Watt, 2006) and thus CSCs may also be found in the basal compartments of those SCC tumors retaining a stratified architecture. Accordingly, an HNSCC PDX showed CD44⁺ staining to be most intense in the basal layer a well-differentiated tumor (Prince et al., 2007). CD44 costained with the basal cytokeratin CK5(14) in this primary HNSCC, with involucrin staining being mutually exclusive with these markers. Cells that were positive for CD44 and nuclear Bmi1 were also mainly localized to basal regions; coexpression of CD44 and nuclear Bmi1 however was most prominent in poorly differentiated tumors (Prince et al., 2007). Sterz et al., observed that CD44 colocalized with the matrix metalloproteinase, MMP-9 within a basal-cell-like compartment at the invasive front of HNSCC tissues (Sterz et al., 2010). CD44 also showed strongest staining in the basal layer of well-differentiated ESCC specimens (Zhao et al., 2011). Interestingly, Krishnamurthy et al. observed that in primary HNSCC tumors, ALDH^{high} cells were found mainly in close proximity to blood vessels, suggesting a potential perivascular CSC niche (Krishnamurthy et al., 2010).

Coexpression of CSC markers Oct4 and Nanog is increased in cisplatin-resistant tumors (Tsai et al., 2011). Increased expression of Oct4, Nanog, and CD133, individually or in combination, was observed in association with higher grade in HNSCC (Chiou et al., 2008). Importantly, expression of one or more of these markers more strongly correlated with poor prognosis, which in HNSCC is not clearly associated with grade, and coexpression of all three predicted the worst overall survival. It was further noted that CD133⁺ cells in tumors are not consistently positive for either Oct4 or Nanog (Chiou et al., 2008), which likely reflects the inability of surface markers in current use to fully capture CSCs. S100A4 expression also appears to have prognostic significance, correlating with moderate to poor differentiation and worse overall survival in HNSCC (Lo et al., 2011).

S100A4 is also found coexpressed with Oct4 and Nanog (Lo et al., 2011). GRP78 similarly correlates with poor prognosis in HNSCCs, and co-expression with Nanog further increases its negative prognostic value (Wu et al., 2010). ABCG2, which is often highly expressed in SCC CSCs, may also be an independent prognostic factor associated with poor survival in ESCC (Tsunoda et al., 2006).

VII. Discussion

It is evident that CSC populations with self-renewal and differentiation capacities exist within head and neck and esophageal SCCs. To date, few studies go further than isolating such populations and supporting their stemness based on sphere formation and clonogenicity *in vitro*, tumorigenicity in xenograft models, and gene expression profiling. A deeper

understanding of the mechanisms that govern the dynamics of SCC CSCs may be critical for deciphering their roles in SCC progression and for targeting for therapeutic benefit. Key questions include (1) the extent to which CSCs as currently defined contribute to intrinsic drug resistance, relative to subpopulations in the non-CSC pool (2) the detailed intrinsic resistance mechanisms in CSCs (3) developmental relationships between CSCs and non-CSCs that determine the outcome of successful CSC targeting (4) developmental relationships between CSCs and other potentially related subpopulations with therapy resistance, including those defined by hypoxia, autophagy, and/or quiescence.

The diverse methods and markers discussed here provide tools for studying CSCs but likely fail to capture all (or the only) tumor-propagating cells within a population. CD44, to date the most broadly applicable marker of CSCs in primary human SCCs, is not without its caveats. The frequency of CD44⁺ cells varies greatly between tumors, with reported frequencies up to 80% in aggressive primary HNSCCs (Joshua et al., 2012), making it unlikely to reflect the heterogeneity most relevant to determining the treatment response in these tumors. Additionally, the level of ERK1/2 activation in a given HNSCC directly regulates CD44 surface expression, *in vitro* growth, and engraftment efficiency (Judd et al., 2012) in a manner that may not necessarily be linked to CSC frequency.

Though evidence supports CD44 as a CSC marker in SCCs, it is unlikely that a CD44⁺ subpopulation is comprised exclusively of highly tumorigenic stem-like cells. Indeed, CD44⁺ populations exhibit heterogeneity in expression of other CSC markers, proliferation, and tumor formation/propagation and can be subdivided using additional markers like ALDH1 and c-Met to enhance tumorigenicity and stemness (Krishnamurthy et al., 2010; Sun & Wang, 2011). The apparent heterogeneity of SCC CSCs presents the challenge of systematically delineating the transition between these subpopulations and their individual roles in progression and therapy resistance. This complexity has been nicely illustrated by Biddle et al, who subdivide CD44^{high}-expressing HNSCC cells by ESA level to reveal two biologically distinct phenotypes: an epithelial-like CSC population (CD44^{high}/ESA^{high}) and a mesenchymal-like CSC population (CD44^{high}/ESA^{low}). Moreover, ALDH activity could predict the bipotent capacity of the CD44^{high}/ESA^{low} population; CD44^{high}/ESA^{low}/ALDH^{high} cells were bipotent, whereas CD44^{high}/ESA^{low}/ALDH^{low} cells were not (Biddle et al., 2011). These data support a model of cell type regulation in HNSCC with a dynamic component. Biddle et al. propose these HNSCC tumor cells exhibit a phenotypic plasticity in which CD44^{high}/ESA^{high} CSCs can self-renew, produce terminally differentiated CD44^{low} cells, or undergo EMT generating CD44^{high}/ESA^{low} CSCs. The mesenchymal-like CSCs (CD44^{high}/ESA^{low}) with high ALDH1 activity can, in turn, self-renew, differentiate to a unipotent ALDH^{low} state, or undergo mesenchymal to epithelial transition, regenerating the epithelial-like CSCs (CD44^{high}/ESA^{high}).

Further evidence of nonhierarchical differentiation by CSCs is provided by the capacity of both CD44⁺ and CD44⁻ HNSCC cells from primary tumors to form spheres, suggesting that the negative population can also enter a self-renewing state (Lim et al., 2011). In addition to highly tumorigenic CD49^{high}/ALDH^{high} cells in a HNSCC cell line, the CD49^{low}/ALDH^{low} population was shown to have latent tumorigenic potential (Bragado et al., 2012). Existence of phenotypic plasticity between CSCs and non-CSCs have garnered increasing support in other tumor types (Roesch et al., 2010; Gupta et al., 2011; Quintana et al., 2010).

The resistance of SCC CSCs to chemotherapy and radiation remains to be precisely defined at the mechanistic level; several inherent properties of stem cells appear to play a role, including altered expression of drug transporter molecules, evasion of apoptosis, and EMT-based shifts in gene expression. Altered cell signaling, including those mediated by the PI3K/Akt pro-survival pathway and others, may confer resistance to cytotoxic and targeted

therapies. For instance, a recent study using cutaneous SCC cell lines found CSC features localizing to a subpopulation (1.3%) with low cell surface EGFR expression (Le Roy et al., 2010), suggesting potential resistance to the EGFR-targeted therapies in current clinical use. Additional hallmarks of stem cells, such as alteration of DNA repair pathways or maintenance of a quiescent state, may play important roles in the survival of therapy-resistant CSCs.

Normal epithelial tissues contain slow-cycling stem cells that divide asymmetrically, giving rise to new stem cells that retain their quiescence as well as actively cycling transit-amplifying cells. Similarly, quiescence has been linked to CSCs in various tumor types (Moore & Lyle, 2001). Label-retaining methods, in which low-turnover cells are identified by retention of a fluorescent vital membrane dye that dilutes as a cell divides, have identified subpopulations of cells with tumor-forming and/or propagating capacities in melanoma, glioblastoma, and ovarian, breast, colon, and pancreatic cancers (Roesch et al., 2010; Deleyrolle et al., 2011; Kusumbe & Bapat, 2009; Fillmore & Kuperwasser, 2008; Moore et al., 2011; Dembinski & Krauss, 2009). Like CSC populations, label-retaining cells (LRCs) can possess an inherent resistance to chemotherapy (Kusumbe & Bapat, 2009; Fillmore & Kuperwasser, 2008; Moore et al., 2011; Dembinski & Krauss, 2009). Little is known about slow-cycling subpopulations of cells in head and neck and esophageal SCCs. A recent study revealed that a slow-cycling subpopulation of HNSCC cells, defined by retention of the fluorescent label CFSE, display enhanced proliferative potential and produced heterogeneous tumors in xenografted mice (Bragado, 2012). These LRCs are also enriched for CD49f, a marker of normal stem cells. Investigation into how these quiescent cells as well as CSCs defined by other methods differentially regulate cell cycle progression may provide important insight into their roles in tumorigenesis and drug resistance. Furthermore, using quiescence to identify CSC subpopulations in SCCs may shed light on the heterogeneity of CSCs and offer novel markers for these cells.

VII. Conclusion

Significant evidence supports a role for CSCs in intrinsic SCC therapy-resistance, though the specific mechanisms by which these subpopulations escape treatment are not currently understood. In this regard, there exist a number of ongoing challenges. Current CSC models are limited in their ability to encompass all drug-resistant SCC cells with a single molecular state or marker. Going forward, the detailed methods used to identify SCC CSCs also merit increased attention, as differences in engraftment host and assay conditions can greatly impact *in vivo* tumorigenicity. Understanding the roles of SCC CSCs in regulating tumor heterogeneity and therapy-resistance is increasingly complicated by evidence for multidirectional state transitions between CSC and non-CSC subpopulations. Nevertheless, advancing understanding of CSC biology in SCC is likely to ultimately impact the development of novel therapeutic strategies.

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Abbreviations

AML	Acute myeloid leukemia
ABC	Adenosine triphosphate binding cassette

ALDH	Aldehyde dehydrogenase
CSC	Cancer stem cell
ESC	Embryonic stem cell
EGFR	Epidermal growth factor receptor
ESA	Epithelial specific antigen
EMT	Epithelial to mesenchymal transition
ESCC	Esophageal squamous cell carcinoma
5-FU	5-fluorouracil
HNSCC	Head and neck squamous cell carcinoma
SP	Side population
SCC	Squamous cell carcinoma

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Figure 1. Proposed biological properties of HNSCC CSCs. HNSCC CSCs are defined primarily by their capacities for self-renewal and differentiation. They also can possess several additional CSC traits (high tumorigenicity, low-turnover, high invasion/migration, evasion of apoptosis), some of which may contribute to their resistance to chemo- and radiotherapies.

Figure 2.
Model and proposed mechanisms of CSC-mediated therapy resistance.

Table 1

CSCs in squamous cell carcinoma of the esophagus, head and neck

Assay/marker(s)	Description	Origin of SCC	Stem-like properties
Sphere-forming	Single cells that can form spheres in an <i>in vitro</i> assay are considered to possess both the ability to self-renew and differentiate.	HNSCC * (Lim et al., 2011), laryngeal (Chen et al., 2011b), tongue (Chen et al., 2011b; Chiou et al., 2008), gingival (Chiou et al., 2008)	self-renewal (serial propagation), differentiation, expression of stemness markers, increased colony formation/invasion, tumor-forming, propensity for quiescence (G ₀ /G ₁ , Ki-67)
Side population (SP)	Small populations of cells that do not accumulate appreciable levels of Hoechst 33342 dye, likely due to increased efflux.	tongue (Tabor et al., 2011; Sun et al., 2010; Loebinger et al., 2008), laryngeal (Yamamoto et al., 2011), buccal (Yajima et al., 2009), esophageal (Li et al., 2011), oropharyngeal (Clay et al., 2010)	self-renewal (sphere-formation), differentiation (reproduce heterogeneity), increased proliferation/colony formation, expression of stemness marker and drug resistance genes, tumor-forming
ALDH ^{high}	Cell that have high levels of aldehyde dehydrogenase activity measured by its conversion of a fluorescent substrate to a negative product which is retained intracellularly, marking the ALDH-expressing cells.	HNSCC * (Chen et al., 2010), buccal, retromolar trigone (Chen et al., 2009), laryngeal (Clay et al., 2010; Chen et al., 2011b), tongue (Clay et al., 2010; Chen et al., 2011b) oropharyngeal (Clay et al., 2010)	self-renewal (sphere-formation), increased proliferation/colony formation/invasion, expression of stemness markers, tumor-forming
CD44 ⁺	Cell surface glycoprotein; most frequently expressed surface marker on CSLCs.	HNSCC * (Prince et al., 2007), hypopharyngeal (Chikamatsu et al., 2012; Okamoto et al., 2009), gingival (Chikamatsu et al., 2012), esophageal (Zhao et al., 2011)	self-renewal (sphere-formation, serial propagation), differentiation (reproduce heterogeneity), increased proliferation/invasion/migration, expression of stemness markers and drug resistance genes, tumor-forming
CD133 ⁺	Cell surface glycoprotein; marker for CSLCs in some solid tumor types.	tongue (Zhang et al., 2010; Chen et al., 2011), buccal (Zhang et al., 2010), gingival (Chen et al., 2011)	self-renewal (sphere-formation), differentiation (reproduce heterogeneity), increased colony formation/invasion, expression of stemness markers, tumor-forming
GRP78 ^{green+}	Glucose regulated protein78; mediator of endoplasmic reticulum homeostasis, anchored at the plasma membrane.	tongue, gingival (Wu et al., 2010)	self-renewal (sphere-formation), differentiation (reproduce heterogeneity), increased colony formation/invasion, expression of stemness markers, tumor-forming
c-Met ⁺	Tyrosine kinase receptor for hepatocyte growth factor.	oropharyngeal, buccal, tongue (Sun & Wang, 2011)	self-renewal (serial transplantation), differentiation (reproduce heterogeneity), increased colony formation, expression of stemness genes, tumor-forming
p75 ^{NTR+}	Low-affinity neurotrophin receptor; mediates neuronal survival, differentiation, and apoptosis.	esophageal (Huang et al., 2009)	self-renewal (sphere-formation, serial passage), differentiation (reproduce heterogeneity), tumor-forming

* specimen(s) of unknown origin

Table 2

Differentially expressed markers in squamous cell carcinoma CSCs

Positive markers		
Oct4, Sox2, Nanog, Nestin, Bmi1, Klf4, Notch, p63, hTERT, β -catenin	Stemness	(Lim et al., 2011; Wu et al., 2010; Chiou et al., 2008; Tsai et al., 2011; Chen et al., 2010; Chen et al., 2009; Tabor et al., 2011; Yamamoto et al., 2011; Prince et al., 2007; Chikamatsu et al., 2012; Zhao et al., 2011; Zhang et al., 2010; Krishnamurthy et al., 2010)
ABCG2, ABCB1, ABCA3, ABCA5, ABCC1	Drug resistance	(Lim et al., 2011; Chiou et al., 2008; Tsai et al., 2011; Chen et al., 2009; Li et al., 2011; Tabor et al., 2011; Yamamoto et al., 2011; Sun et al., 2010; Yajima et al., 2009; Zhao et al., 2011; Okamoto et al., 2009)
BCL2, BCL2A1, BCL2L1, BNIP1, NAIP, CFLAR	Apoptosis	(Yajima et al., 2009; Chikamatsu et al., 2012)
Snail, Twist, vimetin, N- Cadherin, EpCAM(ESA) *	EMT (mesenchymal)	(Chen et al., 2009; Tabor et al., 2011; Chen et al., 2011a; Chen et al., 2011b; Lo et al., 2011, Biddle et al., 2011)
Negative markers		
CK5(14), CK4(13), CK18, involucrin	Differentiation	(Lim et al., 2011; Wu et al., 2010; Zhao et al., 2010)
E-cadherin, EpCAM(ESA) *	EMT (epithelial)	(Chen et al., 2009; Chen et al., 2011a; Lo et al., 2011, Biddle et al., 2011)

* CD44^{high}ESA^{high}, CD44^{high}ESA^{low}ALDH^{high} denote distinct, dynamic epithelial-like and mesenchymal-like CSC populations, respectively (Biddle et al., 2011)

Table 3

Therapy-resistance in squamous cell carcinoma CSCs

Treatment	CSC population(s) with increased resistance	References
5-fluoruracil	tumor spheres, SP, CD44 ⁺	(Lim et al., 2011; Tabor et al., 2011; Yamamoto et al., 2011, Yajima et al., 2009; Okamoto et al., 2009)
cisplatin	tumor spheres, SP, CD44 ⁺	(Lim et al., 2011; Li et al., 2011; Yajima et al., 2009; Okamoto et al., 2009; Sun & Wang, 2011)
carboplatin	SP, CD44 ⁺	(Yajima et al., 2009; Okamoto et al., 2009)
paclitaxel	tumor spheres, CD44 ⁺ , CD133 ⁺	(Lim et al., 2011; Okamoto et al., 2009; Zhang et al., 2009)
docetaxel	tumor spheres, CD44 ⁺	(Lim et al., 2011; Okamoto et al., 2009)
bortezomib	SP	(Li et al., 2011)
mitoxantrone	SP	(Loebinger et al., 2008)
radiation	tumor spheres, CD44 ⁺ , ALDH ^{high}	(Chiou et al., 2008; Chen et al., 2009; Chikamatsu et al., 2012)