The biology of head and neck cancer stem cells

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Abstract

Emerging evidence indicates that a small population of cancer cells is highly tumorigenic, endowed with self-renewal, and has the ability to differentiate into cells that constitute the bulk of tumors. These cells are considered the "drivers" of the tumorigenic process in some tumor types, and have been named cancer stem cells. Epithelial-mesenchymal transition (EMT) appears to be involved in the process leading to the acquisition of stemness by epithelial tumor cells. Through this process, cells acquire an invasive phenotype that may contribute to tumor recurrence and metastasis. Cancer stem cells have been identified in human head and neck squamous cell carcinomas (HNSCC) using markers such as CD133 and CD44 expression, and aldehyde dehydrogenase (ALDH) activity. The head and neck cancer stem cells reside primarily in perivascular niches in the invasive front where endothelial-cell initiated events contribute to their survival and function. In this review, we discuss the state-of-the-knowledge on the pathobiology of cancer stem cells, with a focus on the impact of these cells to head and neck tumor progression.

Keywords

Oral cancer; Tumorigenesis; Epithelial-mesenchymal transition; EMT; Self-renewal; Stemness; Perivascular niche; Squamous cell carcinoma; Angiogenesis

Introduction

Head and neck cancer is a major health problem throughout the world. In 2008, 263 900 new cases of head and neck cancer were diagnosed, and 128 000 deaths related to this malignancy have occurred worldwide.[1] In the United States alone, there were 49 260 new cases and 11 480 deaths that were attributed to head and neck cancer in 2010.[2] The standard of care for patients with head and neck squamous cell carcinomas (HNSCC) includes platinum-based chemotherapeutic drugs, surgery, and radiotherapy.[3] However, the 5-year

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survival rate for these patients has remained in the range 50–60% for the last 3 decades.[4] It is becoming increasingly evident that an improvement in the survival of head and neck cancer patients will require deeper understanding of the mechanisms underlying the initial steps of the tumorigenic process, as well as the strategies employed by cancer cells to disseminate to local lymph nodes and distant sites. Recent studies on the pathobiology of HNSCC have led to the discovery of a small population of cancer cells that is highly tumorigenic, capable of self-renewal, and behave as tumor progenitor cells.[5] Such behavior is consistent with the features of cancer stem cells (CSC). Notably, cancer stem cells appear to play a major role in tumor recurrence and metastatic spread, common causes of the high morbidity and ultimately the death of the majority of patients with HNSCC. Therefore, targeted elimination of these cancer stem cells has been considered a new conceptual framework for head and neck cancer treatment. This review discusses the putative role of stem cells in tumorigenesis, the biological process that leads to the acquisition of stem cell properties, and the potential impact of the cancer stem cell hypothesis to the management of patients with head and neck cancer.

Cancer stem cells

According to the developmental status, physiological stem cells can be classified as embryonic or adult stem cells. Embryonic stem cells are derived from the inner mass of the mammalian blastocyst, have the ability to differentiate into cells of all three germ layers, and develop to all tissues and organs of the organism.[6,7] In contrast, adult stem cells are undifferentiated cells with more limited self renewal and a differentiation potential that is more restricted to cell types of the tissue from where they are found. Adult stem cells play a major role in tissue homeostasis and regeneration. Stem cells also play a major role in the biology of several diseases, including cancer.[8,9] Cancer stem cells are functionally defined as a subset of tumor cells that exhibit the ability of self-renewal and multipotency, serving as progenitor cancer cells.[9,10] In low attachment culture conditions, cancer stem-like cells tend to form spheroids, named orospheres (Figure 1). At least two different hypotheses have been proposed to explain the heterogeneity of tumor-initiating capacity of tumor cells, the cancer stem cell hypothesis[9,11] and the clonal evolution hypothesis.[12,13]

Nowell proposed the clonal evolution hypothesis in 1976, stating that most neoplasms arise from a single cell, and that tumor progression results from acquired genetic variability within the original clone allowing sequential selection of more aggressive sub-lines.[13] Tumor cell populations are apparently more genetically unstable than normal cells. Fearon and Vogelstein proposed a clonal evolution model for colon cancer, in which the progression from early adenoma to invasive carcinoma reflects the stepwise acquisition of mutations in specific cancer genes.[14]

Dick and collaborators provided early evidence for cancer stem cells using leukemia models. [10,11] They induced leukemia by transplanting human acute myeloid leukemia (AML) cells into non-obese diabetic severe combined immunodeficient (NOD/SCID) mice, and showed that primarily CD34+CD38- cells, but not CD34+CD38+ or CD34- cells, initiated leukemia. In addition, they showed that these progenitor cells could be serially transplanted into second recipients. Of note, serial transplantation in vivo has become accepted as an important criterion for the definition of cancer stem cells, and has been used experimentally as a means to propagate cells in an undifferentiated state. The Clarke laboratory unveiled the presence of cancer stem cells in solid tumors, i.e. breast cancer.[8] In xenograft experiments, breast cancer cells sorted for CD44 and CD24 were transplanted into the mammary pads of NOD/SCID mice. These investigators observed that only the CD44+CD24- fraction initiated tumors, whereas 100-fold more CD44+CD24+ or CD44- cells did not. They did not find obvious morphologic and immunophenotypic distinctions

between the tumorigenic and non-tumorigenic breast cancer cells. Notably, the CD44+DC24- cells showed evidence of self-renewal in serial transplantation studies. Since then, cancer stem cells were found in several other cancers, including head and neck,[5] brain,[15,16] lung,[17,18] prostate,[19] colorectal,[20,21] pancreas,[22] liver,[23] and melanoma.[24]

It is important to point out that the cancer stem cell hypothesis has been challenged by findings in some tumor types, as for example melanoma. The Morrison research group demonstrated that 25% of unselected melanoma cells are able to create tumors in immunodeficient mice, which is consistent with the stochastic tumorigenesis model.[25] However, evidence to the contrary has also been seen. CXCR6 discriminated high tumorigenic from low-tumorigenic cells in melanoma models.[26] In an independent study, CD271+ melanoma cells generated more tumors than CD271- cells.[27] In head and neck cancer, several lines of evidence point to the function of a small group of cells with distinct tumorigenic potential. Seminal work by the Prince and colleagues revealed that CD44 expression discriminates a sub-population of progenitor cells.[5] In a follow-up study, the Prince laboratory showed that aldehyde dehydrogenase (ALDH) activity also distinguishes a small group of highly tumorigenic cells.[28] The ability to identify cancer stem cells was further enhanced by the combined use of both markers (ALDH and CD44) that revealed that 1–3% of the cells from primary human HNSCC are uniquely capable of generating tumors.[29] Collectively, these studies suggest that the role of progenitor (stem-like) cells in the tumorigenic process is tumor-type and context dependent.

Development and cancer stem cells

In development, a highly orchestrated and hierarchical process is observed in which a stem cell progressively looses multipotency giving rise to restricted progenitor cells, which in turn differentiate into the cells that constitute the bulk of tissue or organ. In cancer, the cell of origin is the cell that receives the first oncogenic hit(s). A candidate cell of origin is the stem cell, which has the inherent potential of self-renewal and longevity, and therefore is more susceptible to acquired genetic or epigenetic changes that result in transformation. On the other hand, it is not clear if cancer stem cells originate solely from the transformation of normal stem cells. Cancer stem cells may also arise from restricted progenitors or differentiated cells that have acquired self-renewal properties as a consequence of genetic or epigenetic alterations.[30] The plasticity of this system is exemplified by the observation that stem cells can derive from reprogramming of differentiated or somatic cells.[31–33] In 2006, Takahashi and Yamanaka showed that Oct3/4, Sox2, c-Myc and Kif4 induce pluripotency in fibroblasts, generating "induced pluripotent stem (iPS)" cells.[34] Transplantation of iPS cells into nude mice generates tumors that contain cells from all of the three germ layers. The same group also generated iPS cells from adult human fibroblasts.[35] The Thomson research group showed that Oct4, Sox2, Nanog and Lin28 are sufficient to reprogram human somatic cells to pluripotent stem cells that exhibit the essential characteristics of embryonic stem cells.[36] Of note, one of the hallmarks of cancer is the marked phenotypic, functional, proliferative and genetic heterogeneity of the cells.[30,37] This suggests that the cell of origin is capable of generating a highly heterogeneous progeny.

It is becoming increasingly evident that the same pathways that are critical for physiological development also play a role in the early stages of tumorigenesis. For example, Wnt signaling is critical for embryonic development and controls homeostatic self-renewal.[38] On the other hand, somatic mutations of the Wnt pathway are associated with the etiology of several tumors, including intestinal cancer.[38,39] Mutations in adenomatous polyposis coli (APC) in crypt stem cells have been clearly associated with neoplastic transformation.[40] Barker and colleagues identified that Lgr5-cells located at crypt bottom as stem cells that function as cells-of-origin of intestinal cancer.[40,41] Another example is the transcription

factor Sox2, which is essential to maintain the pluripotent phenotype in embryonic stem cells.[42] However, Sox2 efficiently generates iPS cells [34,36] and is amplified in lung and esophageal squamous cancers.[43,44] And finally, it is clear that the Notch1 signaling pathway plays a major role in embryogenesis, as demonstrated by the observation that homozygous mutant embryos died before 11.5 days of gestation.[45] Conversely, Notch signaling is required for the generation and self-renewal of cancer stem cells in several tumor types, including colon cancer.[46] Interestingly, it has been recently demonstrated that Notch1 mutations are frequently found in HNSCC,[47,48] suggesting a potential role for this pathway in the biology of cancer stem cells and in the etiology of head and neck cancer. Collectively, these studies suggest that there are important lessons to be learned from developmental studies that could help identifying processes that result in the malignant transformation of epithelial cells and head and neck cancer initiation.

EMT and cancer stem cells

Epithelial-mesenchymal transition (EMT) is the process that allows a polarized epithelial cell to assume a mesenchymal cell phenotype, which is characterized by enhanced motility and invasiveness.[49] EMT plays a critical role in embryogenesis, and is involved in several pathologies, including fibrosis[49] and cancer.[50–53] An example of this process in physiological settings is the ovarian epithelium that undergoes an EMT-like process during postovulatory wound healing. In this case, EMT is induced by epidermal growth factor (EGF) and involves the activation of metalloproteases and ERK.[54] Key features of EMT are summarized in Table 1.

A critical step in EMT is the loss of cell polarity. Three protein complexes (Par, Crumbs, Scribble) participate in establishing and maintaining apico-basal polarity in epithelial cells.[55] Snail alters epithelial cell polarity by repressing the transcription of Crumbs3 and abolishing the localization of both Par and Crumbs complexes at the junctions.[56] Another hallmark of EMT is the loss of E-cadherin, which appears to be correlated with tumor progression. The loss of E-cadherin is considered a crucial step in the progression of papilloma to invasive carcinoma,[57] and is regulated by a number of transcription factors such as Snail,[58,59] Twist,[60] and ZEB1.[61] The transcription factor Snail controls EMT by repressing E-cadherin expression.[62] Increased Twist expression is found in metastatic breast cancer and is required for EMT and breast cancer metastasis.[60] Importantly, tumors undergoing EMT acquire resistance to chemotherapy.[63–65] Colorectal cancer-derived epithelial cell lines expressing EMT markers exhibit mesenchymal morphology and resistance to oxaliplatin.[63] Twist mediates EMT in breast cancer cells and enhances resistance to paclitaxel.[64] Notably, the deletion of Twist can partially reverse multidrug resistance in breast cancer cells.[65] These data show that the acquisition of a mesenchymal phenotype correlates with increased invasiveness of tumor cells, leading to recurrence/ metastasis and poor clinical prognosis.

Recent reports have suggested that EMT is involved in the acquisition of cancer stem cell properties.[59,66–69] In a seminal publication, the Weinberg research group showed that human mammary epithelial cells undergoing EMT acquire stem cell properties, as demonstrated by the ability of $CD4^{high}CD24^{low}$ cells to form mammospheres in vitro and tumors in vivo. $CD44^{+}CD24^{-/low}$ cells possessing cancer stem-like properties can be generated from $CD44^{low}CD24^{+}$ non-tumorigenic mammary epithelial cells through activation of the Ras/MAPK signaling pathway and induction of EMT.[69] Furthermore, in nasopharyngeal carcinomas, miR200a regulates EMT and induction of stem-like characteristics by targeting E-cadherin repressor ZEB2 via β-catenin signaling.[70] It induces stem-like traits, including CD133+ side population, sphere formation capacity, increased

Oct4 and ALDH expression in tumor spheres and tumor tissues, and tumorigenicity in vivo.[70]

In head and neck cancer, Twist1 induces Bmi-1 (B-cell specific Moloney murine leukemia virus insertion site 1), which in turn downregulates E-cadherin. Bmi-1 has an essential role in the regulation of self-renewal of stem cells.[71–74] Patients with high Twist1 and Bmi-1 tend have worst prognosis.[75] Upregulation of Bmi-1 induced EMT and enhanced the motility and invasiveness of human nasopharyngeal cancer cells, whereas silencing endogenous Bmi-1 reversed EMT and reduced motility.[76] Bmi-1 transcriptionally downregulated expression of tumor suppressor PTEN via direct association with PTEN locus, ablation of PTEN expression partially rescued the migratory/invasive phenotype of Bmi-1 silenced cells.[76]

It has been reported that hypoxia or overexpression of HIF-1a induces EMT and metastasis in head and neck cancer cells.[77] HIF-1α regulates the expression of Twist by binding to the hypoxia-response element (HRE). Notably, siRNA-mediated repression of Twist in hypoxia or HIF1-α overexpression reversed EMT and metastasis.[77] Co-expression of HIF-1α, Twist and Snail in human head and neck tumors correlates with metastasis and poor prognosis.[77] Overexpression of TrkB, a 145-KDa receptor tyrosine kinase, results in EMT and enhances invasion of human HNSCC.[78] Downregulation of TrkB suppressed tumor growth.[78] ALDH + cells from HNSCC cell lines showed enhanced invasion, a phenotype consistent with EMT, and spheroid formation.[79] Cells in spheroids reveal high level of the stemness-related transcription factors Oct3/4, Sox2 and Nanog, upregulation of Snail, Twist, alpha-SMA and Vimentin, and downregulation of E-cadherin.[79] Collectively, these studies suggest that EMT may play a role in the acquisition of stem-like properties in HNSCC, which may ultimately contribute to local invasion and metastatic spread frequently observed in patients with head and neck cancer (Figure 2).

Stem cell niches

Niches are specialized local microenvironments where stem cells reside. They appear to contribute to the survival and stemness of stem cells.[80] It has also been postulated that a niche should shown the capacity to take up and maintain newly introduced stem cells upon depletion.[80] For example, the crypt bottom is considered the niche for stem cells in normal small intestine and colon.[41] It is also the niche for stem cells in intestinal cancer.[40] The perivascular niche is the microenvironment of preference of brain cancer stem cells.[81] It prevents the apoptosis of brain cancer stem cells and maintains an adequate balance between self-renewal and differentiation.[81] When brain cancer stem cells were implanted together with endothelial cells in immunodeficient mice, tumor growth was accelerated.[81,82] This suggests that factors secreted by normal cells surrounding and infiltrating tumors may promote the growth and progression of tumors.[83]

In head and neck tumors, the vast majority of the stem cells are found within a 100 μm-radius of a blood vessel, suggesting the existence of a perivascular niche.[29] Using the SCID mouse model of human tumor angiogenesis,[84] it was observed that specific ablation of tumor-associated endothelial cells with an inducible Caspase-9 results in the decrease in the fraction of head and neck cancer stem cells.[29] It is becoming increasingly evident that the molecular crosstalk between HNSCC and endothelial cells is mutually relevant.[85,86] Tumor cell-secreted factors activate Stat3, AKT and ERK signaling and enhance the survival and angiogenic potential of endothelial cells.[85] Whereas endothelial cell-secreted factors (e.g. IL-6, CXCL8) enhance the migration of tumor cells and protect them against anoikis.[86] Notably, endothelial cell-secreted factors promote the survival and self-renewal of cancer stem cells in HNSCC via upregulation of Bmi-1 expression.[29] These studies demonstrate the

existence of a functionally relevant perivascular niche in head and neck cancer, and suggest that targeted disruption of the crosstalk between endothelial cells and cancer stem cells might be beneficial for the treatment of head and neck cancer patients.

Stem cell markers

It has been recognized that cancer stem cells share many features with physiological stem cells. This constitutes a major difficulty for experimental cancer stem cell research, as well as for the development of targeted therapies. A strategy commonly employed by investigators is the use of molecular markers for the identification of cancer stem cells. In general, these markers are not unique to cancer stem cells. Therefore, the current trend is to combine markers to achieve higher specificity. Also, it is becoming increasingly evident that the most appropriate combination of markers is tumor type-dependent. The following is a brief discussion of some (but not all) markers of that have been used to identify cancer stem cells), with an emphasis on markers that are relevant to HNSCC.

A) Oct3/4, Sox2, Nanog

The transcription factors Oct3/4,[87,88] Sox2[89] and Nanog[90,91] play essential roles in the maintenance of pluripotency and self-renewal of embryonic stem cells.[92,93] They promote self-renewal by interacting with other transcription factors (Stat3, Hesx1, Zic3) and critical cell signaling molecules (TCF3, FGF2, LEFTY2).[92,93] It has been recently reported that the lamina propria of human oral mucosa contains stem cells, as determined by Oct4, Sox2 and Nanog expression.[94] After treatment with dexamethasone and implantation in immunodeficient mice, these stem cells form tumors composed of ectodermal and mesodermal tissues, such as cartilage, bone, fat, striated muscle and neural tissues.[94] These are interesting findings, since tumors were generated by stem cells retrieved from normal tissues.

The expression level of Oct4, Sox2, and Nanog is higher in poorly differentiated tumors than in well differentiated breast cancers, glioblastomas, and bladder carcinoma.[95] These transcriptional factors are also upregulated in spheroid forming cells (i.e. stem-like cells) sorted from human HNSCC,[96] and correlate with the grade of oral squamous cell carcimonas.[97] Collectively, these data indicate that cells that exhibit stem-like features in cancer express the transcriptional factors Oct4, Sox2, and Nanog. However, the usefulness of these factors for the sorting of cancer stem cells by flow cytometry and posterior culture or implantation in animals is hindered by the fact that they are not expressed in the cell membrane, and therefore would require cell permeabilization.

B) CD133

Human CD133 (prominin-1) is a glycosylated protein with five transmembrane domains and two large extracellular loops.[98,99] It was initially characterized as a marker for hematopoietic stem cells.[97,98] After that, CD133 was also found in epithelial cells[100,101] and in somatic stem cells from neural tissues,[102,103] prostate,[104] and kidney.[105] Interestingly, human CD133+ cells from granulocyte colony stimulating factor-mobilized peripheral blood were able to differentiate into endothelial cells, when cultured in pro-endothelial lineage condition.[106] In brain tumors, CD133+ cells revealed properties of cancer stem cells,[107,108] and in the intestine, this marker identified stem cells that were susceptible to neoplastic transformation.[109]

In human oral squamous cell carcinoma, CD133+ stem-like cells possess higher clonogenicity, invasiveness, and tumorigenesis as compared with CD133- cells.[110] CD133+ cells are resistant to standard chemotherapy with paclitaxel.[110] CD133 has been identified as a marker of cancer stem cells in the human laryngeal tumor Hep-2 cell line.[111] In an in vivo

study, CD133+ cells sorted from the Hep-2 cell line showed higher tumorigenic potential than CD133-or unsorted cells.[112] Notably, CD44+ cancer stem-like cells expressed higher CD133 levels than CD44- cells in HNSCC.[113] In laryngeal squamous cell carcinomas, Bmi-1 is highly enriched in CD133+ cells, induces the proliferation of these cells, and prevents apoptosis.[114] The analysis of these studies reveals that CD133 is an useful cancer stem cell marker in HNSCC, and might serve as a putative biomarker to identify head and neck cancer patients that are resistant to conventional chemotherapy.

C) CD44

CD44 is a cell surface glycoprotein that functions as a receptor for hyaluronic acid (hyaluronan).[115,116] CD44 has affinity with other ligands (e.g. osteopontin)[117,118] and certain matrix metalloproteinases (MMPs).[119] In 1991, a CD44 variant was found to be involved in the metastatic potential of tumor cells.[120] As an adhesion molecule, CD44 provides a cell surface docking receptor that is necessary for MMP-9 activity.[121] Localization of MMP-9 in the cell surface of keratinocytes depends on its interaction with CD44, allows for activation of TGF-β, and is required for the promotion of tumor invasion and angiogenesis.[119] Interestingly, immunohistochemical staining showed that CD44 and MMP-9 co-localize in tumor cells at the invasive front,[122] the area where stem cells are typically found in tumors.

CD44 was the marker used in the first description of cancer stem cells in a solid malignancy (i.e. breast cancer).[8] In 2007, Prince and colleagues unveiled that a subpopulation of CD44+ cells presented cancer stem-like properties in HNSCC.[5] They found that CD44+ cells could be serially passaged in vivo, consistently reproducing the original tumor. CD44+ cells expressed high levels of Bmi-1 (stemness marker) and possessed the capacity of self-renewal and differentiation. Since then, many studies have used CD44 as a marker of cancer stem cells in head and neck cancer models.[29,123,124] However, one must take into account a report that showed that the expression of two variants of CD44 (i.e. CD44s, CD44v6) is found in the majority of the cells in head and neck tissue (including carcinomas), and that this marker by itself was not able to distinguish normal from benign or malignant epithelial cells from the head and neck region.[125] CD44 is considered a predictive marker for local recurrence after radiotherapy in patients with larynx cancer.[126] High levels of CD44, aldehyde dehydrogenase and phosphorylated Stat3 are found in high-grade HNSCC, and are indicative of poor prognosis.[124] Also, a higher frequency of CD44+ cells was observed in HNSCC that recurred than in human tumors without recurrence.[127] Collectively, these studies suggest a direct correlation between CD44 expression, cancer stem cells, and the aggressiveness of head and neck tumors.

D) ALDH

Aldehyde dehydrogenase (ALDH) enzymes constitute a family of intracellular enzymes that are involved in cell differentiation, detoxification and drug resistance via the oxidation of intracellular aldehydes.[128–131] ALDH1 is required to converse retinol (vitamin A) to retinoic acid.[132] ALDH1 is the prototypic member of the ALDH family and is highly expressed in human hematopoietic progenitors or hematopoietic stem cells.[129,131,133] ALDH1 has been characterized as a marker of normal and malignant human mammary stem cells, and a prognostic marker for breast cancer being a strong predictor of metastasis and poor patient outcome.[134,135] In primary non-small cell lung cancer (NSCLC), ALDH-positive cancer cells showed stem-like properties, including tumorigenesis, colony formation and self-renewal.[136]

Several studies have demonstrated that ALDH+ cells have a behavior that is consistent with cancer stem cells in head and neck tumors.[28,29,79,137] ALDH+ cells from patients with

HNSCC showed enhanced tumorigenesis and radioresistance when compared with ALDH-cells.[137] Interestingly, knockdown of Snail decreased the expression of ALDH and inhibited cancer stem-like properties and the tumorigenicity of CD44+CD24-ALDH+ cells.[137] In a study from the Prince laboratory, 500 ALDH^{high} cells from primary HNSCC formed tumors in 24 out of 25 mice, while only 3 out of 37 mice transplanted with ALDH^{low} cells showed tumors.[28] Another study showed that 1000 ALDH+CD44+ cells from primary human HNSCC formed tumors in 13 out of 15 mice, whereas 10 000 ALDH-CD44- cells resulted in only 2 tumors in 15 mice.[29] The self-renewal of ALDH+CD44+ cells was confirmed by colony and spheroid formation.[29] These studies demonstrated that ALDH by itself, or in combination with CD44, is capable of discriminating a sub-population of highly tumorigenic cells that exhibit features of cancer stem cells in HNSCC.

E) Side population

Another strategy that has been used extensively to identify highly tumorigenic cells is based on the ability of such cells to eliminate a DNA dye, Hoechst 33342.[138] Side population (SP) cells are enriched in hematopoietic stem cells[139] and identified from a bone marrow-derived cell population.[138] These cells express high levels of ATP-binding cassette (ABC) transporter family members (e.g. MDR1, ABCG2) that allow for the efflux of Hoechst 33342 and other drugs.[140,141] Using fluorescence-activated cell sorting (FACS), SP cells have been identified in normal tissues (e.g. skin,[142] lung,[143] brain,[144] and liver[145]) and solid tumors (e.g. hepatocellular carcinoma,[146] glioma,[147] gastrointestinal cancer,[148] ovarian carcinoma,[149] neuroblastoma[150] and breast cancer.[150]

In recent years, SP cells have been characterized in HNSCC as highly tumorigenic cells with stem-like phenotype.[151–155] The fraction of SP cells tends to be high in metastatic and aggressive HNSCC cells.[153] In head and neck cancer, SP cells express high levels of ABCG2,[152,153,155] Bmi-1,[152,155] CD44, and Oct4.[152] These cells exhibit abnormal Wnt signaling and are highly invasive[153] and chemoresistant.[153,155] The identification of side populations is technically simple and does not rely on the relative binding efficiencies of antibodies. More research is necessary to demonstrate how it compares against antibody-based approaches to specifically distinguish highly tumorigenic head and neck cancer stem cells, from other cells of HNSCC that present low tumorigenicity.

Final thoughts and future directions

There are many factors that play a role in the study of the tumorigenic potential of cells. Among them, the immunological status of the host appears to have a direct impact on the efficacy of tumor initiation in murine experimental models. It has been reported that only 1 in one million acute myeloid leukemia (AML) cells generates a tumor when transplanted into NOD/SCID mice.[156] However, the frequency of tumor-initiating cells is higher when these cells are transplanted into histocompatible mice. Indeed, 1 out of 10 lymphoma cells or AML cells can form tumors when injected into such mice.[157] In melanoma, it was demonstrated that the frequency of tumor-initiating cells is less than 1 per 10^6 cells when transplanted into NOD/SCID mice.[25] However, when melanoma cells were transplanted into highly immunocompromised NOD/SCID interleukin-2 receptor gamma chain null (Il2rg(−/−)) mice, the fraction of tumorigenic melanoma cells was increased by several orders of magnitude. In this case, 27% of unselected melanoma cells generated tumors.[25] Collectively, these studies highlight the impact of the experimental model on the results of studies exploring the tumorigenic potential of cells. They constitute an important reminder that one should use caution while interpreting the results of laboratory studies involving the transplantation of human cells into murine hosts.

A critical issue that remains unanswered is what is the frequency of tumor-initiating cells in head and neck squamous cell carcinomas. The seminal publication by Prince and collaborators showed that CD44 expression distinguished a highly tumorigenic sub-population of cells (that behave as cancer stem cells) from another cell population that had low tumorigenic potential.[5] A recent report revealed that tumor-initiating cells are rare (<1 in 2500 cells) in primary pancreas, lung, or head and neck tumors.[158] And in a serial dilution assay, we observed that the transplantation of as low as 1 ALDH+CD44+ cell/SCID mouse consistently formed tumors (unpublished observations), while transplantation of 10,000 ALDH-CD44- generate tumors in only 13.3% of the mice.[29] These studies, and many others not described in detail here,[28,110,127,152] suggest that head and neck squamous cell carcinomas follow the cancer stem cell hypothesis. On the other hand, it was recently reported that all single-cell clones randomly isolated from certain HNSCC cell lines can form tumors when xenotransplanted to NOD/SCID mice.[159] This study indicated that essentially any cell from HNSCC cell lines has the ability to form tumors. Further studies focused on the identification of the nature, frequency, and characteristics of the cells capable of generating HNSCC are certainly warranted.

The analysis of the existing literature suggests a hypothetical model for head and neck tumor progression (Figure 3). The crosstalk between HNSCC cells and other cells of the tumor microenvironment results in EMT, which enhances the motility of carcinoma cells and endows them with stem cell properties. The invasive phenotype of cells that have undergone EMT allows them to penetrate the lymphatic and/or angiogenic vasculature. And the highly tumorigenic nature of cancer stem cells enables some of them to initiate tumors in regional lymph nodes or in distant sites (e.g. lungs). According to this hypothetical model, patients with HNSCC might benefit from therapeutic strategies that inhibit EMT by blocking the crosstalk between tumor and stromal cells, or therapies that directly target the cancer stem cell.

In conclusion, the discovery that heterogeneous HNSCC tumor cells exhibit a spectrum of tumorigenic potentials has brought significant interest to the application of stem cell biology concepts to the understanding of the pathobiology of head and neck cancer. Much work remains to be done to more fully understand the biology of cancer stem cells in HNSCC. For example, whether cancer stem-like cells exist in premalignant lesions and what is their behavior and function through the multi-step process of disease progression remains largely unclear. However, what is clear is that the development of mechanism-based therapies for head and neck cancer will require deeper understanding of the biological processes that generate the cells that drive recurrence and metastatic spread. It is tempting to speculate that the combination of therapies aimed at debulking the tumor (e.g. surgery, conventional chemotherapy, radiotherapy) together with targeted therapies aimed at the elimination of the cancer stem cells might have a positive impact on the long-term outcome of patients with head and neck cancer in the future.

Acknowledgments

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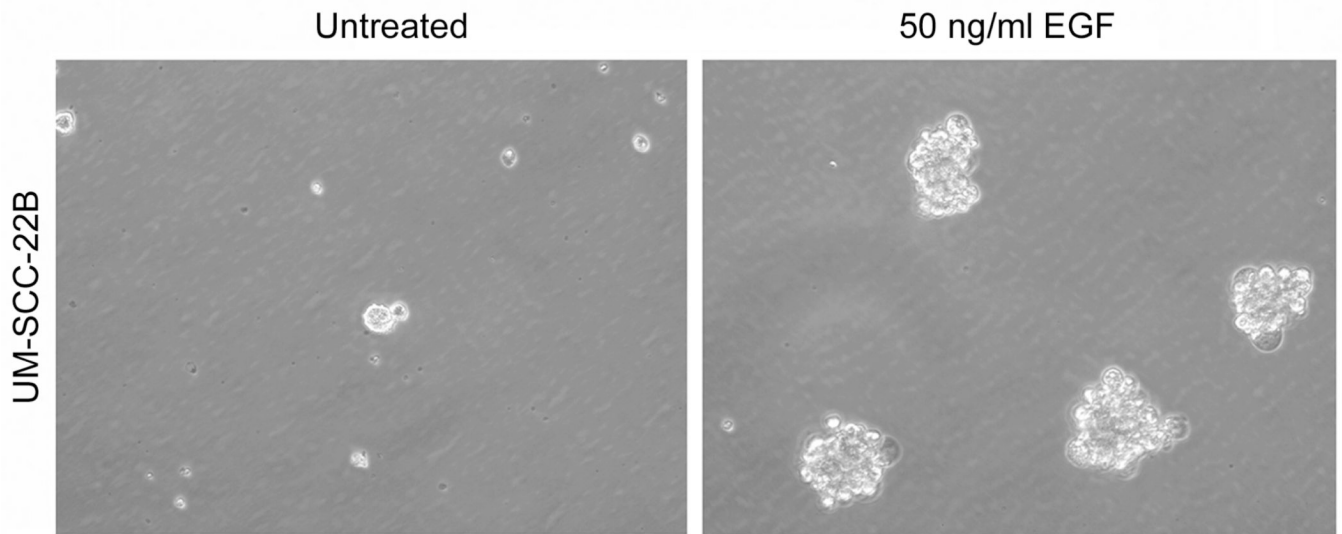
Figure 1.
Orosphere assay to study the acquisition of a cancer stem-like phenotype *in vitro*. UM-SCC-22B is a cell line derived from the metastatic lymph node of a patient with HNSCC in the hypopharynx. We have recently reported that UM-SCC-22B contains a sub-population of cells that exhibit cancer stem-like characteristics.[160] Photomicrographs (200x) of UM-SCC-22B cells cultured with serum-free medium in ultra-low attachment plates in presence of 0 or 50 ng/ml EGF for 5 days. The formation of spheroid-like colonies containing at least 25 cells (named orospheres) was enhanced by EGF treatment. Growth of carcinoma cells in suspended spheres under low (or no serum) culture conditions has been considered indicative of acquisition of a stem-like phenotype *in vitro*.
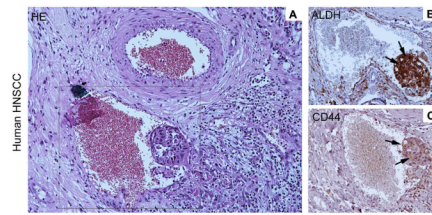
Figure 2.
Cancer stem-like cells (ALDH+CD44+) inside a blood vessel in a primary human head and
neck cancer. **(A)** Highly aggressive human primary HNSCC cells invade a blood vessel
(H&E). **(B,C)** Close-up view of the area limited by a square in panel A, showing cancer
cells inside a blood vessel with positive staining for ALDH1 (B) and CD44 (C), as
determined by immunohistochemistry. Please note strong cytoplasmic staining for ALDH1
and typical cell membrane staining for CD44 in the cells located inside the blood vessel
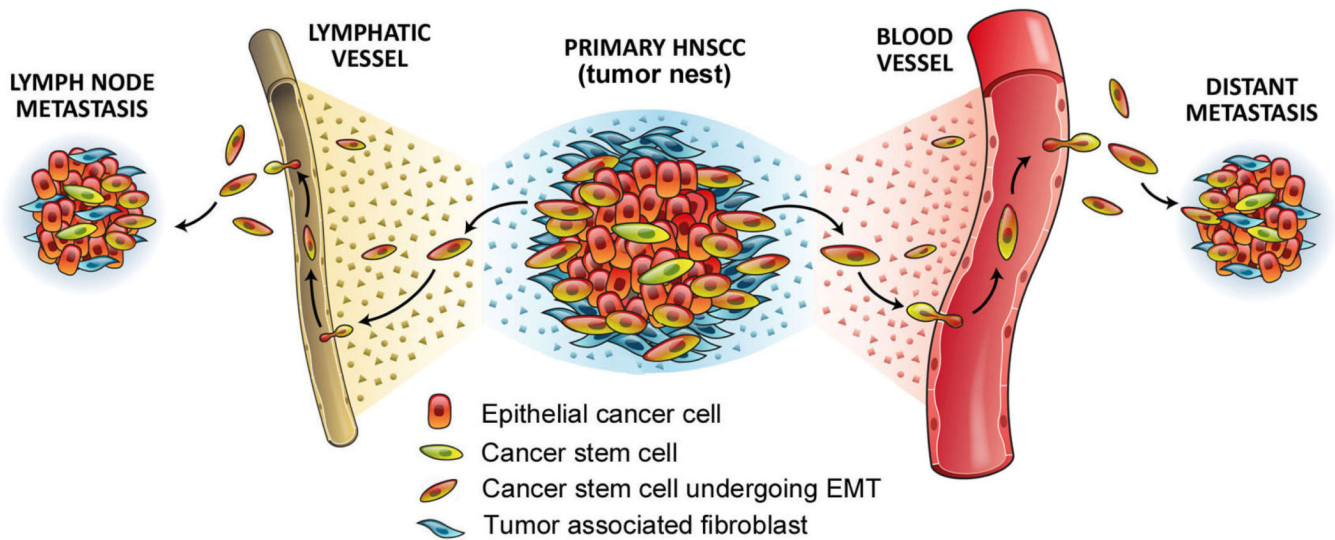(arrows).

Figure 3.
Diagram depicting a putative model for the role of EMT and acquisition of cancer stem cell phenotype in the metastatic spread of HNSCC. The epithelial tumor is a complex organ that contains carcinoma cells, fibroblasts, immune cells, blood vessels, lymphatics, and a small population of cancer stem cells. Through EMT, cancer stem cells become invasive and acquire characteristics that enable them to metastasize to regional lymph nodes (through lymphatic vessels) and to distant sites, such as lungs (through blood vessels). Notably, it is unlikely that every single cancer stem cell has the ability to generate a viable metastasis.

Table 1

Characteristics of normal epithelial and mesenchymal cells

	Epithelial cells	Mesenchymal cells
Morphology	Cobblestone polarized	Elongated Non Polarized
Behavior	Non-motile Non-invasive	Migratory Invasive
Markers	E-cadherin Desmoplakin Cytokeratin	Vimentin N cadherin Snail