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ABSTRACT

Most cancers contain a small sub-population of cells that are endowed with self-renewal, multipotency, and a unique potential for tumor initiation. These properties are considered hallmarks of cancer stem cells. Here, we provide an overview of the field of cancer stem cells with a focus on head and neck cancers. Cancer stem cells are located in the invasive fronts of head and neck squamous cell carcinomas (HNSCC) close to blood vessels (perivascular niche). Endothelial cell-initiated signaling events are critical for the survival and self-renewal of these stem cells. Markers such as aldehyde dehydrogenase (ALDH), CD133, and CD44 have been successfully used to identify highly tumorigenic cancer stem cells in HNSCC. This review briefly describes the orosphere assay, a method for *in vitro* culture of undifferentiated head and neck cancer stem cells under low attachment conditions. Notably, recent evidence suggests that cancer stem cells are exquisitely resistant to conventional therapy and are the “drivers” of local recurrence and metastatic spread. The emerging understanding of the role of cancer stem cells in the pathobiology of head and neck squamous cell carcinomas might have a profound impact on the treatment paradigms for this malignancy.

KEY WORDS: head and neck squamous cell carcinoma, perivascular niche, tumorigenesis, therapy, angiogenesis, oral cancer.

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Head and Neck Cancer Stem Cells

INTRODUCTION

Tumors are not insular masses of proliferating cells (Hanahan and Weinberg, 2000). Instead, a tumor can be seen as an “organ” composed of transformed cells that interact with stromal cells within the tumor microenvironment (Fig. 1). The understanding of cancer as a complex tissue where tumor cells rely on interactions with stromal cells to progress toward malignancy and overcome host defenses has been solidified by extensive research in the last decade (Hanahan and Weinberg, 2011). Collectively, this work suggested that not all tumor cells are equal. Indeed, malignant cells with varying tumorigenic potential have been found in many tumor types. The possibility of identifying those cells with higher tumorigenic potential, and selectively eliminating them, is the clinical rationale underlying this review.

According to the stochastic hypothesis, tumor cells are homogeneous, *i.e.*, every cancer cell has equal propensity to initiate and propagate tumors and metastasize. In effect, it means that there is no selectivity between the cancer cells in a tissue; each cell has the same potential to initiate tumors. The heterogeneity in tumors is explained by spontaneous shifts in cell phenotypes (Albers *et al.*, 2011). However, emerging evidence from a variety of tumors suggests an alternative explanation, called the cancer stem cell hypothesis. It states that the tumor tissue has a distinct hierarchy of cells, and only a small sub-population of cells within the tumor is capable of initiating cancers. These are called cancer stem cells or “tumor-initiating cells” (Reya *et al.*, 2001). The bulk of the tumor tissue, however, is composed of rapidly proliferating cells, called transit-amplifying cells and post-mitotic differentiated cells, which do not contribute to tumor initiation. These cells are derived from the cancer stem cells through their differentiation, but do not cause tumor initiation by themselves.

Extensive work has been done in recent years to understand the applicability of these two hypotheses to different tumor types. Recently, it was demonstrated in melanomas that about 25% of unselected melanoma cells were able to create tumors in immunocompromised mice, suggestive of a more stochastic model (Quintana *et al.*, 2008). However, in recent literature, evidence to the contrary has been seen. CD271+ cells sorted from melanomas generated more tumors than the CD271- cells, supporting the cancer stem cell hypothesis (Civenni *et al.*, 2011). In another independent study, CXCR6+ cells created more aggressive tumors than CXCR6- cells in melanoma models (Taghizadeh *et al.*, 2010). This suggests the existence of primitive melanoma cells capable of phenotypic plasticity, self-renewal, and immune evasion (Girouard and Murphy, 2011). Several features of HNSCC can also be explained by the stochastic model. The existence of large pre-neoplastic areas

beyond the surgical margins results in local recurrences and secondary cancers, which are explained by the stochastic model (Albers *et al.*, 2011). In contrast, the heterogeneity seen in the head and neck cancers and distant metastasis supports the cancer stem cell hypothesis (Prince *et al.*, 2007; Chen *et al.*, 2009).

One of the earliest studies exploring the tumorigenic potential of cells showed that a single tumor cell from one mouse generated a tumor in a secondary recipient mouse (Furth and Kahn, 1937). The idea of tumor-initiating cells was further explored in leukemia, where low numbers of leukemic cells generated tumors in mice (Hewitt, 1958; Bonnet and Dick, 1997). The tumors generated in these studies were heterogeneous, with a hierarchical organization suggestive of cells with various degrees of tumorigenicity. Importantly, a study involving the labeling of mouse squamous cell carcinoma cells with tritiated thymidine showed that undifferentiated malignant cells are capable of generating non-tumorigenic keratinocytes (Pierce and Wallace, 1971). These results suggested that not all progeny of malignant cells are tumorigenic. Further support for the presence of a small sub-population of cells with higher tumorigenic potential came from studies which reported that only 1-4% of lymphoma cells formed colonies in the spleen, and only about 0.02-0.1% of solid tumor cells formed colonies (Hamburger and Salmon, 1977).

In the late 1970s, however, the focus of cancer biology shifted to the concept of clonal evolution, as mutations in oncogenes and tumor suppressors were found to cause human cancers. Tumor cell populations were considered genetically unstable, and acquisitions of genetic mutations created human malignancies. The step-wise genetic mutations that certain genes acquired in colon cancer were well-documented by the Vogelstein laboratory (Fearon *et al.*, 1990). However, in the early 1990s, technological advances allowed for studies that again suggested a hierarchy of tumorigenic potential of tumor cells. Such studies were facilitated with the advent of technologies such as the Fluorescence Activated Cell Sorting system (FACS), which enabled researchers to study specific cell-surface markers in individual cells and use these markers to sort predetermined sub-populations of cells. For example, FACS helped in identifying that leukemic engraftment was possible primarily with CD34+CD38-expressing cells (Bonnet and Dick, 1997). Xenograft assays further demonstrated that only one in a million cells was capable of initiating tumors in acute myeloid leukemia (Lapidot *et al.*, 1994). Collectively, these studies suggest that the stochastic theory and the cancer stem cell model are not mutually exclusive. Rather, these two theories may have a different impact in different stages of tumor progression, and certainly are tumor-type-specific.

Figure 1. Cancer tissue is a complex "organ". The tumor tissue microenvironment is composed of a variety of cells, including tumor cells, cancer stem cells, inflammatory cells, and cancer-associated fibroblasts, along with blood vessels. The cancer stem cells are rare cells found primarily in the invasive edge of tumors close to blood vessels, *i.e.*, in supportive perivascular niches.

CANCER STEM CELL HYPOTHESIS

It is well known that most human tissues (*e.g.*, brain, skin, and intestine) contain stem cells (Blanpain *et al.*, 2004; Vries *et al.*, 2010). The cancer stem cell hypothesis is fundamentally based on the application of stem cell concepts derived from embryogenesis to understanding of the tumorigenic process. The following are key features of the cancer stem cell hypothesis: "(1) Only a small fraction of the cancer cells within a tumor have tumorigenic potential when transplanted into immunodeficient mice; (2) the cancer stem cell sub-population can be separated from the other cancer cells by distinctive surface markers; (3) tumors resulting from the cancer stem cells contain the mixed tumorigenic and non-tumorigenic cells of the original tumor; and (4) the cancer stem cell sub-population can be serially transplanted through multiple generations, indicating that it is a self-renewing population" (Prince and Ailles, 2008). Therefore, cancer stem cells are capable of self-renewal and differentiating into other distinctive cells that make up the tumor mass (Reya *et al.*, 2001).

The fundamental concept underlying the cancer stem cell hypothesis is that not all tumor cells in a cancer are equal. Indeed, in a landmark publication, Clarke's laboratory showed that breast tumors were heterogeneous, and as few as 100 CD44⁺CD24^{-/low} cells isolated from primary breast cancers were capable of initiating tumors, whereas tens of thousands of phenotypically different cells did not (Al-Hajj *et al.*, 2003). An important observation of this study was that the resulting xenografts had distinct sub-populations of cells reproducing the heterogeneity of the original tumors (suggestive of the multipotency of the tumor-initiating cells), and consistently expressed specific cell-surface markers that were used to identify the cancer stem cells (indicative of self-renewal capability of these cells). In recent years, head and neck squamous cell carcinomas have

Figure 2. Spatial relation between putative head and neck cancer stem cells and blood vessels. ALDH1+ cells are localized close to vessels in a squamous cell carcinoma surgically removed from the tongue of a 77-year-old Caucasian male (T2N1M0). **(A)** Light photomicrograph after immunohistochemistry for ALDH1 (red staining; black arrows). **(B)** Confocal microscopy after immunohistochemistry for ALDH1 (green) to identify putative cancer stem cells (white arrowheads), von Willebrand Factor (red) to identify blood vessels, and DAPI (blue) to identify the cell nuclei.

been studied in great detail to identify whether they abide by the cancer stem cell hypothesis or the stochastic model. Such knowledge has major implications for cancer therapy, as will be discussed later.

CANCER STEM CELLS IN HEAD AND NECK TUMORS

The immense cancer mortality rate worldwide requires that strategies be developed to detect cancers earlier, understand them better, treat them more effectively, and prevent their recurrence. Head and neck squamous cell carcinoma (HNSCC) ranks sixth worldwide for cancer-related mortality, with an estimated 500,000 new cases diagnosed every year (Vermorken *et al.*, 2007). More than 30,000 new cases of head and neck cancers are diagnosed in the United States alone (Jemal *et al.*, 2007). For the past several decades, the mainstay of treatment for HNSCC has been surgery and radiation. Though the standard therapy cures significant numbers of patients with Stage I disease, more than 23% develop secondary primaries, relapse, and die (Larson *et al.*, 1990). For more advanced stages of the disease, the survival rate has not improved significantly in the last couple of decades. The use of platinum-based therapy has improved local control of the disease, but the incidence of distant metastases appears to be in the rise in recent years (Sano and Myers, 2007; Forastiere, 2008). It is possible that cancer stem cells participate in the processes that lead to resistance to therapy and the establishment of distant metastases.

In a landmark publication, Prince and collaborators (2007) unveiled the presence of highly tumorigenic, stem-like, cells in HNSCC. They analyzed HNSCC using FACS sorting for expression of CD44, and were able to generate tumors with as few as 5×10^3 cells of CD44+ cells, whereas higher numbers of CD44- cells failed to create tumors. They were also able to show that the resultant xenografts were heterogeneous (Prince *et al.*, 2007). In our own work, we demonstrated that implantation of 1000

ALDH+CD44+ cells sorted from primary human HNSCC generated 13 tumors out of 15 implantations, whereas 10,000 ALDH-CD44- cells created only 2 tumors out of 15 implantations (Krishnamurthy *et al.*, 2010). In addition, we were consistently able to serially passage the tumor xenografts generated by ALDH+CD44+ cells, but not ALDH-CD44-, showing self-renewal properties for the double-positive cells. Notably, the xenografts resembled the primary tumors histologically, with distinctive sub-populations of cells, thus accounting for the tenets of the cancer stem cell hypothesis. Analysis of these data, collectively, lends support to the concept that HNSCC follows the cancer stem cell hypothesis, where sub-populations of cancer cells have significantly higher tumorigenic potential than others.

IDENTIFICATION OF CANCER STEM CELLS IN HEAD AND NECK TUMORS

Identification and isolation of cancer stem cells constitute a major experimental challenge. Researchers attempt to isolate these cells by identifying properties that distinguish stem cells from their differentiated progeny and from stromal cells. These properties include the efflux of vital dyes by multidrug transporters (*e.g.*, ABC transporters), enzymatic functions (*e.g.*, aldehyde dehydrogenase activity), the sphere-forming capacity in low attachment conditions, and the expression of cell-surface antigens.

CD44, a cell-surface glycoprotein, functions as a receptor for hyaluronic acid and is involved in cell adhesion and migration (Gao *et al.*, 2011). The Prince laboratory demonstrated that CD44 serves as a cancer stem cell marker in HNSCC (Prince *et al.*, 2007). Twenty out of 30 implantations of CD44+ cells generated tumors in immunodeficient mice, whereas only one of 40 implantations of CD44- cells generated tumors. Following this work, several independent groups confirmed that CD44 either alone or in combination has the properties of a cancer stem cell marker and being a tumor initiator (Baumann and Krause, 2010; Chikamatsu *et al.*, 2011). Emerging literature is revealing a role for CD44 in tumor metastasis. Indeed, it has been described that certain forms of CD44 (*i.e.*, v3, v6, v10) are associated with tumor progression and metastatic spread of HNSCC (Wang *et al.*, 2009). It has also been shown that CD44+ cells express high levels of Bmi-1 (Prince *et al.*, 2007), a self-renewal protein found in embryonic stem cells (Bracken *et al.*, 2006).

The transmembrane glycoprotein CD133 has also been investigated as a putative marker for cancer stem cells (Wu and Wu, 2009). CD133 or Prominin-1, originally found on neuroepithelial stem cells in mice, was isolated in human hematopoietic stem cells by a monoclonal antibody recognizing a specific epitope called the AC133 (Shmelkov *et al.*, 2005). In some HNSCC cell lines (*e.g.*, hep-2), CD133+ cells were found to have increased clonality when compared with CD133- cells (Zhou *et al.*, 2007). Oral cancer stem-like cells from cell lines and primary tumors were found to have an increased expression of CD133, and displayed increased migration and tumorigenicity as compared with controls (Chiou *et al.*, 2008). In fact, correlation of Oct-4, Nanog, and CD133 status showed a poorer prognosis for oral cancer patients with increased CD133 expression (Chiou *et al.*, 2008). Recently, CD133+ cells were found to

possess increased clonogenicity, invasiveness, and tumorigenicity as compared with CD133⁺ cells, along with resistance to paclitaxel (Zhang *et al.*, 2010).

Aldehyde dehydrogenase (ALDH) is an intracellular enzyme that is involved in converting retinol to retinoic acid (Sobreira *et al.*, 2011). In cancer, ALDH⁺ cells were identified in the breast and the brain (Ginestier *et al.*, 2007; Rasper *et al.*, 2010). In these tumors, ALDH⁺ cells were characterized as highly tumorigenic cells that can self-renew, which are hallmarks of cancer stem cells. In HNSCC, ALDH enriches for cancer stem cells and is involved in epithelial-to-mesenchymal transition (EMT) (Chen *et al.*, 2009). Our laboratory demonstrated that cells with high ALDH activity exhibit a stem cell-like behavior in HNSCC. We observed that 13 (out of 15) implantations of 1000 ALDH⁺CD44⁺ cells sorted from primary HNSCC generated tumors, while only 2 (out of 15) implantations of 10 times more (*i.e.*, 10,000) ALDH[−]CD44[−] cells generated tumors (Krishnamurthy *et al.*, 2010). Interestingly, a recent report demonstrated that as few as 500 ALDH⁺ cells were able to create tumors, unlike the ALDH[−] cells (Clay *et al.*, 2010).

The ability of cells to actively pump out dyes like the Hoechst 33342 by the ATP-binding cassette transporter (ABC) has also been used to identify cells with increased longevity. Since stem cells and cancer stem cells can remain quiescent for long terms, Hoechst 33342 has been used to identify putative cancer stem cells. Side-population cells in HNSCC have been shown to increase clonality and tumorigenic potential (Zhang *et al.*, 2009).

STEM CELL NICHE

Physiological stem cells and cancer stem cells depend on their immediate microenvironment or niche for their survival and function (Borovski *et al.*, 2011). The cellular and non-cellular components of the niche provide cues that regulate proliferative and self-renewal signals, thereby helping cancer stem cells maintain their undifferentiated state (Kuhn and Tuan, 2010). Non-epithelial stromal cells, inflammatory cells, and the vasculature have been proposed as key components of the niche that support and sustain cancer stem cells (Fuchs *et al.*, 2004). This knowledge raised the hypothesis that one could suppress the survival of cancer stem cells by disrupting the interactions with

Figure 3. Possible implications of the cancer stem cell hypothesis for therapy. **(A)** Conventional chemotherapy targets primarily the highly proliferative cells that constitute the bulk of the tumor. With suitable microenvironments, the cancer stem cells proliferate, and the tumor recurs. **(B)** Direct cancer stem cell targeting or **(C)** indirect cancer stem cell targeting via disruption of their perivascular niche can potentially eliminate cancer stem cells. Ablation of the stem cells may inhibit the regeneration of the tumor and ultimately result in tumor regression.

their supportive niche. Indeed, it has been recently suggested that the hematopoietic stem cell niche is a potential therapeutic target for metastatic bone tumors (Shiozawa *et al.*, 2011).

A seminal work demonstrated that endothelial cells present in the perivascular niche provide critical survival and self-renewal cues for cancer stem cells in glioblastomas (Calabrese *et al.*, 2007). In head and neck squamous cell carcinomas, the majority of the stem cells are localized close to blood vessels and depend on interactions with components of the niche for their survival (Krishnamurthy *et al.*, 2010). In addition to providing nutrients and oxygen to cells, endothelial cells secrete factors that promote the self-renewal and survival of head and neck cancer stem cells. Fig. 2 depicts photomicrographs of a primary HNSCC showing ALDH⁺ cells close to blood vessels. Collectively, these studies suggest the existence of a perivascular niche for head and neck

Figure 4. Orosphere assay. **(A)** Representative photomicrograph of an orosphere (black arrowhead). ALDH+CD44+ cells were sorted from a head and neck squamous cell carcinoma cell line (UM-SCC-74A) and cultured in an ultra-low-attachment plate. **(B)** Confocal microscopy of an orosphere stained for the stem cell markers, ALDH1 and CD44, along with DAPI.

cancer stem cells. However, it is still not clear if the perivascular stem cell niche is a viable therapeutic target for head and neck cancer.

THERAPEUTIC IMPLICATIONS AND SIGNIFICANCE OF CANCER STEM CELLS IN HNSCC

The cancer stem cell hypothesis has significant implications for the management of patients with malignancies. It tells us that the tumor tissue is heterogeneous, and that the sub-population of cancer stem cells is primarily responsible for tumor initiation. It also implies that the bulk of the tumor tissue might be relatively innocuous compared with the highly tumorigenic cancer stem cells. Importantly, cancer stem cells tend to be very resilient and resistant to conventional therapies (chemotherapy and radiation therapy) that are targeted to highly proliferative cells (Chikamatsu *et al.*, 2011). It has been postulated that cancer stem cells can remain quiescent for extended periods of time, and therefore escape from conventional treatment protocols. However, these cells have the potential to become activated, differentiate, and proliferate, leading to the establishment of local recurrences or distant metastases.

In Fig. 3, we propose a hypothetical model for the response of HNSCC to different therapeutic strategies. HNSCC is represented as a complex tissue where the cancer stem cells constitute a relatively small number of cells that are capable of undergoing self-renewal and differentiating into a complex and heterogeneous tumor. Conventional chemotherapeutic drugs are successful in de-bulking the tumor. However, it is proposed that slow-growing cancer stem cells evade conventional therapies, and, with the passage of time, these cells are activated and regenerate tumors locally or at distant sites (Fig. 3A). This might help to explain the relatively high recurrence rates in patients with HNSCC. In contrast, targeting the cancer stem cells either directly (Fig. 3B) or *via* their niche (Fig. 3C) could lead to a more definitive response, since the cancer stem cells are the putative drivers of recurrence and metastatic spread. An

emerging concept is the combined use of conventional chemotherapy and cancer-stem-cell-targeted therapy. This drug combination is appealing, since such strategy could potentially allow for tumor debulking (with conventional drugs) and prevention of recurrence/metastases (cancer-stem-cell-targeted drugs).

Extensive work is being done to understand the molecular mechanisms that might be playing a role in the pathobiology of cancer stem cells but not in normal cells, which would allow for specific targeting of pathological cells. One such target could be Bmi-1, present in high levels in cancer stem cells (Hayry *et al.*, 2010). Several other signaling pathways (*e.g.*, Wnt, PTEN, Notch, Hedgehog) are also currently being explored as potential

therapeutic targets (Pannuti *et al.*, 2010; Takahashi-Yanaga and Kahn, 2010; Takezaki *et al.*, 2011). Various laboratories are isolating cancer stem cells and performing gene array analyses to understand how cancer stem cells might be differentially regulated compared with the rest of the tissue. Recent studies also suggest a role for micro-RNA like the Let7, MicroRNA-200c in the regulation of the tumorigenicity of cancer stem cells (Lo *et al.*, 2011; Yu *et al.*, 2011). The ability to selectively target cancer stem cells, while sparing normal stem cells, appears to be critical for the future application of cancer stem cell therapy in the clinic.

Another important conceptual strategy for targeted elimination of cancer stem cells is through the disruption of their supportive niche. In glioblastoma models, the use of anti-angiogenic therapies correlated with a decrease in cancer stem cell fraction (Calabrese *et al.*, 2007). Our own work in HNSCC showed that the cancer stem cells rely on their interactions with the perivascular niches for survival and self-renewal (Krishnamurthy *et al.*, 2010). Using a caspase-based artificial death switch (*i.e.*, iCaspase-9), we selectively eliminated tumor-associated blood vessels in xenografts and observed a reduction in the fraction of cancer stem cells in xenograft HNSCC models. Collectively, these results demonstrate that by interfering with the cancer stem cell microenvironment (*e.g.*, the perivascular niche), one can compromise the ability of these cells to survive and/or to behave as a stem cell. Analysis of these data suggests that anti-angiogenic therapies might have the unexpected, yet most welcome, effect of decreasing the presence of highly tumorigenic cancer stem cells in HNSCC. However, anti-angiogenic therapy has to be considered with caution, since there is recent evidence demonstrating that anti-angiogenic therapy (especially anti-VEGF-based therapy) may influence the malignant progression of tumors. It has been hypothesized that tumor cells may acquire an invasive phenotype in an attempt to escape from the unfavorable tumor microenvironment generated by the effects of anti-angiogenic drugs *via* a phenomenon called 'evasive resistance' (Paez-Ribes *et al.*, 2009; Keunen *et al.*, 2011). More studies are therefore necessary to understand the possible effects of

anti-angiogenic therapy on cancer stem cells, and their ultimate consequence for head and neck tumor progression.

CHALLENGES FACING HEAD AND NECK CANCER STEM CELL RESEARCH

One of the biggest challenges in cancer stem cell research has been the development of methods for culture, expansion, and analyses of undifferentiated cancer cells *in vitro*. The property of surviving in suspension (anchorage independence) has been used for this purpose (Jensen and Parmar, 2006). The method of enriching for cancer stem cells by sphere generation under low-attachment culture conditions has been proposed and used in various cancer models such as breast, neural, and prostate (Dontu *et al.*, 2003; Pastrana *et al.*, 2011) and adapted for HNSCC (Krishnamurthy *et al.*, 2010). In this case, putative head and neck cancer stem cells are cultured either in a matrix-based assay like soft agar or in ultra-low-attachment plates. The cancer stem cells grow in suspension and form independent spheres or colonies (Fig. 4). In this Fig., ALDH+CD44+ cells sorted from a head and neck cancer cell line (*i.e.*, UM-SCC-74A) and cultured in low-attachment conditions were able to create spheres. This assay is derived from the work on breast and brain tumor models (*i.e.*, mammospheres, neurospheres) and was named the “orosphere assay” to reflect the fact that these spheres were generated from either primary tumors from the oral cavity or from oral squamous cell carcinoma cell lines.

Despite intense research in the area of cancer stem cell biology in recent years, the understanding of the impact of cancer stem cells on the pathobiology of HNSCC is still quite primitive. One of the reasons for this is the need to perform studies with primary HNSCC specimens, which are difficult to obtain. There is still controversy over the existence of cancer stem cells in cell lines, despite the fact that independent reports have described them in detail (Locke *et al.*, 2005; Harper *et al.*, 2007). In addition, the expansion of cancer stem cells is frequently performed *in vivo*, which is time-consuming and expensive. However, existing *in vitro* methods offer limited capacity for expansion of cancer stem cells in an undifferentiated state. It has become clear that the development of improved methods for isolation and expansion of head and neck cancer stem cells is imperative for the acceleration of the pace of discovery in this area.

CONCLUSIONS

The discovery of a small sub-population of cells that possess exquisitely high tumorigenic potential, associated with the possibility of identifying these cells in clinical settings, provides a new conceptual target for cancer therapy. It is well-known that a frequent cause of failure of conventional therapy in HNSCC is the high incidence of local recurrence and distant metastasis. Notably, it has been hypothesized that conventional therapies do not eliminate the slow-growing cancer stem cells, which appear to be the “drivers” of tumor recurrence and metastases. Therefore, the recent observation that HNSCC might follow the cancer stem cell hypothesis suggests that targeted elimination of these tumor-initiating cells will prevent tumor regrowth and

distant disease. The authors are cautiously optimistic that the development of cancer-stem-cell-based therapies will have a positive impact on the survival of patients with head and neck cancer in the future.

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