Cancer Stem Cells: A Review of the Literature and the Implications in Head and Neck Cancer

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Abstract: In the last few decades, stem cells have been the focus of researchers in an effort to understand the molecular pathways involved in tissue regeneration. By studying normal cell interactions, researchers have since identified cancer stem cells and demonstrated their role in tumorigenesis and metastasis. The authors aimed to review the major molecular pathways involved in tumorigenesis, the role of cancer stem cells, and emerging therapies that target these pathways in squamous cell carcinoma of the head and neck.

Keywords: Stem cells, cancer stem cells, head and neck, squamous cell carcinoma, hypoxia inducible factor, curcumin, cisplatin.

INTRODUCTION

Over the last two decades, research in regenerative medicine has become one of the leading topics in medicine. Experiments in regenerative medicine are based on three different approaches. The first is to boost endogenous repair mechanisms, the second is to engineer artificial bioscaffolds that are subsequently grafted into the desired location, and the third is to control the differentiation of stem cells [1]. This paper will focus on the third approach to tissue regeneration, the molecular pathways involved, and their implications in cancer, particularly squamous cell carcinoma of the head and neck.

STEM CELLS AND CANCER STEM CELLS

Stem cells, by definition, have the ability to self-renew by making one identical copy of itself, and to differentiate into a mature, functional cell. Recent research has focused on stimulating embryonic stem cells to differentiate into the desired tissue, and on isolating adult multipotent cells from different organs. Embryonic cells have become increasingly popular because of their pluripotent nature, meaning they are capable of differentiating into any cell or tissue type based on their microenvironment. The advantages to understanding the factors involved in this differentiation process are infinite and would provide a mechanism for stimulating new growth and development [2-5]. Somatic stem cells, on the other hand, are multipotent, meaning they are capable of generating many differentiated cells but are restricted to one particular tissue type. They are thought to be involved in the day-to-day regeneration of damaged tissues and organs *in vivo,* as seen in the heart, lungs, and central nervous system [6-8]. Understanding the molecular mechanisms of pluripotency and multipotency, and how to maintain this state, has been the main focus of research in the last few years in an attempt to control this process, and therefore to gain the ability to regenerate new tissue to replace damaged organs.

Induced pluripotent stem (iPS) cells are somatic cells that have been reprogrammed into a pluripotent cell similar to embryonic stem cells. A few groundbreaking studies by Yamanaka utilizing a mouse model demonstrated that four transcription factors played a critical role in this transformation: Oct4, Sox2, Kfl4, and c-Myc [9-12]. Further research has shown that Oct4 and another transcription factor, Nanog, seem to be critical for the maintenance of pluripotency [13-18]. Despite these advances in understanding, limitations to using iPS cells has led researchers to focus instead on altering and amplifying the environment of somatic stem cells. The complex nature of the differentiation process, however, has also slowed progress in understanding the molecular mechanisms responsible for controlling the somatic stem cell microenvironment.

Of the advantages gained from studying the molecular microenvironment, one of the most important is the cancer stem cell (CSC) theory. Researchers hypothesized that if such mechanisms existed in normal somatic stem cells, these same pathways may exist in the growth and propagation of tumors. CSCs, also referred to as tumor initiating cells (TIC), are similar to somatic stem cells based on their ability to

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ISSN: 1929-2260 / E-ISSN: 1929-2279/13

self-renew and to differentiate, but due to an acquired defect, they differentiate into and propagate a tumor phenotype. CSCs were first isolated and described by researchers studying leukemia. Dick *et al.* demonstrated that a few leukemic cells could cause tumor occurrence and growth in non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice [19-21]. Additionally, they demonstrated that the differentiation of cancer cells shared cell-surface markers that are also present in normal hematopoietic stem cells, suggesting that cancer stem cells arise from an aberrant pathway of differentiation [20, 21]. Since then, researchers have applied this model to study and identify cellular markers involved in a variety of other human cancers.

CANCER STEM CELL MARKERS IN HNSCC

The existence of CSCs and the overexpression of the transcription factors involved in maintaining pluripotency as described above have also been implicated in the growth of solid tumors [12]. CSCs were first described in solid tumors using breast cancer as a model. In 2003, Al-Hajj *et al.* identified and isolated $CD44^+$ tumor initiating cells that were able to generate new tumors in mice with as few as 100 cells, while alternative phenotypes failed to produce tumor growth [22]. Researchers expanded on the model proposed by Al-Hajj *et al.* to identify the molecular signals and cell markers involved in tumorigenesis of head and neck squamous cell carcinoma (HNSCC). HNSCC is the sixth most common cancer worldwide, accounting for approximately 500,000 new cases annually, with over 40,000 cases occurring in the United States [23]. Despite the advances made recently, the five-year survival rates have not improved for more than 3 decades, making HNSCC a major cause of morbidity and mortality [24]. The poor prognosis of advanced HNSCC increases the importance of determining new markers for targeted therapy.

Currently, although no single predominant CSC has been identified in HNSCC, a number of cellular markers have been identified that play a role in tumorigenesis and metastasis. CD44, previously identified in other solid tumors such as breast, colon, prostate and pancreatic cancer, is a transmembrane protein cell surface receptor for hyaluronan, which is involved in cell adhesion and migration [25]. In a study by Prince *et al.,* the authors identified $CD44^+$ cells in HNSCC tumors, and demonstrated their ability to initiate tumorigenesis in mice. More importantly, cells that demonstrated high expression of CD44 regenerated the heterogeneous tumor phenotype while those cells with low expression of CD44 did not [25, 26]. The authors also demonstrated that the gene *BMI1*, previously identified as an important gene in self-renewal and tumorigenesis, was expressed in higher levels in $CD44^+$ cells. Studies have shown that cells that overexpress *BMI1* exhibit stem-like properties as well as chemo-radioresistance and increased metastasis [26]. In a study by Davis *et al.,* the authors further demonstrated that mice injected with $CD44^{high}$ cells produced tumors *in vivo* whereas no mice (0 out of 17) injected with $CD44^{low}$ cells developed lesions [27]. Their study also revealed that although $CD44^{high}$ cells were more motile than $CD44^{low}$ cells, they were not more invasive *in vitro*. They attributed this to faults in their *in vitro* model and did not believe it was an indication of the cells metastatic potential. They also suggested that the increased motility seen in $CD44^{high}$ cells supports the epithelial-mesenchymal transition (EMT) hypothesis, which would account for the results seen in the *in vivo* arm of their experiment [27]. Their theory was further supported by Takahashi *et al.* who showed that tumor necrosis factor (TNF) induced EMT, causing an interaction between CD44 and hyaluronan, thereby mediating cell-cell dissociation, actin remodeling, and therefore enhanced motility [28]. Although a number of studies have supported the original work of Prince and his colleagues, only one study to date presents an alternate perspective. A study by Oh *et al.* revealed that both $CD44^+$ and $CD44^-$ cells possessed stem-like characteristics with the ability to propagate the tumor and resist the chemotherapeutic agent cisplatin. Based on their findings, they concluded that CD44 could not be used as a selective marker of CSCs in HNSCC [29]. The evidence suggests that further research is required to determine CD44 role in tumorigenesis as a CSC marker in HNSCC, although the majority of researchers would agree that inhibition of this cell surface marker might provide significant improvements in targeted therapy [30].

Another functional marker linked to $CD44^+$ cells is aldehyde dehydrogenase 1 (ALDH1). ALDH1 is normally responsible for maintaining cellular homeostasis by detoxifying intracellular aldehydes through the oxidation and conversion of retinol to retinoic acid [25, 31]. ALDH1 is highly expressed in hematopoietic stem cells, as well as in malignant

mammary stem cells. It has been used as a prognostic indicator of metastases and poor survival [32]. ALDH1A1 was first identified by Visus *et al.* as a premalignant marker in HNSCC, but it was not until a study by Chen *et al.* that revealed the link between $ALDH^+$ cells and CSCs in HNSCC [33, 34]. The authors demonstrated that $ALDH1+CD44^+$ cells were more radioresistant than their $ALDH^-$ counterparts. Clay *et al.* also showed that $ALDH^+$ cells propagated tumors, indicating these cells had stem-like characteristics [35]. Further research by Krishnamurthy and colleagues demonstrated that the combination of $ALDH1^+$ and $CD44^+$ cells were a more selective CSC marker than either marker individually. Furthermore, Snail, a zinc-finger transcription factor, is highly expressed in $ALDH+CD44^+$ cells, causing increased tumorigenesis, metastasis, and chemo-radioresistance [25].

Two additional cell surface markers, CD133 and CD147, have been identified in specific types of HNSCC. CD133 was identified as a marker in laryngeal HNSCC both *in vitro* and *in vivo*, and in oral cavity tumors with higher cloning and invasive potential [34]. Additionally, as with CD44, co-expression with *BMI1* also promotes stem-like properties and enhanced proliferative capacity [35]. CD147, identified and upregulated in oral cavity cancers, was also shown to have increased invasive and metastatic potential in HNSCC, although more research is needed to determine its exact role.

Twist, another transcription factor that controls gene expression during embryonic development, promotes epithelial-mesenchymal transition (EMT) and regulates *BMI1*, contributing to tumor invasion and metastasis [36]. Nanog and Oct4, as described above, have also been shown to confer increased resistance to chemotherapy and radiation in HNSCC. c-Met, a tyrosine kinase receptor for growth factor, is also overexpressed in HNSCC, causing increased tumor growth, angiogenesis, invasiveness and metastasis [37]. The authors also determined that c-Met overexpression led to cisplatin-resistance, implying that knockout of this receptor may increase the effectiveness of cisplatin [37].

Finally, Prince and his colleagues have identified side population (SP) cells that exhibit features similar to CSC cells, and maintain a variety of highly tumorigenic and therapy-resistant properties. First, SP cells exhibit chemo-resistance by overexpressing ABCG2, an ATP-binding cassette membrane transporter that acts by pumping cytotoxic agents from the cell [38]. SP cells were also found to express high levels of *BMI1*, CD44, and Oct4 in HNSCC, contributing to their tumorigenic capacity [32, 39]. They also reported that SP cells had an increased resistance to 5-FU based on its CSC-like properties. Additionally the Wnt/β-catenin pathway, as described below, was abnormally activated in SP cells causing tumor cell proliferation, metastasis, and drug resistance [40]. The SP had lower activity of β-catenin compared to non-side population cells, with the latter being more sensitive to 5-FU. In normal cells, higher β-catenin activity promotes DNA synthesis and cell proliferation, allowing cytotoxic drugs to exhibit their effect [28]. Evidence suggests that SP cells serve as another potential target for new therapy based on their stem-like, tumorigenic capabilities.

SIGNALING PATHWAYS INVOLVED IN MAINTAINING CANCER STEM CELLS IN HNSCC

As researchers identified specific markers, it became evident that specific signaling pathways existed to maintain these CSCs. Understanding these pathways is essential to developing new targeted therapies because it explains why highly aggressive tumors are resistant to cytotoxic chemotherapy and radiation. It was hypothesized that CSCs are protected from destruction by their microenvironment, or niche. This niche protects CSCs against differentiation and apoptosis and maintains their self-renewal capacity. Evidence also supports the hypothesis that the niche contributes to CSCs ability to metastasize and to resist therapy [40].

Head and neck cancers tend to be highly vascular tumors, and it was found that most CSCs are found in close proximity to blood vessels [32]. Studies have shown that there are specific molecular mechanisms in place which enhance endothelial and tumor-cell maintenance, and that ablation of specific endothelial cells associated with metastases decreases the number of CSCs in the tumor. In fact, *BMI1* expression is upregulated by endothelial cell-secreted factors and promotes CSC proliferation and regenerative capacity in HNSCC [32]. In addition to the endothelial cell promotion of the “perivascular niche” [32], there are many other signaling pathways involved in maintaining the stem cell niche. Three pathways, in particular, have emerged as important foundations in HNSCC.

Hypoxia Inducible Factors

Hypoxic stress is one important factor in the niche [41]. Numerous studies have demonstrated that tumor hypoxia is an independent factor leading to increased

metastatic potential, malignancy, and resistance to treatment. Hypoxia is thought to contribute to the malignant transformation of cells by increasing expression of drug-resistant genes, facilitating tumor invasion and metastasis, reducing expression of DNA repair genes, and decreasing genomic stability [41]. It was also shown that tumor hypoxia may regulate cell differentiation, supporting the theory that it may facilitate the maintenance of cancer stem cells [42]. The primary mediator of hypoxia-induced cell signaling is hypoxia-inducible factor-1 (HIF-1), which is often correlated with tumor development and decreased patient survival. HIF proteins play a critical role in angiogenesis, facilitating the growth of vasculature within tumors. In a study involving glioma cells, knockdown of HIF-1 reduced tumor growth [43]. Furthermore, studies have shown that hypoxia can increase CD133 expression in glioma cells, a finding that has clinical implications for treatment [44, 45]. In a study by Bao *et al.,* the authors revealed that $CD133^+$ tumor cells were more effectively able to repair radiation-induced DNA damage than $CD133^-$ cells in the same tumor. Therefore targeting HIF to decrease CD133 expression may lead to decreased radio-resistance and therefore better tumor control [46].

Wnt/β-Catenin

Another important pathway involved in maintaining the stem cell niche and tumorigenesis is Wnt/β-catenin signaling, normally involved in regulating pluripotency in embryonic and somatic stem cells, and intimately involved in tissue homeostasis [47]. There are three different pathways involved in Wnt signaling: the canonical Wnt/β-catenin cascade, the non-canonical planar cell polarity (PCP) pathway, and the Wnt/Ca^{2+} pathway. The canonical pathway is the one implicated in tumorigenesis. Wnt receptor complexes are bound by Wnt ligand, leading to intrinsic kinase activity of the APC complex to inhibit β-catenin. Specifically, Wnt stabilizes β-catenin and enhances ABCB1/MDR-1 transcription, which are multidrug resistance genes expressed in stem cells [48]. Furthermore, β-catenin accumulates and translocates to the nucleus to bind the N terminus of LEF/TCF transcription factors. Binding subsequently causes activation of *Myc, AXIN2* and *CYCLIND1* genes [49]. Wnt, through the activation of β-catenin, also plays a role in metastasis and epithelial –mesenchymal transition (EMT). When this pathway becomes dysregulated, neoplastic proliferation occurs [1, 50]. Inhibiting these pathways is another potential target of therapeutic agents [51].

Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) plays a significant role in normal development and wound healing by inducing cellular changes leading to breakdown of cell-to-cell interactions. Breaking cell communications is a critical step in transforming adherent, stationary epithelial cells into migratory cells [52]. Based on its role in normal tissues, researchers have studied and proven how EMT transforms an epithelial cell into a cancer cell capable of migration, leading to increased metastasis and progression of solid tumors [53-55]. Both HIF and Wnt as described above induce EMT, promoting metastasis in HNSCC [54]. Brabletz *et al.* hypothesized that there were two types of CSCs: stationary CSCs (sCSCs) and migrating CSCs (mCSCs). sCSCs are embedded in the epithelia and are nonmobile, while mCSCs mediate tumor cell metastasis. They proposed that mCSCs were derived from sCSCs who underwent EMT [56]. In other words, EMT was an essential component involved in promoting metastasis from the CSCs protected within the niche. In EMT, E-cadherin and β-catenin are down-regulated while vimentin, fibronectin, and N-cadherin are up-regulated [57]. Mani *et al.* demonstrated this up-regulation in mammary epithelial and breast cancer cells [58]. Similar to EMT role on normal epithelial cells, the Weinberg group further described how the EMT transcription factors give mesenchymal traits to carcinoma cells, enhancing their ability to invade and metastasize [55, 58]. Other researchers have identified that EMT induction also contributes to drug resistance. Identifying the exact mechanism of this drug resistance would provide a critical target for new therapy as would specific targets to HIF, Wnt, and EMT.

NEW TARGETED THERAPY

Once tumors progress from an organ-confined disease into locally invasive and metastatic cancers, conventional therapies become less effective. This is in part due to genetic abnormalities causing overexpression of oncogenic signaling pathways as well as down-regulation of tumor suppressor gene products such as p53, PTEN or Rb in cancer cells [59]. Furthermore, CSCs' quiescent state makes them resistant to standard chemotherapy. Chemotherapeutic agents normally act on rapidly dividing cells that are actively synthesizing DNA. In comparison to other cells within the tumor, because CSCs are not rapidly synthesizing DNA, they are relatively protected from the toxicity of chemotherapy, leading to resistance [17,

28]. In fact, CSCs are thought to be one of the main factors causing relapse and locally advanced and metastatic cancers. Therefore, based on the identification of the above cell surface and functional markers, researchers started developing targeted therapies to attack these cells normally resistant to conventional therapy.

Cisplatin is currently a first-line treatment of HNSCC, although tumor cells are increasingly resistant leading to disease relapse. Platinum-based therapies, such as cisplatin, cause DNA cross-links and double-strand breaks during the process of replication, inducing cell cycle arrest and apoptosis [60]. As described above, overexpression of cMET, a tyrosine kinase receptor identified in HNSCC CSCs, leads to cisplatin-resistance. Therefore tyrosine kinase receptors are a potential target of new therapies. In a study by Yilmaz *et al.,* a novel agent AZ64 was shown to modulate the tyrosine kinase receptor and overcome cisplatin resistance in HNSCC [61]. Future studies involving other novel agents offer promising treatment strategies to overcome cisplatin resistance.

Another novel approach to overcoming cisplatin resistance is through curcumin treatment. Curcumin has been previously established as a chemosensitizer through inhibition of EGFR, NF- κB, IL1a, and TNFα [62]. Murali *et al.* showed that pre-treatment with curcumin sensitized ovarian cancer cells to cisplatin at doses ten times lower than in cells without pretreatment, with no change in effectiveness. Davis and colleagues then demonstrated that a novel curcumin analog FLLL32, which targets signal transducer and activator of transcription (STAT) 3, sensitized HNSCC tumor cells to cisplatin therapy [63]. STAT proteins are cytoplasmic transcription factors involved in modulating gene expression, and are critical for cell growth, differentiation, apoptosis, and metastasis. HNSCC lines express STAT3, and the curcumin analog FLLL32 targeted STAT3. They showed that FLLL32 down-regulated STAT3 and increased the number of apoptotic cells through cisplatin sensitization, producing a significant antitumor effect [64]. Similar to the study by Murali *et al.,* they demonstrated that the same tumor size reduction could be achieved with a 4-fold lower dose of cisplatin. The evidence from this novel approach in decreasing both the morbidity and mortality associated with HNSCC and cisplatin treatment has significant implications for the future. Curcumin has also been shown to suppress growth of $CD44^{high}$ cells in HNSCC using *in vitro* models, but data are still limited and would be an important potential target in the future [65].

Using an alternative approach to increasing cisplatin sensitivity, Lim *et al.* demonstrated how all-trans-retinoic acid (ATRA) inhibits growth of HNSCC by suppressing the Wnt/β-catenin pathway [66]. First, they showed that ATRA induced CSC differentiation by decreasing stem cell marker expression of Oct4, Sox2, Nestin, and CD44. Furthermore, they showed that ATRA increased HNSCC tumor cell susceptibility to cisplatin therapy. They hypothesized that this effect was mediated by caspase-3 expression, a key mediator of apoptosis, which was greatly increased with the combination of ATRA and cisplatin in comparison to either agent alone. Furthermore, the authors demonstrated that tumor volume was significantly smaller following ATRA treatment in comparison to controlled mice. Additionally they found that ATRA suppressed β-catenin expression in HNSCC CSCs, although further research is needed to elucidate the exact relationship between them [66]. Regardless, their study is evidence that novel therapies targeting CSC cell markers offer promising new strategies to treat drug-resistant HNSCC.

In addition to the novel biologic therapies, nanotechnology and specific delivery methods to administer chemotherapeutic drugs to a tumor offer a new treatment strategy. As previously established, the upregulation of the anti-apoptotic gene sphingosine kinase (SphK1) is associated with radioresistance in HNSCC cells. Using a biocompatible gold nanorod (GNR) as a transporter of short interfering RNA (siRNA), Masood and colleagues targeted the SphK1 gene, which led to a 50% reduction in tumor size following radiation therapy. Also, the radiation dose required was much lower following treatment with GNR than without [67]. Successful targeting of the SphK1 gene to increase radiosensitivity suggests that similar mechanisms can be employed to target CSCs to modify gene expression and to reduce chemotherapy resistance in HNSCC cells.

CONCLUSION

In the last few decades, stem cells have been the focus of researchers in an effort to understand the molecular pathways involved in tissue regeneration. By studying normal cell interactions, researchers have since identified cancer stem cells and demonstrated their role in tumorigenesis and metastasis. Isolating specific cellular markers of CSCs has also been a

major focus of research in the last decade, and has led to new, targeted therapies in head and neck squamous cell carcinoma.

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Received on 15-04-2013 Accepted on 07-05-2013 Published on 01-07-2013

DOI: http://dx.doi.org/10.6000/1929-2279.2013.02.03.4