Targeting Head and Neck Cancer Stem Cells and Endothelial Cell Interaction

by

Hong Sun Kim

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Doctoral Committee:

Professor Jacques E. Nör, Chair
Professor Colin S. Duckett
Professor Yvonne L. Kapila
Professor Weiping Zou

To my dear husband, **Thomas Kwun**, who gave me courage, laughter and love daily.

To my lovely daughter, **Abigail Kwun**, who is the greatest blessing in my life.

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ABSTRACT

Tumor recurrence and loco-regional metastases are the major clinical challenges in the management of patients with head and neck squamous cell carcinoma (HNSCC). A subpopulation of cells, called cancer stem cells, has been identified in HNSCC and characterized as cells with uniquely high tumor-forming ability and resistance to conventional chemotherapies. Multiple reports have shown that head and neck cancer stem cells drive the metastatic process. How tumorigenic cells are displaced from the tumor "island", travel through connective tissues, and get into blood vessels is still not well understood. We hypothesized that endothelial cell-secreted factors generate a chemotactic gradient that attracts head and neck cancer stem cells towards blood vessels. Head and neck cancer stem cells reside in perivascular niches. Close proximity between cancer stem cells and blood vessels may facilitate cancer stem cells to invade into the bloodstream and initiate metastasis. Our group previously has shown that endothelial cell-secreted factors, specifically interleukin-6 (IL-6), potentiate the self-renewal and tumorigenicity of head and neck cancer stem cells. Here, we assessed the role of endothelial cell-initiated IL-6 pathway activation in head and neck cancer stem cell motility. Endothelial cell-secreted IL-6 induced the expression of the mesenchymal marker Vimentin and epithelial-mesenchymal transition-activating transcription factor Snail in head and neck cancer stem cells. When endothelial cell-secreted IL-6 was inhibited, we observed decreased motility in head and neck cancer stem cells. Xenograft HNSCC tumors vascularized with IL-6 knockout human endothelial cells grew slower than tumors vascularized with control endothelial cells. Notably,

tumors grown with IL-6 knockout endothelial cells had smaller fraction of cancer stem cells than those with control endothelial cells. In addition, anti-IL-6 receptor (IL-6R) antibody, tocilizumab, also decreased cancer stem cell population. Tissue microarray analysis of 80 HNSCC patient samples revealed that high IL-6R or its co-receptor gp130 expressions correlated with low patient survival. Taken together, these results highlight the contribution of endothelial cell secreted factors on the migratory behavior of head and neck cancer stem cell that ultimately result in dissemination of tumor cells. Further, we show the therapeutic potential of tocilizumab targeting cancer stem cells in head and neck cancer.

CHAPTER I

INTRODUCTION

Head and neck cancer is the sixth most common cancer worldwide with more than half a million new cases diagnosed every year. Tobacco, alcohol and human papillomavirus infection are the common risks of head and neck cancer. Approximately 90% of head and neck cancer is squamous cell carcinoma (HNSCC). Standard of care for head and neck cancer includes surgery, chemotherapy and radiation. Cetuximab, anti-EGFR inhibitor, is the only targeted therapy that is approved for head and neck cancer. Approximately 60% of the head and neck cancer patients present locally advanced disease (stage III/IV) (Bhave et al., 2011). These patients often develop distant metastasis or tumor relapse after receiving initial treatments. When the tumor relapses or metastasizes, the median overall survival drops down to 3 to 4 months (Gold et al., 2009). However, our understanding of mechanisms related to tumor metastasis and recurrence is still limited.

A tumor consists of heterogeneous population of tumor cells. Heterogeneity within a tumor was well recognized by earlier studies dating back to 1970s. For example, clonally expanded metastatic tumor cells had varied rate of generating metastasis, suggesting different population of cells has varied tumor forming ability (Fidler and Kripke, 1977). Seminal work by Bonnet and Dick showed that $CD34^{+}CD38^{-}$ cells from acute myeloid leukemia exhibited robust tumor forming ability in immunocompromised mouse (1997). This initial discovery led to

identification of a subpopulation of cells, called cancer stem cells, from various cancer types. Cancer stem cells have high tumorigenicity, resistance to chemotherapy and self-renewal ability. Our group has previously reported that HNSCC cells with high aldehyde dehydrogenase (ALDH) activity and high CD44 expression ($ALDH^{high}CD44^{high}$ cells) have self-renewal and superior tumor-forming ability (Krishnamurthy et al., 2010). Recent studies revealed that cancer stem cells are the key players of metastasis in head and neck cancers (Davis et al., 2010; Chinn et al., 2015). However, the molecular mechanism that enhances cancer stem cells metastatic ability is still unclear.

Both normal and cancer stem cells need to be protected to retain their survival and self-renewal abilities. Like normal stem cells, cancer stem cells reside in a protective environment. Brain tumor stem cells reside in the perivascular niche to self-renew and initiate rapid tumor growth (Calebrese et al., 2007). Head and neck cancer stem cells also reside close by the blood vessels, and endothelial cell-secreted factors enhance the self-renewal and survival of head and neck cancer stem cells (Krishnamurthy et al., 2010). Our group showed that endothelial cell-secreted factors induce migration and epithelial-mesenchymal transition in HNSCC cells (Neiva et al., 2008; Zhang et al., 2014). Specifically, we found that endothelial cell-secreted interleukin-6 (IL-6) enhances survival and tumorigenic potential of head and neck cancer stem cells (Krishnamurthy et al., 2014). Such observations suggest that the endothelial cell-secreted factors, such as IL-6, may induce migratory behavior of cancer stem cells to initiate metastasis.

Identification of cancer stem cells opened up the possibility of developing targeted therapies against cancer stem cells. Cancer stem cell hypothesis explains the current failure of cancer treatment is due to inability to ablate cancer stem cells that are resistant to conventional

chemotherapies. Therefore, the combination of existing drug and cancer stem cell-targeting agent may result in complete remission of tumor.

In this Ph.D. dissertation, we gave an overview of cancer stem cells as potential therapy target in Chapter II and described the method to isolate cancer stem cells and grow orospheres to assess cancer stem cell targeting agents in Chapter III. In Chapter IV, we hypothesized that endothelial cell secreted factors enhance the aggressiveness of head and neck cancer stem cells. Specifically we addressed the role of endothelial cell-secreted IL-6 on the head and neck cancer stem cell motility and epithelial-mesenchymal transition induction. Finally, we tested the therapeutic potential of IL-6 pathway inhibiting agent to target head and neck cancer stem cells.

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CHAPTER II

Cancer Stem Cells in the Biology and Treatment of Head and Neck Squamous Cell Carcinoma

Abstract

Emerging evidence has demonstrated that the pathobiology of head and neck squamous cell carcinomas (HNSCC) is defined by the function of cancer stem cells. These cancer stem cells constitute a small fraction of the overall tumor cells, typically ranging between 1-5% of the overall tumor mass. They share the properties of multipotency and self-renewal with physiological stem cells. However, cancer stem cells are endowed with high tumorigenic potential, which makes these cells an integral part of tumor initiation and progression towards metastasis. These findings have provided strong rationale for targeted elimination of cancer stem cells in the treatment of patients with head and neck cancer. Recent studies demonstrated that the cancer stem cells are highly resistant to conventional chemotherapy, which may explain why so many head and neck cancer patients experience tumor recurrence. Therefore, the elimination of these cancer stem cells will likely require the development of therapies specifically targeted to these cells. In this chapter, we will review the evidence on head and neck cancer stem cells and the perivascular niche where these cells typically reside and will also discuss initial attempts to overcome resistance to treatment by combining debulking therapies with cancer stem cell-targeted therapies.

Introduction

Head and neck cancer stem cells are characterized by multipotency, self-renewal and high tumorigenic potential. These cells are typically found in close proximity to blood vessels, in a dynamic and protective microenvironment named the perivascular niche. It is believed that the crosstalk of cancer stem cells with stromal cells (e.g. endothelial cells, cancer-associated fibroblasts) provides molecular cues that maintain the stem cell pool, and the crosstalk with other tumor cells regulates the processes that eventually lead to their differentiation into rapidly proliferative tumor cells. Emerging knowledge of the biology of cancer stem cells has provided the scientific rationale for the targeted elimination of these cells in the treatment of patients with head and neck cancer.

Cancer stem cells in head and neck squamous cell carcinoma

The experimental identification of cancer stem cells is typically done by the use of markers that enable the identification of cells with enhanced ability to self-renew, differentiate into other cell types and generate new tumors. *In vitro* characterization of the stem cell phenotype is performed by culturing the cells in serum-free, ultra-low attachment conditions. This assay exploits the fact that stem cells are capable of anchorage-independent growth, whereas normal differentiated cells cannot grow under these conditions (Dontu and Wicha, 2005; Reynolds et al., 1992). Growth and passaging of these spheres is an indication of the cells ability to self-renew. *In vivo* characterization of the cancer stem cell phenotype is performed by serially transplanting fluorescence-activated cell sorting (FACS)-sorted cell populations into immunodeficient mice (Fig. 2.1). Differences in tumorigenic potential correlate with the level of cancer stem cell enrichment within the sorted populations. Further passaging of these FACS-sorted cells is a measure of the cells ability to self-renew. Generation of cells with different marker

subpopulations indicates that the original cell subpopulation that generated the tumor is multipotent and able to give rise to a diversity of cells types that make up the complexity and heterogeneity typically observed in tumors.

Using these experiments, cancer stem cells were first isolated in HNSCC by Prince and collaborators (2007). In this groundbreaking work, Prince and colleagues sorted varying dilutions of lineage-negative CD44+ or CD44- primary HNSCC cells and implanted them subcutaneously in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice or Rag2/cytokine receptor common γ-chain double knockout (Rag2γDKO) mice. They found that

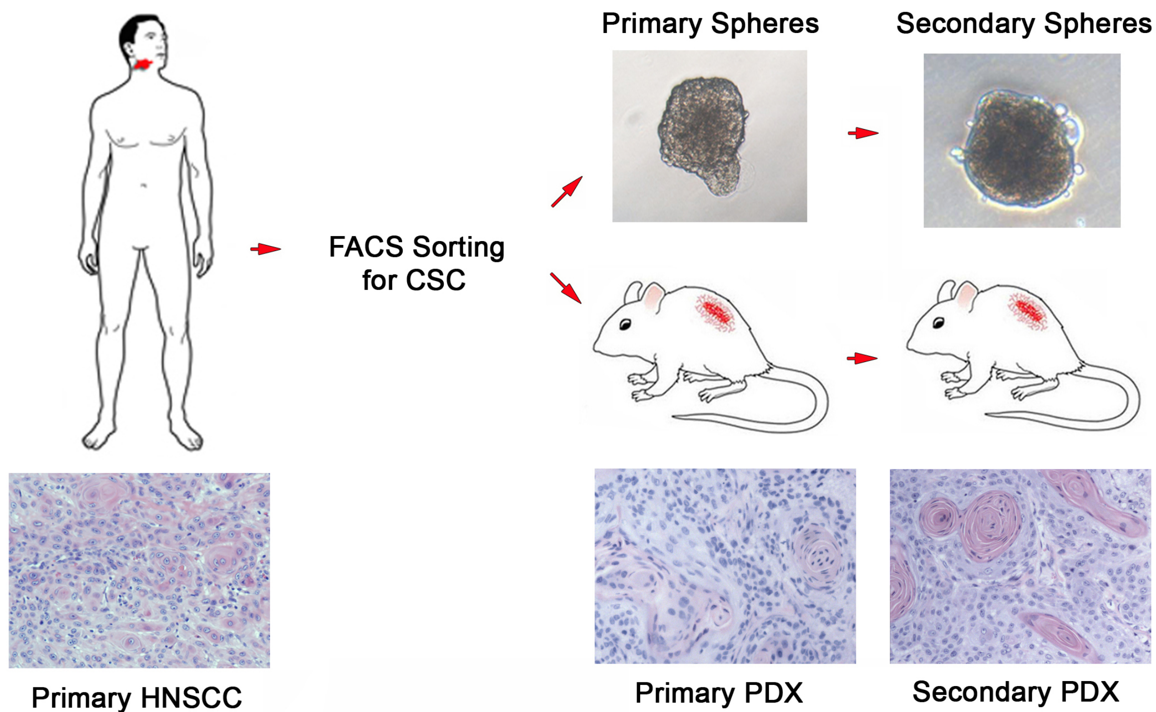

Figure 2.1. Experimental strategies for mechanistic and developmental therapeutic studies of head and neck cancer stem cells. The HNSCC from a patient is digested immediately after surgery and sorted for cancer stem cellS. Following sorting, the cells are transplanted subcutaneously in immunodeficient mice to generate patient-derived xenograft (PDX) tumors. Sorted cells can also be plated in low-attachment culture conditions to generate orospheres and maintain the cancer stem cell phenotype. Both, the orospheres as well as the PDX tumors can be passaged for studies of self-renewal properties of the cancer stem cells.

20 of the 31 transplantations of the CD44+ cells yielded tumors where as only 1 of the 40 CD44- transplantations formed tumors suggesting that the CD44+ are more tumorigenic than the CD44- cells. As few as 5,000 CD44+ primary tumor cells were able to generate tumors. In contrast, a minimum of 500,000 CD44- primary cells was necessary for tumor growth. Tumors generated from CD44+ tumor cells were serially passaged, showing that these cells are capable of self-renewal. When the tumors were digested and analyzed by FACS, both CD44+ and CD44- cells were present, suggesting that the CD44+ cells are capable of differentiation. Primary CD44+ cells showed a significant upregulation of Bmi-1 expression. Sections taken from primary human tumors showed significant co-staining of CD44 with the squamous epithelial stem cells markers Cytokeratin 5/14 further suggesting that these cells do indeed display a stem cell-like phenotype (Prince et al., 2007). Collectively, this work unveiled the presence and function of a subpopulation of tumor cells with uniquely high tumorigenic potential, self-renewal and multipotency in HNSCC.

In another important study, Clay and colleagues showed that cells isolated based on high aldehyde dehydrogenase (ALDH) activity also can be used to enrich for cancer stem cells in HNSCC. In this study, Clay and colleagues (2010) found that FACS-sorted ALDH^{high} primary HNSCC cells were significantly more tumorigenic when compared to the ALDH^{low} primary HNSCC cells when transplanted into NOD/SCID mice. Primary HNSCC cells sorted for ALDH^{high} cells were able to form tumors in 24 of the 45 transplantations, while the cells sorted for ALDH^{low} cells activity formed only 3 tumors out of 37 transplantations. Importantly, as few as 50-100 ALDH^{high} cells were able to form tumors. Notably, ALDH^{high} cells were able to generate tumors that showed a similar histology when compared to the original unsorted tumors

and were able to replicated the original tumor heterogeneity for ALDH activity suggesting that ALDH^{high} cells are have both self-renewal and multipotency (Clay et al., 2010).

As both CD44 expression and ALDH activity have been described to enrich for cancer stem cells in HNSCC, Krishnamurthy and colleagues combined the two markers to determine if they could further enrich for this cell population. In these studies, it was found that ALDH+CD44+ cells were capable of forming colonies in soft agar more efficiently than ALDH-CD44+ and ALDH-CD44- cells, suggesting that ALDH+CD44+ cells are predominately stem-like compared to the other subpopulations. These results led them to perform further *in vivo* experiments where they FACS-sorted 1,000 ALDH+CD44+ cells and 10,000 ALDH-CD44- cells and co-transplanted them subcutaneously in immunodeficient mice together with human endothelial cells in biodegradable scaffolds (Krishnamurthy et al., 2010). They found that the ALDH+CD44+ cells were able to generate tumors in 13 of the 15 total transplants, while the ALDH-CD44-Lin- cells were only able to for tumors in 2 out of the 15 transplants. To investigate whether these cells were capable of self-renewal, they digested the tumors into single cell suspensions and serially transplanted into immunodeficient mice. ALDH+CD44+ cells were able to generate secondary tumors whereas the ALDH-CD44- cells were unable to form secondary tumors again suggesting that these cells are capable of self-renewal. The fraction of ALDH+CD44+ cells remained low in both the primary and secondary tumors suggesting once again that these cells are multipotent (Krishnamurthy et al., 2010).

In addition to ALDH and CD44, HNSCC cancer stem cells can also be isolated using a DNA binding dye called Hoechst 33342. When taken up by the cell, this dye binds the DNA and can be seen in FACS analysis. However, cells that up-regulate drug resistant cell transporter proteins, such as ABCG2, exclude the dye and can be sorted out by FACS. These cells are

termed side population (SP) cells. In a study by Song and colleagues (2010), they found that SP cells were able to form significantly more spheres in clonogenic soft agar assays when compared to non-SP cells. When SP and non-SP cells were transplanted *in vivo,* the SP cells were able to form tumors using as few as 100 cells, while 10,000 non-SP cells were required to initiate tumor growth (Song et al., 2010). Tabor and colleagues (2011) also found this SP cells in HNSCC cells lines. When they sorted the SP cells and re-plated the sorted cells into new tissue culture flasks, the SP cells were able to differentiate and generate non-SP cells suggesting that SP cells are multipotent. In addition to multipotency, they also saw that SP cells showed an increased ability to formed spheres under non-adherent conditions suggesting that SP cells are also capable of self-renewal. When SP cells were transplanted into mice, they were able to generate tumors using 5,000 SP cells. In contrast, no tumors were observed using 5,000 non-SP cells suggesting that SP cells are uniquely tumorigenic (Tabor et al., 2011).

Several studies have also proposed the use of other cancer stem cell markers in HNSCC. One study suggested that cells containing low levels of reactive oxygen species are uniquely tumorigenic (Chang et al., 2014). Other markers that have been suggested include CD10 and CD271 (Fukusumi et al., 2014; Murillo-Sauca et al., 2014). In their studies, Fukusumi and colleagues found that CD10+ HNSCC cells were significantly more sphere forming *in vitro* and tumorigenic *in vivo* (2014). Using HNSCC cell line Detroit562, they were able to generate tumors in all of the six CD10+ transplants, whereas only 2 of 6 CD10- transplants developed tumors. However, this difference in tumorigenicity was not seen in the FaDu HNSCC cell line, suggesting that CD10 maybe a cell line-specific cancer stem cell marker. Murillo-Sauca and colleagues sorted CD271+ cells alone or in combination with CD44+ cells and transplanted these cells subcutaneously into $Rag^{-/-}\gamma c^{-/-}$ mice. When 10,000 CD271+ and 10,000 CD271- cells were

transplanted, they were able to generate tumors in 3 of 5 CD271+ implants, whereas no tumors were generated in the CD271- cells. When CD271+CD44+ cells were transplanted, they were able to generate tumors using as few as 100 cells. The CD44-CD271- cells were only able to generate tumors when 1,000 cells were transplanted.

The search for the ideal marker(s) for head and neck cancer stem cells is far from being complete. Ideally, a specific marker or marker combination would select for highly tumorigenic cancer cells, and absence of these markers would identify cancer cells that have not tumorigenic potential at all. Such specificity has not been achieved yet. Further, it will be critical to understand if cancer stem cell markers have only the ability to identify stem cells, or if these markers actually play a functional role in the making of a cancer stem cell. This is perhaps relatively clear when one thinks about SP cells, where the function of a key drug resistant cell transporter protein is up-regulated. This might explain, at least in part, the observation that cancer stem cells are highly resistant to chemotherapeutic drugs such as cisplatin (Nör et al., 2014). On the other hand, a possible functional role for other cancer stem cell markers (e.g. CD10, CD44) appears less clear. Nevertheless, this is an area of intense research that should yield more conclusive results in the upcoming years.

Signaling pathways in head and neck cancer stem cells

Much research has been done to characterize the pathways that regulate the cancer stem cell phenotype in HNSCC. For example, a recent study showed that the Wnt/β-catenin signaling axis is critical for the maintenance of the stem cell phenotype (Lee et al., 2014). Wnt signaling plays an important role in normal stem cell function during embryonic development by modulating differentiation, migration and proliferation. In their investigations, Lee and colleagues found that both cytoplasmic and nuclear β-catenin were seen in a small subpopulation of HNSCC cells.

This staining overlapped with ALDH1 and CD44 staining, suggesting that the β-catenin activity is active and primarily restricted to the cancer stem cells. Indeed, when β-catenin was overexpressed in HNSCC cell lines, they saw an increased sphere formation as well as an increase in expression of stem cells markers Oct4, Sox2 and CD44. Importantly, overexpression of β-catenin caused an increased in expression of ABC transporters as well as significantly increased chemoresistance to cisplatin treatment. In contrast, when β-catenin was knocked down, they saw a significant reduction in sphere growth and a decreased expression of Oct4, Sox2, CD44 and the ABC transporters. Importantly, knockdown of β-catenin significantly reduced the tumorigenic potential of HNSCC cells *in vivo*. Interestingly, overexpression of Oct4 restored the tumorigenic potential *in vivo* upon knockdown of β-catenin, suggesting that β-catenin regulation of the cancer stem cell phenotype happens in part through Oct4 (Lee et al., 2014). In agreement with this study, work by Song and colleagues found that SP cells have significantly higher Wnt/β-catenin signaling than non-SP cells. In their studies, they used a TOPFLASH luciferase reporter with wild-type β-catenin binding sites and found an increased activity of β-catenin-dependent transcription in SP cells. They also found that DKK1 and AXIN2, two critical Wnt/β-catenin target genes, were up-regulated using PCR analysis (Song et al., 2010). Collectively, this work provides strong support for the functional role of the Wnt/β-catenin signaling pathway in the pathobiology of head and neck cancer stem cells.

Several other studies have elucidated the importance of Oct4 in HNSCC cancer stem cells function. Ventelä and colleagues (2014) found that cells expressing Oct4 possess a less differentiated phenotype and are more resistant to chemotherapy. Indeed, patients with high Oct4 expression have lower survival than those patients who do not express Oct4 (Ventelä et al., 2014). In agreement with this study, Koo and colleagues found that overexpression of Oct4 in

several HNSCC cells lines significantly increased cell proliferation and sphere formation. Notably, cells overexpressing Oct4 were more resistant to cisplatin treatment. These cells showed a greater expression of the stem cell markers Sox2 and Nanog, as well as the ABC transporter protein. Oct4 overexpression also increased the invasive potential of these cells and elevated the levels of Slug, an important epithelial to mesenchymal transition (EMT) transcriptional factor. Notably, $Oct4^{high}$ cells showed increased tumorigenic potential *in vivo,* when compared to $Oct4^{low}$ cells (Koo et al., 2014).

Another important signaling pathway in cancer stem cell biology is mediated by interleukin-6 (IL-6), an important inflammatory cytokine. This pathway was first characterized in breast cancer stem cells by a study from Sansone and colleagues (2007). In their investigations they found that antibody blockage of the IL-6 binding to the IL-6 receptor (IL-6R) significantly decreased secondary mammosphere formation in low-attachment conditions, suggesting that this ligand to receptor interaction is important in the self-renewal of breast cancer cells. Conversely, when IL-6 was added to primary sphere cultures, these investigators observed an increase in secondary mammosphere production further supporting their hypothesis. Interestingly, the MCF-7 cell line-derived mammospheres showed an increased expression of IL-6 mRNA when compared to the normal attachment MCF-7 cells suggesting that the breast cancer stem cells significantly up-regulate IL-6 when compared to the bulk tumor cells. Further experiments suggested that IL-6 binding activates the Notch-3 pathway, an important signaling axis in the regulation of stem cell function (Sansone et al., 2007). The Poliak laboratory further elucidated the role IL-6 plays in the function of breast cancer stem cells (Marotta et al., 2011). In their study they found that *IL6* is important in many stem cell self-renewal pathways. In particular, IL-6

reduction lead to a reduction in phosphorylated STAT3, which has been shown to be important for maintaining stemness in murine embryonic stem cells.

IL-6 was also found to be important in HNSCC specifically in predicting the recurrence and survival rates among HNSCC patients. In an epidemiological study by Duffy and colleagues (2008), they compared pretreatment IL-6 serum levels and correlated these data to the post-treatment clinical outcomes of the patient. They found that patients with high IL-6 pretreatment serum levels had lower survival and a higher rate of disease recurrence, suggesting that IL-6 may be an important biomarker for HNSCC aggressiveness and risk for recurrence (Duffy et al., 2008). IL-6 signaling was also found to be important in HNSCC cancer stem cells. Krishnamurthy and colleagues found that IL-6R was significantly up-regulated in the $ALDH^{high}CD44^{high}$ cancer stem cell population, when compared with controls (Krishnamurthy et al., 2014). Importantly, tumors in IL-6 wild-type mice grew significantly faster than tumors grown in IL-6 knockout mice. Notably, HNSCC xenograft tumors generated in the IL-6 wild-type mice showed higher fraction of $ALDH^{high}CD44^{high}$ cells, suggesting that stromal IL-6 plays an important role in the maintenance and self-renewal of head and neck cancer stem cells *in vivo*. This effect was in part mediated by activation of STAT3 signaling. Interestingly, studies from Islam and colleagues (2014) suggested that inhibition of RhoC expression down-regulates the STAT3 pathway, indicating that this protein may also be involved in IL-6-driven maintenance of the cancer stem cell phenotype. In these studies, knockdown of RhoC suppressed sphere formation, decreased the percentage of $ALDH^{high}$ cells and decreased the level of phospho-STAT3. Addition of IL-6 to the RhoC knockdown cells restored levels of phospho-STAT3 in HNSCC cell lines. They concluded that RhoC activates downstream pathways (possibly NF-κB)

that induce transcription of IL-6, which then goes on to activate downstream STAT3 signaling and maintenance of the cancer stem cell phenotype (Islam et al., 2014).

Bmi-1, a member of the polycomb-repressor 1 complex, is another protein that plays an important role in normal stem cells function (Park et al., 2003). It was also found to be important for the maintenance of cancer stem cells in HNSCC, particularly upon treatment with a chemotherapeutic drug (cisplatin) (Nör et al., 2014). Treatment of HNSCC with cisplatin significantly increased the population of $ALDH^{high}CD44^{high}$ cells in a dose-dependent manner and increased their ability to form spheres *in vitro*. Interestingly, this increase in the cancer stem cell fraction correlated with an increase in Bmi-1 expression. A study by Giudice and colleagues (2013) further elucidated the role of Bmi-1 in HNSCC. They showed that HNSCC cells are typically hypoacetylated. Chemical inhibition of histone-deacetylase significantly decreased sphere formation and the fraction of $ALDH^{high}CD44^{high}$ cancer stem cells. Paradoxically, chromatin hyperacetylation induced Bmi-1 expression and EMT, suggesting that the regulation of Bmi-1 through histone acetylation in HNSCC tumor cells may be important for transitioning from a more stem-like state to a more motile and invasive state (Giudice et al., 2013).

Cancer stem cell niche

Stem cell niche is the specific microenvironment that allows stem cells to retain their stemness and give rise to progenitor cells. Like normal stem cells, cancer stem cells are known to reside in niches (Fig. 2.2). Stem cell niches consist of endothelial cells, fibroblasts, immune cells, signaling molecules secreted from different types of cells and extracellular matrix (Korkaya et al., 2011). Cancer stem cell niches allow cancer stem cells to maintain its population and act as protective environment against cancer therapies (Hovinga et al., 2010; Folkins et al., 2007).

Here, we discuss the role of fibroblasts and endothelial cells within head and neck cancer stem cell niches.

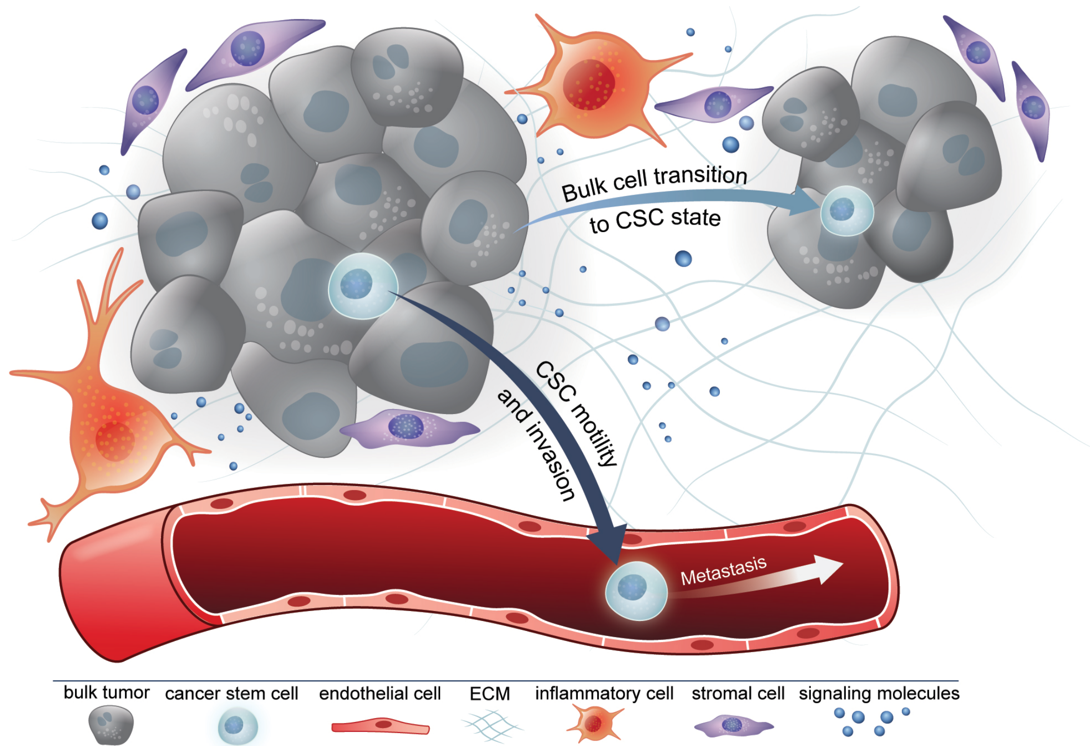

Figure 2.2. Cancer stem cell niche. The cancer stem cell niche is a protective environment including multiple cell types where cancer stem cells reside. The interaction between cancer cells and the stromal cells allows the cancer stem cell population to retain stemness. Such interactions might also enhance invasiveness of cancer stem cells enabling them to enter into the blood stream and disseminate through the process of metastases. It has been hypothesized that environmental cues might enable the de-differentiation of more differentiated tumor cells back to a cancer stem cell state.

Cancer-associated fibroblasts

Emerging knowledge supports the concept that stromal cells are important components of most tumor microenvironments and play a key role in the pathobiology of cancer. Factors secreted by tumor cells result in cancer-associated fibroblasts (CAFs) that are phenotypically distinct from normal fibroblasts (reviewed in Kalluri and Zeisberg, 2006). CAFs, along with other cells within the cancer stem cell niche, activate stemness-related pathways. Vermeulen and colleagues found that stromal myofibroblasts activated canonical Wnt pathway to regulate the stemness of cancer

cells (Vermeulen et al., 2010). In lung cancer, CAFs activated IGF-II/IGFR signaling pathway enhances the stemness of cancer cells (Chen et al., 2014). Stromal contribution in head and neck cancer has also recently been proposed to play a role in tumor cell invasion (Markwell and Weed, 2015). Stromal cell-derived factor (SDF-1) secreted by fibroblasts induces migration of head and neck cancer stem cells to supportive niche. SDF-1 is also involved in podia formation, which is needed for cell interaction with the microenvironment (Faber et al., 2014). SDF-1 is a strong chemoattractant that plays an important role in tumor metastasis (Geminder et al., 2001; Taichman et al., 2002; Phillips et al., 2003). Collectively, these findings suggest that cancer stem cells acquire enhanced stemness and motility through CAF-induced molecular signaling. Such stromal cell-tumor cell interactions may ultimately contribute to tumor progression and dissemination.

Perivascular niche

Existing knowledge that normal neural stem cells reside near blood vessels inspired the existence of a cancer stem cell niche. Endothelial cells secrete factors that allow neural stem cells to maintain self-renewal ability (Jin et al., 2002; Ramirez-Castillejo et al., 2006). As it is observed in normal neural stem cells, brain cancer stem cells in glioblastoma multiforme are in close proximity with endothelial cells (Calabrese et al., 2007). When patient derived brain cancer stem cells were injected with vascular endothelial cells to immunodeficient mice, the cancer stem cells were able to maintain their stemness and tumorigenicity (Calabrese et al., 2007).

In HNSCC, cancer stem cells reside in perivascular niche (Krishnamurthy et al., 2010). Close proximity between cancer stem cells and blood vessels enables active crosstalk between the two cell types. Factors secreted by endothelial cells potentiate self-renewal ability and survival of cancer stem cells (Krishnamurthy et al., 2010). Specifically, endothelial cell-secreted

IL-6 is important in maintaining tumor-initiating ability of cancer stem cells as well as in maintenance of cancer stem cell population within the tumor (Krishnamurthy et al., 2014). In addition to IL-6, endothelial cells also secrete high levels of epithelial growth factor (EGF). EGF enhances orosphere formation and increases motility of HNSCC *in vitro* (Zhang et al., 2013). In addition, specific silencing of EGF expression in tumor-associated endothelial cells decreases the fraction of cancer stem cells and the tumorigenic potential in preclinical models of HNSCC. Collectively, these findings suggest that factors secreted by the perivascular niche contribute to the maintenance of cancer stem cell population and the acquisition of a more aggressive phenotype by HNSCC cells.

Epithelial-mesenchymal transition and cancer stem cells

Epithelial-mesenchymal transition (EMT) happens when cell of epithelial-origin acquires phenotypes resembling mesenchymal cells. Cells that have undergone EMT present enhanced migratory and invasive ability as well as resistance to apoptosis (Kalluri and Neilson, 2003). EMT is involved in cancer initiation and progression in many different cancer types (reviewed in Thiery, 2002; De Craene and Berx, 2013). Several studies linked EMT with conversion of non-cancer stem cells into cancer stem cells. Mani and colleagues (2008) were the first to report that induction of EMT results in increased proportion of cancer stem cells, sphere forming ability and tumorigenicity in preclinical models of breast cancer. Head and neck cancer stem cells are reported to express more EMT related genes (La Fleur et al., 2012). Moreover, EGF induces EMT in HNSCC and increases the proportion of cancer stem cells *in vitro* (Zhang et al., 2013). IL-6 is also shown to induce EMT in HNSCC and transform non-metastatic tumors into metastatic tumors *in vivo* (Yadav et al., 2011). Collectively, these studies suggest that non-cancer stem cells might be capable of converting to cancer stem cells by undergoing EMT mediated by

molecular crosstalk with other cells from the tumor environment. However, this is an area of much investigation and the relative contribution of EMT to the tumorigenic process is likely tumor-specific.

Cancer stem cells as therapy target

A continuing challenge in the management of patients with cancer treatment is tumor relapse and metastasis resulting from therapy resistance. Potential cause of tumor recurrence is that conventional therapies target highly proliferative cells, but miss slow growing cancer stem cells that can repopulate the tumor and result in tumor relapse (Schatton et al., 2009; Kaiser et al., 2015). It is also found in HNSCC that cisplatin treatment, most commonly used chemotherapy for head and neck cancer, results in enhanced fraction of cancer stem cells (Nör et al., 2014). Therefore, growing understanding of cancer stem cell contribution in tumor progression sparked much interest in developing ways to use cancer stem cells as anticancer therapy target.

The Notch signaling pathway is important in maintenance of cancer stem cell population. Preclinical study showed that inhibition of Notch pathway resulted in reduced number of breast cancer stem cells and tumor growth (Schott et al., 2013). When the inhibition of Notch pathway was combined with docetaxel, the tumor growth inhibition was much more effective (Schott et al., 2013), suggesting that targeting both bulk tumor cells and cancer stem cells might lead to a more pronounced and long lasting anticancer effect.

Cancer stem cell markers themselves can serve as potential targets for cancer therapy. CD133 is one of the well-studied cancer stem cell markers in many tumor types, including glioblastoma, colon cancer, ovarian cancer, and head and neck cancer. Wang and colleagues targeted CD133-positive glioblastoma cells by designing CD133 antibody combined with nanomaterial that results in photothermolysis (Wang et al., 2011). The antibody was able to

selectively target CD133-positive cancer stem cells *in vitro* and inhibit the tumor growth ability of glioblastoma cells by targeting CD133-positive cells *in vivo* (Wang et al., 2011).

Cancer stem cells can also be targeted with immunologic approaches. Ning and colleagues reported that pulsing dendritic cells with cancer stem cell population from murine melanoma and squamous cell carcinoma model results in antitumor immunity (Ning et al., 2012). A recent phase I clinical trial was performed with nasopharyngeal cancer stem cell vaccine to evaluate its safety and efficacy (Lin et al., 2015). As it was reported in the animal study, the sera from vaccinated patients had cytotoxic effects on target cancer stem cells *in vitro*, and the vaccination had very little side effects. Clinical trials on cancer stem cell vaccines are ongoing for many other cancers including pancreatic, hepatocellular, colon, breast, and ovarian cancer. Considering the prominent immune suppressive effect observed in head and neck cancer, strategies to boost the patients' immune system have recently become a subject of much interest and investigation.

Despite tremendous progress on the understanding of the biology of cancer stem cells, the direct therapeutic targeting of these cells remains challenging. This is largely due to the fact that cancer stem cells share many characteristics with normal stem cells. Therefore, cancer stem cell-targeted therapies result in the elimination of normal stem cells and significant toxicities. In recent years, it has been proposed that cancer stem cells could be targeted indirectly, by disrupting the cancer stem cell niche (Calabrese et al., 2007; Yang and Wechsler-Reya, 2007; Ritchie and Nör, 2013). In HNSCC, when perivascular niche was disrupted by inducing apoptosis in endothelial cells, the fraction of cancer stem cells decreased (Krishnamurthy et al., 2010). Therefore, anti-angiogenic modalities, such as VEGF inhibitors, can be used to destroy the tumor-associated blood vessels and indirectly target cancer stem cells. Preclinical data

showed that combination of anti-angiogenic agent with conventional chemotherapy reduces the proportion of glioma cancer stem cells (Folkins et al., 2007).

An alternative way to target cancer stem cell is to block key molecular pathways that are involved in the crosstalk between cancer stem cells and the microenvironment. Endothelial cell secreted IL-6, EGF, and IL-8 are important factors that induce migratory phenotype of HNSCC and make cancer cells resistant to anoikis (Neiva et al., 2009). Targeting inhibition of IL-6 signaling with tocilizumab, a humanized anti-IL-6R antibody, also resulted in a sharp decrease in the fraction of cancer stem cells in preclinical models of HNSCC (Krishnamurthy et al., 2014). IL-6 is primarily secreted by endothelial cells, while cancer stem cells express higher levels of IL-6R as compared to non-cancer stem cells. Collectively, these studies suggest at least two distinct strategies to target indirectly cancer stem cells: A) With a blood vessel-disrupting approach with an anti-angiogenic drug, which leads to a decrease in tumor microvessel density. Or B) With a crosstalk-targeting drug, which may maintain tumor angiogenesis but block critical signaling pathways between endothelial and cancer stem cells. Preclinical evidence suggests that both strategies might be effective in reducing the fraction of cancer stem cells. However, clinical trials will be needed to determine the impact of either strategy, particularly when used in combination with a tumor-debulking strategy (*e.g.* chemotherapy, radiation therapy) on the long-term survival of patients with head and neck cancer.

Cancer stem cell targeting agent alone may not be able to eradicate the tumors due to possible conversion of non-cancer stem cells into cancer stem cells. In addition, since cancer stem cells constitute a small fraction of the overall tumor mass, the responses to a pure cancer stem cell-targeted therapy would most likely be slow and not be measurable in short-term. Therefore, the field is rapidly moving towards combination therapies involving a cancer stem

cell-targeting agent and conventional therapy (e.g. chemotherapy, radiation therapy) that targets the bulk tumor cells (Kaiser, 2015). Much work ahead is warranted in the search of the appropriate combination of therapies that are able to effectively target bulk and cancer stem cells in different tumor types.

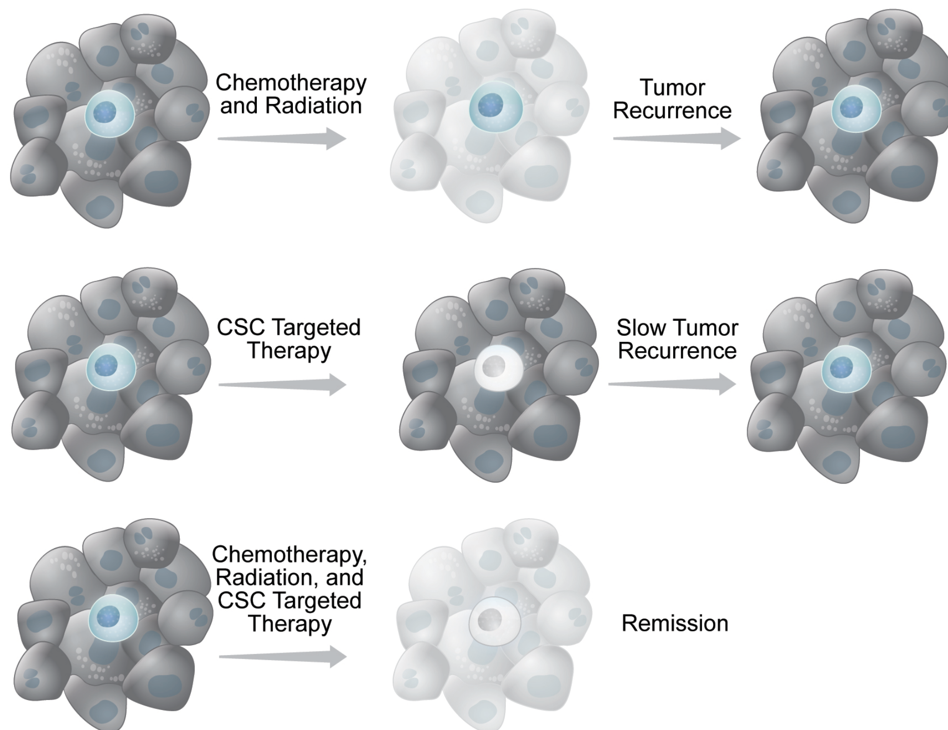

Figure 2.3. Hypothetical impact of the cancer stem cell model in cancer therapy. Conventional cancer therapies target fast-growing bulk tumor cells and leave behind cancer stem cells. In this case, the tumor shrinks, but cancer stem cells can repopulate the tumor and enabling tumor recurrence. Another therapeutic strategy is to use a cancer stem cell-targeting agent to eradicate these stem cells. In this case, the tumor undergoes modest short-term response, since the cancer stem cells constitute a very small fraction of the tumor mass. However, cancer stem cells can be repopulated with time by conversion of non-cancer stem cells to cancer stem cells and tumor recurrence might be observed. On the other hand, targeting both cancer stem cells and the bulk tumor cells might effectively eradicate the entire tumor, leading to a long lasting response or cure. Adapted from Schatton et al., (2009) and Kaiser et al., (2015).

Conclusions

Over the last decade, independent investigators have demonstrated rather unequivocally the heterogeneity of cancer cells within each tumor. Not only these cells are heterogeneous, but also

exhibit significant plasticity, being able to change phenotypic characteristics from time-to-time depending on the tumor microenvironment changes and tumor cell needs. Strong evidence demonstrates that certain tumors, including breast cancer and HNSCC, contain a subpopulation of uniquely tumorigenic cells that can be therapeutically targeted. It has been proposed that while chemoradiotherapy debulk the tumor, it does not eliminate the cancer stem cells and the tumor recurs (Fig. 2.3). On the other hand, cancer stem cell-targeted therapies might eliminate temporarily cancer stem cells, but the inherent plasticity of tumor cells enables the repopulation of cancer stem cells, eventually leading to tumor recurrence. A more long-lasting response might be obtainable if a debulking therapy is combined with a cancer stem cell-targeted therapy, enabling more long-lasting response and perhaps even cancer cure. We predict that future work will be focused on how to best target the cancer stem cells in combination with therapies that debulk the more differentiated tumor cells. Head and neck cancer patients might one day benefit from such combination therapies and experience better quality of life and extended survival.

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CHAPTER III

Isolation and Characterization of Cancer Stem Cells from Primary Head and Neck Squamous Cell Carcinoma Tumors

Abstract

Drug resistance remains a significant problem in the treatment of patients with head and neck squamous cell carcinoma (HNSCC). Recent reports showed that a subpopulation of highly tumorigenic cells, called cancer stem cells, is uniquely resistant to chemotherapy, suggesting that these cells play an important role in the relapse of HNSCC. The development of methods for the isolation and culture of cancer stem cells is a key step to enable studies exploring the mechanisms underlying the role of these cells in chemoresistance. Here, we describe a method to isolate cancer stem cells from primary head and neck tumors and for the generation of orospheres.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with approximately 500,000 cases diagnosed annually (Kamangar et al., 2006). In the past, treatment of HNSCC was primarily limited to upfront surgical resection when technically feasible. Owing to the high morbidity often associated with radical surgery, most patients today receive radiation combined with chemotherapy as front line treatment for locally advanced HNSCC. Chemotherapy alone is implemented for the treatment of distant metastatic disease, but most patients do not exhibit substantial response. A major difficulty in treating HNSCC is the frequency of recurrent disease, with 20-40% of patients developing loco-regional recurrence and 5-20% developing distant metastatic disease at 2 years (Forastiere et al., 2003). Chemotherapy for HNSCC typically involves the use of platinum, taxane, or pyrimidine analog agents (Pfister et al., 2011). While these agents are effective at indiscriminately debulking tumor cells, recent data report that a subpopulation of cells, called cancer stem cells, is resistant and survive these therapies (Okamoto et al., 2009; Zhang et al., 2010; Nör et al., 2014). Indeed, mounting evidence suggests that chemotherapy enriches the cancer stem cell population (Nör et al., 2014; Reers et al., 2014). Notably, the mechanisms exploited by cancer stem cells to resist chemotherapy are largely unknown. Therefore, the development of methods for the isolation and characterization of cancer stem cells from primary tumors is a critical enabling step to improve the mechanistic understanding of the processes mediating chemoresistance, and for the development of therapeutic strategies to overcome this resistance.

The most widely used method to study cancer stem cell is the sphere assay. The sphere assays were originally used for culturing normal neuronal stem cells (Reynold and Weiss, 1992). Since the initial discovery that culturing cells in ultra-low attachment plates and serum-free (or

low serum) conditions enhance maintains their undifferentiated state, the assay evolved as an important tool to study not only normal stem cells but also cancer stem cells. Multiple reports show that cancer stem cells are anoikis-resistant and able to grow in suspension as spheres, as for example the breast cancer stem cells (Dontu et al., 2003). Spheres from head and neck tumors are called orospheres (Krishnamurthy et al., 2010; Krishnamurthy et al., 2013). The orosphere assay is useful to evaluate the stemness, self-renewal and tumorigenicity of cancer stem cells, but also to study processes involved in the chemoresistance of these cells to drugs (Nör et al., 2014).

This chapter will describe in detail the method for isolation of cancer stem cells from HNSCC and for their culture in suspension as orospheres. There is ongoing discussion about the ideal markers to identify head and neck cancer stem cells. Here, we have used the combination of ALDH activity and CD44 expression to sort these cells from primary tumors (Clay et al., 2007; Prince et al., 2007). The selection of markers can certainly be adapted to the specific tumor of interest. Nevertheless, the overall principles of the assay described here might be germane to other tumor types.

Materials

2.1 Tumor digestion

1. Supplemented media (*see* NOTE 1).
2. Sterile petri dish.
3. Sterile razor.
4. Tumor digestion reagents:
    1. Collagenase/hyaluronidase solution (STEMCELL Technologies, Vancouver, BC, Canada).
    2. Miltenyi Biotech human tumor dissociation kit (Miltenyi Biotech, San Diego, CA, USA).
5. 40 µm nylon cell strainer for 50 mL conical tube.
6. Serum: serum neutralizes the digestion process.
7. Ammonium-Chloride-Potassium (ACK) lysing buffer (Gibco Life Technologies, Grand Island, NY, USA) (*see* NOTE 2).

2.2 Fluorescence activated cell sorting

1. 1xPBS or 1xPBS with 2% fetal bovine serum (staining buffer).
2. 5 mL round bottom flow cytometry tubes.
3. 4-Diethylaminobenzaldehyde (DEAB) (STEMCELL Technologies, Vancouver, BC, Canada).
4. Activated ALDEFLUOR™ (STEMCELL Technologies, Vancouver, BC, Canada).
5. APC CD44 and APC isotype IgG (BD Pharmingen, Franklin Lakes, NJ, USA).
6. Lineage markers: PE-Cy5 CD2, CD3, CD10, CD16, CD18 (BD Pharmingen, Franklin Lakes, NJ, USA).

7. 7-aminoactinomycin (7-AAD) (BD Pharmingen).

8. Aluminum foil.

2.3 Orosphere culture

1. Sphere media: DMEM/F12 medium (500 mL) (Gibco Life Technologies, Grand Island, NY, USA), fibroblast growth factor (FGF) (20 ng/mL), epithelial growth factor (EGF) (20 ng/mL), N2 supplement (Gibco Life Technologies, Grand Island, NY, USA) (*see* NOTE 3).

2. 6-well low-attachment plate (Corning, Corning, NY, USA).

3. 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco Life Technologies, Grand Island, NY, USA).

4. Trypsin neutralizing solution (Lonza, Walkersville, MD, USA).

Methods

3.1 Preparation for digestion

1. Place tumor in supplemented media.

2. Pour off the media and add fresh supplemented media. Centrifuge at 130 g-force for 5 minutes at 4°C. Wash the tumor 3-4 times (*see* NOTE 4).

3. Place tumor with 5 mL of supplemented media on a petri dish.

4. Cut the tumor into small pieces (approximately 4 mm x 4 mm in size) with sterile razor blade (*see* NOTE 5).

5. Collect minced tumor fragments into a 50 mL conical tube. Add 25-30 mL base media without supplements. Centrifuge at 130 g-force for 5 minutes at 4°C.

3.2 Tumor digestion

1. Collagenase/hyaluronidase method

    1. Decant media and place the tumor fragments on a new sterile petri dish. Add 6-10 mL of 1x collagenase/hyaluronidase solution.

    2. Mix the tumor fragments and digestion solution by pipetting several times with 25 mL pipette. Incubate in 37°C for 15 min.

    3. Take out the petri dish from the incubator. Pipet the mixture 2-3 times to mechanically digest the tumor. Repeat step 2 and 3 2 more times (*see* NOTE 6).

    4. Prepare a 50 mL tube with 5 mL serum and place 40 μm nylon mesh on top of the tube. Filter the tumor digestion mix and collect cell suspension. Collect and filter remaining cell/fragment mixture with 5 mL of supplemented media (*see* NOTE 7). Centrifuge at 130 g-force for 5 minutes at 4°C.

5. Decant media. Add 1-5 mL of ACK lysing buffer. Incubate in room temperature for 1 minute. Centrifuge at 130 g-force for 5 minutes at 4°C.

6. Decant ACK lysing buffer and resuspend the cell pellet in PBS (*see* NOTE 8).

2. GentleMACS method

1. Prepare enzyme cocktail mix with RPMI in the appropriate tube for GentleMACS.

2. Transfer minced tissue fragments into the enzyme cocktail mix. Close the tube tightly (*see* Fig. 3.1).

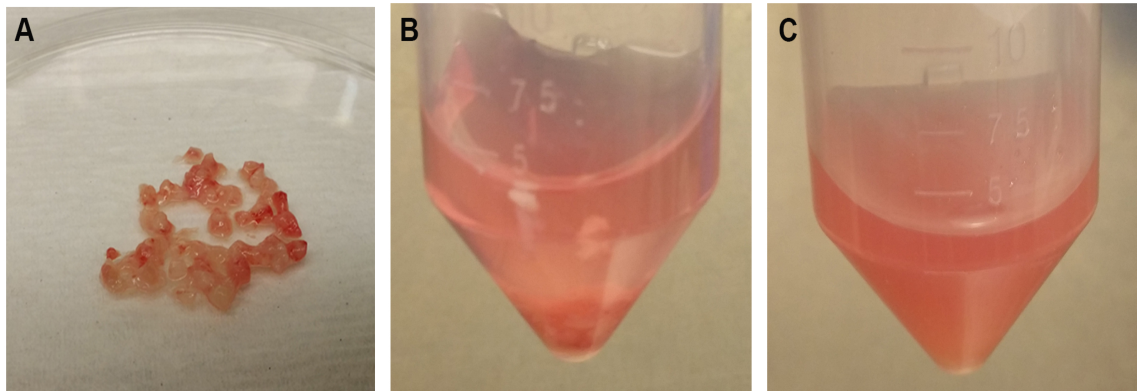

Figure 3.1. Tumor digestion steps. A. Cut tumors into small fragments. B. Before tumor digestion. C. After tumor digestion. Notice the digestion media turned opaque after the digestion process.

3. Mechanically dissociate tumors using GentleMACS homogenizer. Select appropriate tumor dissociation program.

4. Incubate in 37°C for 30 min on a shaker or rotator.

5. Repeat step 3 and 4.

6. Repeat step 3.

7. Transfer the digested tumor to 50 mL tube with 40 μm cell strainer placed on top. Use equal volume of supplemented medium to collect residual tumor cells and undigested tissues. Collect digested cell suspension by filtering with the cell strainer.

8. Centrifuge at 130 g-force for 5 minutes at 4°C.

9. Decant media. Add 1-5 mL of ACK lysing buffer. Incubate in room temperature for 1 minute. Centrifuge at 130 g-force for 5 minutes at 4°C.

10. Decant ACK lysing buffer and resuspend the cell pellet in PBS (*see* NOTE 8).

3.3 Isolation of cancer stem cells

1. Prepare and label tubes.

Unstained

7-AAD

Lineage

APC IgG

APC CD44

DEAB

ALDH

Sample: T1, T2, T3, etc.

2. Count the cells recovered from tumor digestion. Add $1x10^5$ cells to each control tube except DEAB tube. Add $\leq 1x10^6$ cells to the sample tube.

3. Resuspend cells in 1 mL PBS or staining buffer.

4. Add 1 µL of DEAB reagent to the control DEAB tube (*see* NOTE 9). Add 1 µL of ALDEFLUOR™ reagent control ALDH tube. Mix by pipetting once and transfer 500 µL of cells to control DEAB tube (*see* NOTE 10). Mix well.

5. Add 5 µL of ALDEFLUOR™ reagent to each sample tube. Mix well. Protect from light by covering the tubes with aluminum foil. Incubate in 37°C for 30-45 minutes.

6. Centrifuge at 130 g-force for 5 minutes at 4°C. Remove PBS or staining buffer from all the tubes. Resuspend cells in 1 mL PBS or staining buffer.

7. Add 1 µL of APC isotype IgG to control IgG APC tube. Add 1 µL of APC CD44 antibody to control APC tube. Add 5 µL APC CD44 to all the sample tubes.

8. Add 1 µL of CD2, CD3, CD10, CD16 and CD18 to control Lineage tube. Add 5 µL of the same antibodies to the sample tubes.

9. Protect from light by covering the tubes with aluminum foil. Incubate in 4°C for 30 minutes.

10. Add 1 mL of PBS to all of the tubes. Centrifuge at 130 g-force for 5 minutes at 4°C.

11. Aspirate PBS and resuspend cells with 1 mL PBS. Centrifuge at 130 g-force for 5 minutes at 4°C.

12. Aspirate PBS. Add 200 µL PBS to all the control tubes except control 7AAD tube.

13. Prepare 7-AAD solution by adding 5 µL 7-AAD for each 1 mL of PBS. Add 200 µL 7-AAD solution to control 7-AAD tube. Resuspend sample tubes with 500 µL 7-AAD solution.

14. Sort cancer stem cells by fluorescence activated cell sorting (FACS) (*see* Fig. 3.2) (*see* NOTE 11).

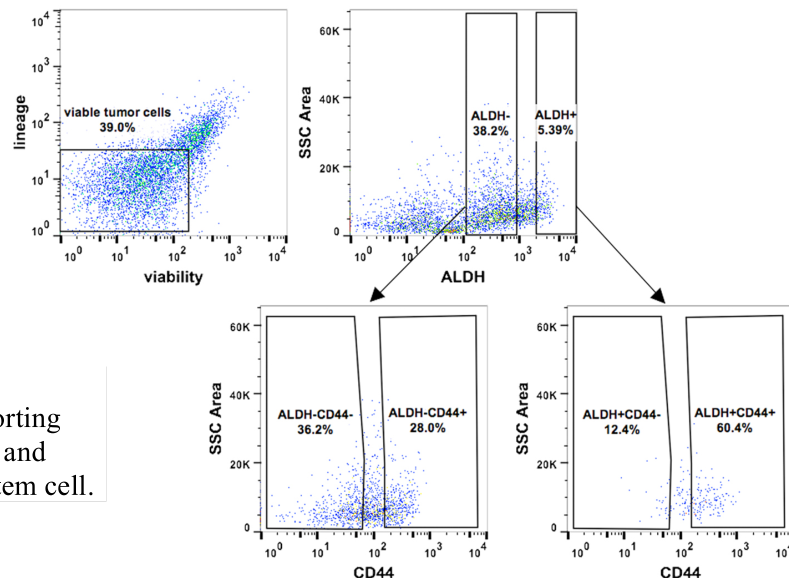

Figure 3.2. Representative fluorescence activated cell sorting scheme from a primary head and neck squamous cell cancer stem cell.

3.4 Orosphere assay

1. Seed sorted cancer stem cells in 6-well low-attachment plate. 5,000 sorted cells are plated in each well of low-attachment plate (*see* NOTE 12). Add 1.5 mL of sphere media in each well (*see* NOTE 13).

2. Feed spheres every 3-4 days by adding 300 μL of sphere media to each well.

3. Count spheres on day 3 and 7 (*see* NOTE 14) (*see* Fig. 3.3).

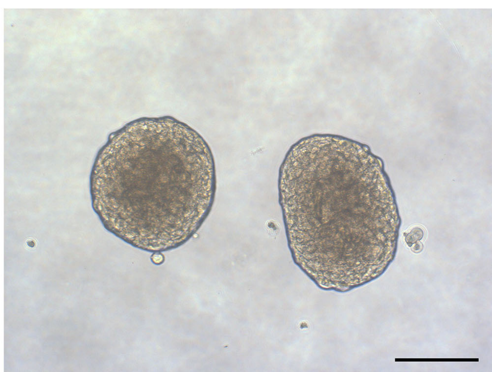

Figure 3.3. Example of orospheres. Scale bar = 100 μm.

4. On day 7, collect spheres in 15 mL tube. Centrifuge at 130 g-force for 5 minutes at 4°C.

5. Wash with PBS. Centrifuge at 130 g-force for 5 minutes at 4°C. Remove supernatant.

6. Add 1 mL 0.05% trypsin/EDTA. Incubate for 5-10 minutes in room temperature. Mechanically dissociate the spheres by pipetting up and down every 2 minutes until spheres are invisible.

7. Add 1 mL trypsin neutralizing solution and mix. Centrifuge at 130 g-force for 5 minutes at 4°C.

8. Count the cells.

9. Seed sorted cells in low-attachment plate. 5,000 sorted cells are plated in each well of low-attachment plate. Add 1.5 mL of sphere media in each well (*see* NOTE 13).

10. Feed spheres every 3-4 days by adding 300 μL of sphere media to each well. Count spheres on day 10 and 14.

Notes

1. Depending on the tumor type, add necessary supplements and growth factors to keep tumor fragment. The media should include antibiotics (*e.g.* amphotericin B, AAA, nystatin, etc.) to prevent bacterial/fungal contamination.
2. ACK lysis buffer eliminates red blood cells from the digested cell pellet.
3. Cancer stem cells from different tumor type require different supplements needed for sphere formation. Here, we describe the supplements needed for orosphere assay.
4. Washing the tumor prevents potential contamination during cancer stem cell culture following isolation of cancer stem cells.
5. Mincing the tumor into smaller pieces is important for good cell recovery from digestion. Too much cutting results in unhealthy cells after digestion, and too little cutting results in poor tumor digestion.
6. As the tumor fragments are digested, the solution will become opaque, and the tumor fragments will be able to pass through 10 mL pipette.
7. Incompletely digested tumor fragments can be stored for future use. Re-suspend the chunks in freeze media that contains 5% DMSO and 5% serum and move gradually to liquid nitrogen for storage.
8. Serum containing staining buffer will enhance the cell viability during the staining procedure.
9. After adding DEAB to the tube, close the lid so the solution will not evaporate.
10. Transfer the cell suspension as soon as possible to minimize the background ALDEFLUOR^{TM} signal.

11. Cancer stem cells are 7-AAD negative, Lineage negative, ALDEFLUOR^{TM} positive, CD44 positive cells.
12. Cell density can affect the number and the quality of orosphere. Optimize the number of cells plated in each well.
13. Add chemotherapeutic reagents to the sphere media to study the chemoresistance of cancer stem cells.
14. Orospheres are considered as non-adherent colony with at least 25 cells.

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CHAPTER IV

Endothelial Cell-Initiated IL-6/STAT3 Pathway Induces Mesenchymal Cell Marker Expressions to Enable Motility in Head and Neck Cancer Stem Cell

Abstract

High incidence of locoregional recurrence and distant metastases are the key clinical challenges in the management of patients with head and neck squamous cell carcinoma (HNSCC). A rare population of cells, called cancer stem cells, has been characterized as cells with high aldehyde dehydrogenase (ALDH) activity and high CD44 expression ($ALDH^{high}CD44^{high}$) in HNSCC. HNSCC cancer stem cells reside in the perivascular niche, and endothelial cell-secreted interleukin-6 (IL-6) enhances the tumorigenic potential and self-renewal of head and neck cancer stem cells. Tissue microarray (TMA) of 80 oral squamous cell carcinomas revealed that high IL-6 receptor (IL-6R) (p=0.0217) or its co-receptor gp130 (p=0.0422) expression correlated with low patient survival. Here, we assessed the role of endothelial cell-secreted IL-6 on the migratory behavior of head and neck cancer stem cells. We observed that endothelial-cell secreted factors increased vimentin and snail in $ALDH^{high}CD44^{high}$ cells. Upon blockade of the IL-6 pathway, endothelial cell-induced vimentin and snail expression were inhibited in these cells. $ALDH^{high}CD44^{high}$ cells were more migratory in the presence of endothelial cell-secreted factors, and again IL-6 signaling blockage inhibited the migration of these cells. Notably, humanized anti-IL-6R antibody tocilizumab decreased the fraction of $ALDH^{high}CD44^{high}$ cells *in vivo*. Taken

together, these findings demonstrate that endothelial cell-secreted IL-6 enhances the aggressive behavior of head and neck cancer stem cells, suggesting that patients with HNSCC may benefit from therapeutic inhibition of IL-6 signaling.

Introduction

Head and neck cancer is the sixth most common cancer worldwide (SEER Cancer Statistics Factsheets, 2014; Curado and Hashibe, 2009). Squamous cell carcinoma (HNSCC) comprises 90% of head and neck cancer. The overall 5-year survival rate is 80% in patients with early stage disease, but the rate drops to 20-50% in late stage patients (Gold et al., 2009). Approximately half of the late stage patients develop locoregional or distant metastasis, which significantly lowers the survival rates of head and neck cancer patients (León et al., 2005). The understanding of mechanisms driving the invasive behavior of tumorigenic HNSCC cells is critical for the development of a mechanism-based therapy that prevents tumor dissemination.

HNSCC contains a subpopulation of cells, called cancer stem cells, that is uniquely tumorigenic, endowed with self-renewal and multipotency. Cancer stem cells are known to be resistant to radiotherapy and chemotherapeutic agents and contribute to metastasis and relapse (Dean et al. 2005). Prince et al. first identified head and neck cancer stem cells using CD44 expression alone (Prince et al., 2007). Subsequent report showed that aldehyde dehydrogenase (ALDH) activity-based cell isolation selects for cancer stem cells in HNSCC (Chen et al., 2009). High ALDH1 expression in the HNSCC in the patient tumor correlates with tumor size and lymph node metastasis (Hildebrand et al., 2014; Michifuri et al., 2012). Our group used the two markers together and observed that the $ALDH^{high}CD44^{high}$ marker combination is highly effective at distinguishing uniquely tumorigenic cancer stem cells from the remaining tumor cells in HNSCC (Krishnamurthy et al., 2010).

Like normal stem cells, cancer stem cells reside in a niche microenvironment to survive and protect their self-renewal ability (Calabrese et al., 2007). Our group has shown that head and neck cancer stem cells reside in perivascular niche (Krishnamurthy et al., 2010). In theory, close

proximity between cancer stem cells and blood vessels makes it easy for the cancer stem cells to migrate and invade into blood vessels to initiate metastasis. Endothelial cells secrete multiple cytokines that affect the behavior of tumor cells. For example, we have shown that endothelial cell-secreted CXCL8 in creases the frequency of local recurrence in preclinical models of HNSCC (Warner et al., 2008). In addition, we observed that endothelial cell-secreted epidermal growth factor induces epithelial-mesenchymal transition (EMT) and migration in HNSCC (Zhang et al., 2014), suggesting the endothelial cell-tumor cell interaction plays a critical role in cancer progression. However, the effect of endothelial cell-secreted factors on the invasive behavior of the highly tumorigenic cancer stem cells remains to be determined.

Interleukin-6 (IL-6) is pro-inflammatory cytokine that activates JAK/STAT3 pathway. IL-6 level has been correlated with tumor progressions in multiple cancer types (Nachbaur et al., 1991; Plante et al., 1994; Zhang et al., 1999; Chung et al., 2003). A prospective cohort study found that serum IL-6 level was predictive marker for recurrence rate and overall survival of HNSCC patients (Duffy et al., 2008). Independent research groups showed that tumor cells acquire metastatic potential through IL-6/STAT3 pathway (Yadav et al., 2011; Xie et al., 2006). IL-6 is secreted by many different cells, including T cells, B cells, monocytes, endothelial cells, fibroblasts and some tumor cells (Kishimoto et al., 1995). Upon inflammatory stimulation, endothelial cells secrete high levels of IL-6 (Makó et al., 2010). We previously have reported that tumor-associated endothelial cells lining tumor blood vessels express more IL-6 than the tumor cells themselves (Krishnamurthy et al., 2014). However, the role of endothelial cell-secreted IL-6 on migratory behavior of head and neck cancer stem cells has not been investigated. Notably, humanized anti-IL-6 receptor (IL-6R) antibody, tocilizumab, has been

approved by the FDA for rheumatoid arthritis since 2010 (Thompson, 2010). However, the effect of tocilizumab in the pathobiology of head and neck cancer has not been evaluated.

Here, we evaluated the significance of endothelial cell-secreted IL-6 on head and neck cancer stem cell motility and the therapeutic potential of targeting IL-6 pathway in HNSCC. We observed that expression of IL-6 receptor or its co-receptor gp130 in the invasive front of primary HNSCC tumors correlated with poor overall patient survival. Endothelial cell-secreted IL-6 induced EMT and enhanced migration in head and neck cancer stem cells. Collectively, these results demonstrate that endothelial cell-secreted IL-6 induces a migratory phenotype in head and neck cancer stem cells, and suggest that the progression of HNSCC towards metastasis or recurrence might be delayed by therapeutic blockage of the IL-6 pathway.

Materials and Methods

Cell culture and reagents

HNSCC cell lines UM-SCC-1 and UM-SCC-22B (Tissue Biorepository, University of Michigan Head and Neck SPORE) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen). Primary human dermal microvascular endothelial cells (HDMECs; Lonza, Walkersville, MD, USA) were cultured in endothelial growth medium-2 for microvascular cells (EGM2-MV; Lonza). Endothelial cell conditioned medium (CM) was prepared by collecting supernatant from 24-hour culture in serum-free endothelial basal medium (EBM2; Lonza). IL-6 signaling pathway was inhibited by treating cells with 2 ug/mL tocilizumab (Genetech, South San Francisco, CA, USA) or with 1 ug/mL anti-IL-6 neutralizing antibody (R&D Systems, Minneapolis, MN, USA).

Tissue microarray, immunohistochemistry and immunofluorescence

The preparation of the tissue microarray (TMA) slides is described elsewhere (Fonseca et al., 2014). Tissue sections were deparafinzed in xylene, washed with 100% ethanol and rehydrated with graded ethanol. Antigen retrieval was performed by boiling the slides in 1X citrate buffer (Thermo Scientific, Fremont, CA, USA) for 20 minutes. After slides were cooled down to room temperature, tissue section was permeabilized for 10 minutes at room temperature, and endogenous peroxidase activity was inhibited by 10-minute incubation with 3% hydrogen peroxide (Fisher, Waltham, MA, USA). Mouse monoclonal anti-human gp130 (1:100 dilution; Santa Cruz) and rabbit polyclonal anti-human IL-6Rα (1:100 dilution; Abcam) were incubated overnight at 4°C. Chromogenic development was achieved by incubating DAB peroxidase substrate (Biocare Medical, Concord, CA, USA) for 1-5 minutes at room temperature. Two

pathologists blind to patient information scored the stained sections based on the staining intensity (1=no staining, 2=moderate staining, 3=intense staining) and percent positive (0= <10%, 1=10-50%, 2= >50%). Final score was calculated by multiplying the intensity and positive scores. Patients were divided to low (score ≤4) and high (score >4) groups.

Same deparafinization and antigen retrieval steps were performed for immunofluorescence assay. Primary antibodies, ALDH1A1 (1:100 dilution; BD Biosciences) and IL-6Rα (1:100 dilution; Santa Cruz), were incubated in 4°C overnight. Sections were washed with PBS (Invitrogen) and incubated in fluorochrome-conjugated secondary antibodies for 20 minutes at room temperature. After another PBS wash, the slide was mounted with DAPI mounting solution and covered with coverslip.

***In vivo* tumor growth experiment**

1-2 X 10^5 UM-SCC-22B cells and 8-9 x 10^5 HDMEC were seeded in a biodegradable scaffold, as we described (Nör et al., 2001). Loaded scaffold was implanted bilaterally in subcutaneous space of the dorsal region of severe combined immunodeficient mouse (CB.17.SCID; Charles River, Wilmington, MA, USA). Tumors were measured weekly and tumor volume was calculated by (width x width x length)/2. 5 mg/kg of tocilizumab or control IgG was given via intraperitoneal (IP) injection. Mouse weight was measured weekly to observe any adverse effect from the treatment.

Fluorescence Activated Cell Sorting (FACS) analysis

Head and neck cancer stem cells were isolated as described previously (Krishnamurthy et al., 2010). Briefly, single cell suspension was prepared from cell culture and resuspended at 1x10^6 cells/mL. 5 uL activated aldefluor (BODIPY-aminoacetate) (Aldefluor kit; Stem Cell Technologies, Vancouver, BC, Canada) or negative control (DEAB; diethylaminobenzaldehyde)

was added and incubated at 37°C for 35 minutes. Then the cells were washed and incubated with 5 uL anti-human CD44 (BD Pharmingen, Pharmingen, NJ, USA) at 4°C for 30 minutes. 5 uL 7-aminoactinomycin (BD Pharmingen) was used to select out dead cells. Head and neck cancer stem cells were defined as $ALDH^{high}CD44^{high}$ and non-cancer stem cells as $ALDH^{low}CD44^{low}$.

Migration assay

Transwell migration assay was performed on HTS transwell 96 well permeable support (Corning, Corning, NY, USA). Inserts were coated with 0.2% gelatin (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes prior to cell loading. $5x10^4$ sorted $ALDH^{high}CD44^{high}$ or $ALDH^{low}CD44^{low}$ cells resuspended in 50 uL DMEM were loaded to each insert. 200 uL of endothelial cell CM was loaded at the bottom well. Transwell plate was incubated in cell culture incubator for 24 hours. Cells that did not migrate were scrapped off from the top of the insert. Migrated cells were stained with 0.5% crystal violet (Sigma-Aldrich) solution in 25% methanol (Fisher Scientific, Fair Lawn, NJ, USA) for 20 minutes at room temperature. Migration was quantified by dissolving the crystal violet staining in 10% acetic acid (Fisher Scientific) and reading absorbance at 560 nm.

The cell migration assay with microfluidics device was performed using the previously published microfluidic migration platform (Chen et al., 2015; Burgos-Ojeda et al., 2015). Diagram of the device layout is shown in Fig. 4E. Prior to cell loading, the devices were coated with collagen solution in 0.2% acetic acid (rat tail collagen type 1; BD Biosciences) for 1 hour to enhance cell adhesion and viability. The devices were rinsed with PBS (Invitrogen) for 1 hour to remove the residual collagen. Sorted $ALDH^{high}CD44^{high}$ or $ALDH^{low}CD44^{low}$ cells were re-suspended to $3x10^5$ cell/mL concentration for loading. After cell loading, the cell suspension in the left inlet was replaced with serum-free DMEM, and HDMEC CM was applied to the other

inlet to induce migration. Migration frontier was measured by taking the average of distances the cells migrated after 24 hours of incubation without media replenishment.

Generation of IL-6 knockout endothelial cells

IL-6 knockout (sgRNA-IL-6) endothelial cells (HDMEC) were generated using CRISPR/Cas9 system (29). lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961). HEK293T cells were transfected with cocktail of pMD2G, psPAX2, and lentiCRISPR v2 with IL-6 guide sequences (5'-GGTCCAGTTGCCTTCTCCCT-3' and 5'-GTTCCTGCAGTCCAGCCTGA-3') using calcium phosphate method. HDMEC were incubated in HEK293T supernatant with 4 ug/mL polybrene (Sigma-Aldrich) overnight and maintained in 1 ug/mL puromycin (InvivoGen, San Diego, CA, USA) EGM2-MV for 2 weeks. IL-6 knockout efficiency was evaluated by performing ELISA (R&D Systems) assay with sgRNA-IL-6 HDMEC CM.

Generation of STAT3 knockdown HNSCC cells

The calcium phosphate method was used to transfect shRNA-control (scramble control) or shRNA-STAT3 constructs (University of Michigan Vector Core) with pMD2G and psPAX2 package vectors into HEK 293T cells. HNSCC cells were infected with transfected HEK293T supernatant overnight with 4 ug/mL polybrene (Sigma-Aldrich). Infected tumor cells were cultured in 1 ug/mL puromycin (InvivoGen) added DMEM medium for 2 weeks.

Western blot

Sorted ALDH^{high}CD44^{high} and ALDH^{low}CD44^{low} cells were plated in 6-well ultra-low attachment plate (Corning) and incubated for 24 hours. Protein lysates were prepared using 1% nonidet P-40 (NP-40) buffer. 10-20 ug protein was loaded in 9-11% SDS-PAGE gel. Primary antibodies include: phosphorylated-STAT3 (1:1000 dilution), STAT3 (1:10,000 dilution), Vimentin (1:500

dilution) and Snail (1:1000 dilution) from Cell Signaling (Danvers, MA, USA); E-cadherin (1:1000 dilution) antibody from Santa Cruz.

Sprouting assay

$5X10^4$ HDMECs per well were plated in 12 well plates (BD Falcon, NJ, USA) coated with growth factor-reduced Matrigel (Corning). After 24 hour incubation in 37°C 5% CO_2, the number of capillary-like sprouts was counted to determine the impact of IL-6 knockout on *in vitro* angiogenic potential.

Statistical analysis

Tumor microarray survival time data was analyzed using log-rank test or multivariate Cox proportional hazards models. Tumor volume growth rate was assessed using linear mixed effect models to account for repeated measurements with an auto-regressive correlation structure assuming more correlation among temporally proximate observations. The tumor size was log transformed to account for exponential tumor volume growth. Model fixed effects included time and IL-6 knockout status, and model random effects included tumor. Survival analysis was performed using the "survival" package and mixed effect regression was performed using the "nlme" package, both in the statistical software program R version 3.1.0. Unpaired t test was used to determine significance. $P \leq 0.05$ was considered significant (n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Comparisons in means were performed using Prism software (GraphPad Software, La Jolla, CA, USA).

Results

Expression of IL-6R or its co-receptor gp130 correlates with HNSCC patient outcome

Tumor specimens (n=80) were divided into low (score ≤4) and high (score >4) IL-6R or gp130 expression groups based on IHC staining (Fig. 4.1A). 40% of the tumor specimens expressed high levels of IL-6R and gp130. We found that high IL-6R expression in the invasive front of the tumor correlated with poor survival (log rank test, p=0.0217; Fig. 4.1B). To control for other known prognostic variables, we performed multivariate regression analysis, and IL-6R maintained significant discriminating ability for overall survival (Cox PH model, p=0.0128) in a model including age, tobacco use, advanced stage at diagnosis, race, sex, and alcohol. gp130 expression also had strong correlation with the patient overall survival (log rank test, p=0.0422; Cox PH model, p=0.0243) (Fig. 4.1C). Immunofluorescence staining of ALDH1A1 and IL-6R in the invasive front of tumors showed that ALDH1-positive cancer stem cells strongly expressed IL-6R in human HNSCC tumors (Fig. 4.1D).

Therapeutic inhibition of the IL-6 pathway decreases the fraction of cancer stem cells

Informed by the TMA results, we assessed the therapeutic potential of IL-6R inhibition with tocilizumab in preclinical models of HNSCC. Unsorted UM-SCC-22B cells were co-transplanted with HDMECs in biodegradable scaffolds to generate xenograft tumors with human vasculature (Nör et al., 2001), which is amenable to the testing of tocilizumab that does not cross-react with mouse cells (Okazaki et al., 2002). Two doses of tocilizumab (5 kg/mg, IP) were administered within a week period before the tumors were surgically removed. Tocilizumab treatment had no effect on overall tumor volume when compared to IgG treated tumors (Fig. 4.2A).

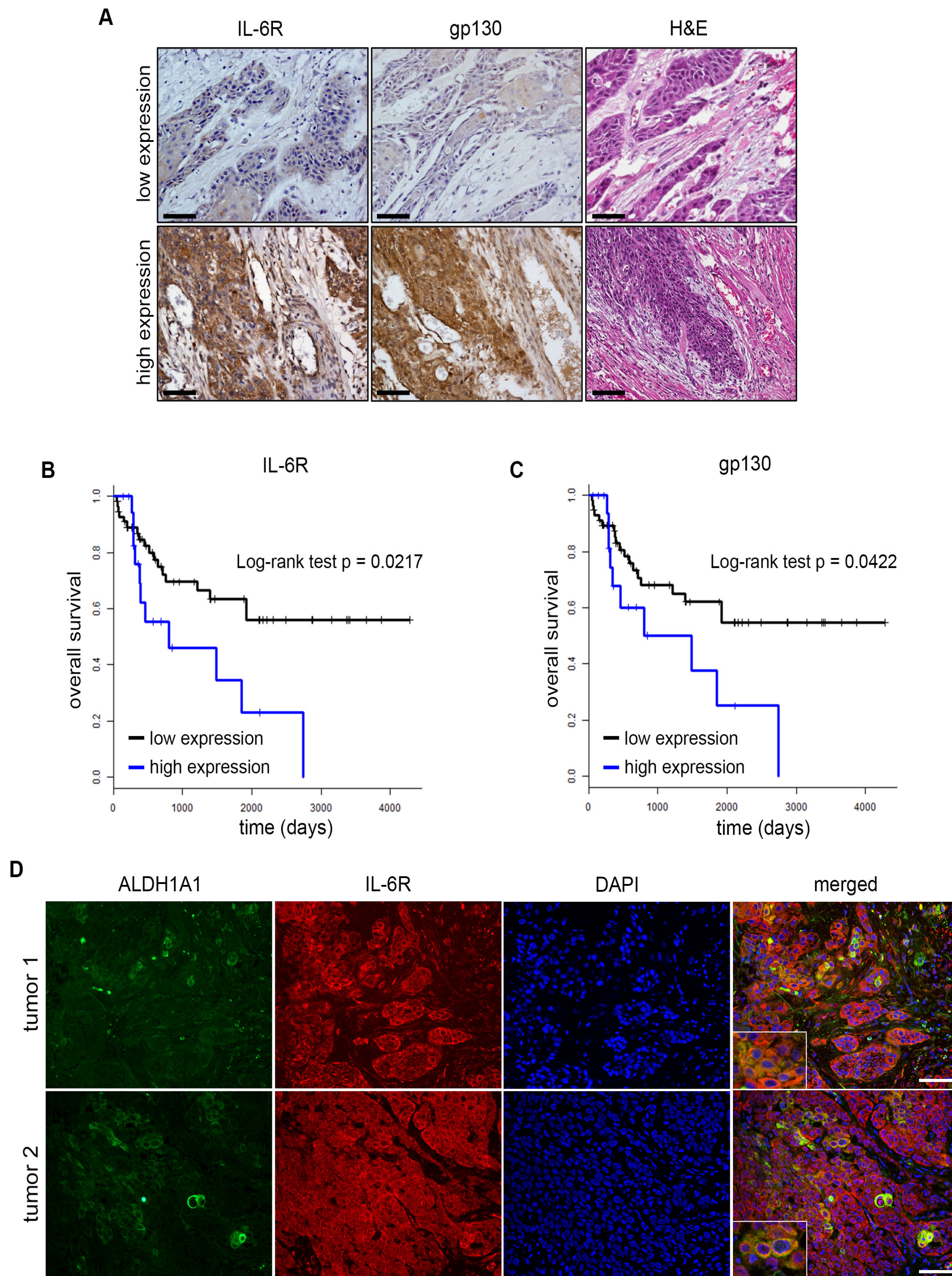


Figure 4.1. Expression of IL-6R or its co-receptor gp130 correlates with HNSCC patient outcome. A, representative immunohistochemistry staining of low and high expression IL-6R and gp130 and corresponding hematoxylin and eosin (H&E) staining. Scale bars=100 um. B and C, Kaplan-Meier curves of oral squamous cell carcinoma patients over IL-6R and gp130 expression. D, immunofluorescence staining of ALDH1A1, IL-6R and DAPI in invasive fronts of two primary patient tumors used in TMA. . Scale bars=100 um.

There was no change in mouse weight, indicating that tocilizumab was well tolerated in mice (Fig. 4.2B). FACS analysis of single cell suspensions prepared from the tumor tissues showed a significant decrease in ALDH^{high}CD44^{high} cell population in tocilizumab-treated tumors compared to tumors treated with IgG (Fig. 4.2C). In accordance with the *in vivo* data, tocilizumab treatment reduced the ALDH^{high}CD44^{high} cell population in UM-SCC-22B cells (Fig. 4.2D). Concentration of tocilizumab used for *in vitro* experiment did not have cytotoxic effect on the tumor cells (Supplementary Fig. 4.1A).

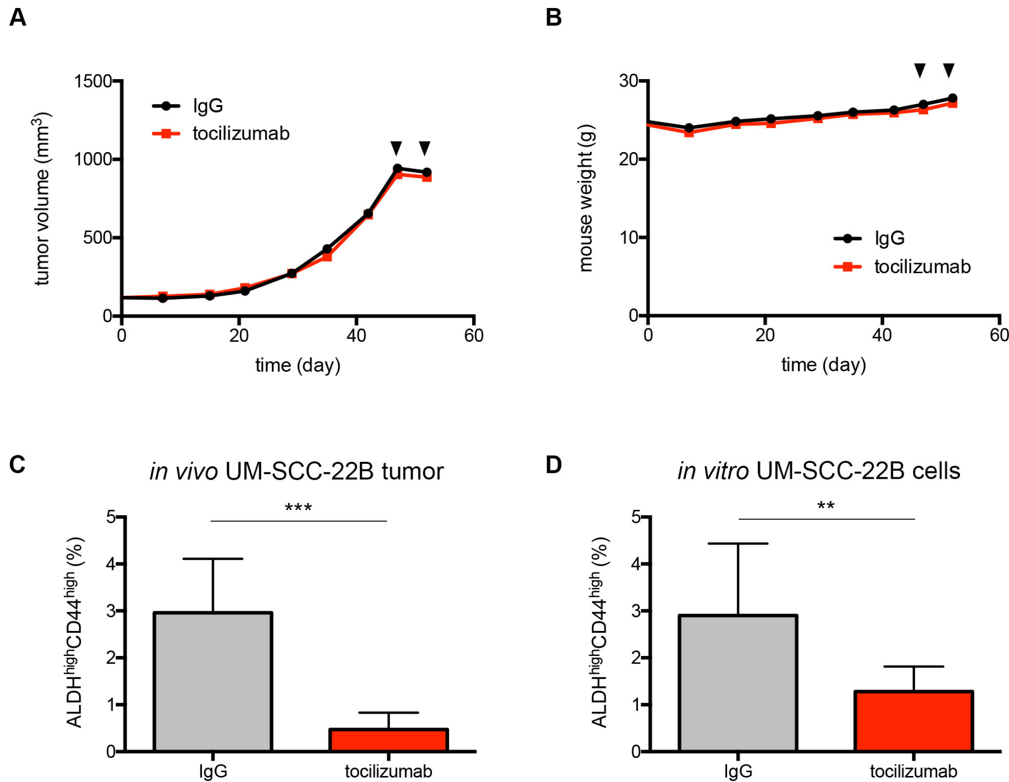


Figure 4.2. Therapeutic inhibition of the IL-6 pathway decreases the fraction of cancer stem cells. A, tumor volume during the course of study. Arrows indicate the two doses of tocilizumab given before tumor resection. B, mouse weight during the *in vivo* study. Arrows indicate the two doses of tocilizumab given before tumors were removed. C, proportion of ALDH^{high}CD44^{high} cells in UM-SCC-22B xenograft tumors after tocilizumab treatment detected by FACS analysis. D, FACS analysis result showing the proportion of ALDH^{high}CD44^{high} cells in UM-SCC-22B cells after tocilizumab treatment for 24 hours *in vitro*. n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Endothelial cell-secreted IL-6 supports cancer stem cells and tumor growth

Our group previously reported that head and neck cancer stem cells reside in close proximity with blood vessels, suggesting functional crosstalk between the two cell types (Krishnamurthy et al., 2010). To test the effect of endothelial cell-secreted IL-6 on the fraction of cancer stem cells *in vivo*, we generated IL-6 knockout endothelial cells (sgRNA-IL-6 HDMEC) using CRISPR/Cas9 system. ELISA assay showed a significant reduction in IL-6 production in sgRNA-IL-6 HDMEC as compared to vector control cells (Fig. 4.3A). The IL-6 knockout did not affect the vessel forming ability of the endothelial cells, as demonstrated in a Matrigel-based capillary sprouting assay (Fig. 4.3B). sgRNA-IL-6 HDMEC were co-implanted with UM-SCC-22B cells to generate xenograft tumors with humanized vasculature. Compared to tumor cells generated with sgRNA-control HDMEC, tumor cells grown with sgRNA-IL-6 HDMEC generated smaller tumors (Fig. 4.3C). Regression analysis of the tumor growth rates showed that IL-6 knockout in the endothelial cells is sufficient to slow down xenograft tumor growth (Fig. 4.3D). FACS analysis revealed that tumors vascularized with sgRNA-IL-6 HDMEC had a lower fraction of $ALDH^{high}CD44^{high}$ cell population than those vascularized with sgRNA-control HDMEC (Fig. 4.3E).

Endothelial cell-secreted IL-6 induces cancer stem cell migration

We tested if cancer stem cells had enhanced motility compared to non-cancer stem cells in the presence of endothelial cell conditioned medium (CM) using transwell migration assay. In the presence of endothelial cell CM, more $ALDH^{high}CD44^{high}$ cells migrated than $ALDH^{low}CD44^{low}$ cells (Fig. 4.4A and 4.4B). In order to evaluate the role of endothelial-cell secreted IL-6 on migration of cancer stem cells, we treated sorted $ALDH^{high}CD44^{high}$ cells with tocilizumab and allowed the cells migrate in the presence of endothelial cell CM. After 24 hours, we found that

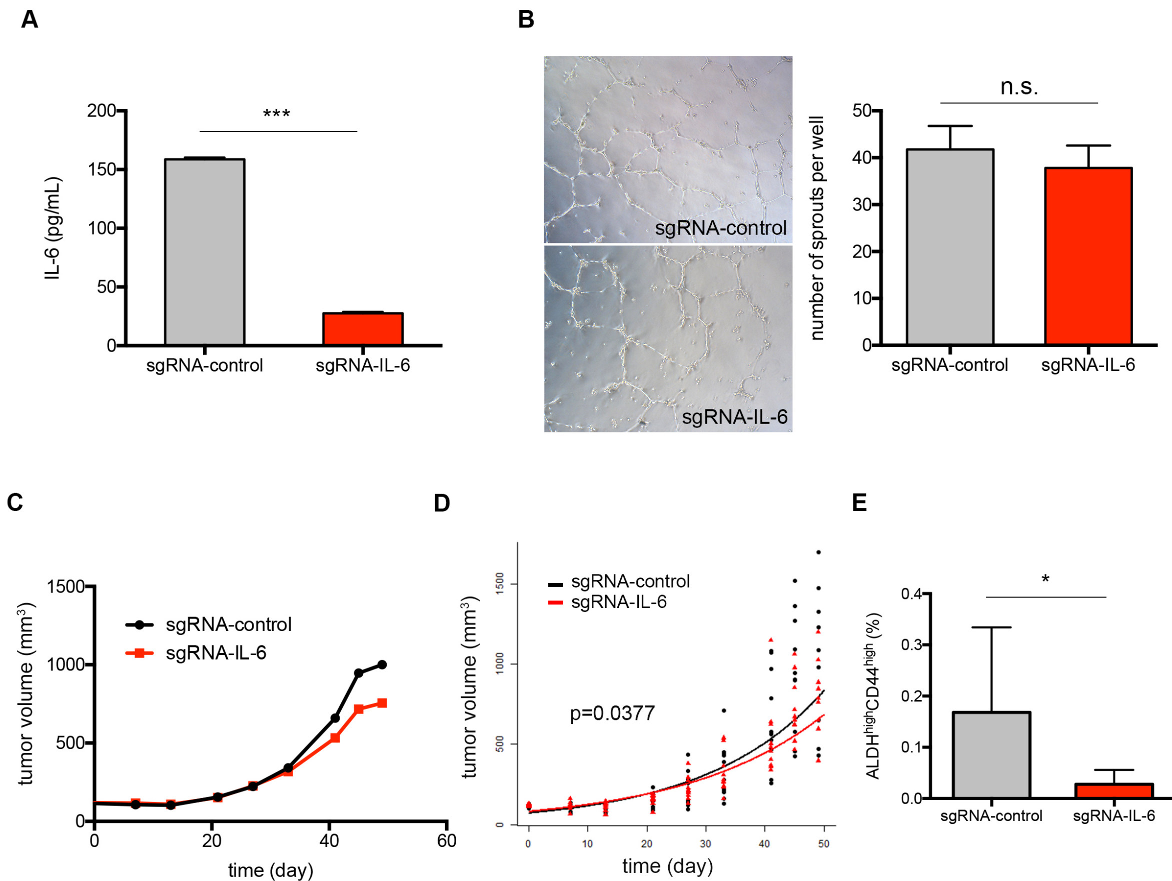


Figure 4.3. Endothelial cell-secreted IL-6 supports cancer stem cells and tumor growth. A, concentration of IL-6 secreted by sgRNA-control or sgRNA-IL-6 HDMEC quantified by ELISA. B representative pictures of sprouts formed by sgRNA-control and sgRNA-IL-6 HDMECs on matrigel. Average number of sprouts per well is shown as bar graph. C, graph depicting the average tumor volumes. D, repeated measures linear regression estimated mean tumor size prediction line sgRNA-control and sgRNA-IL-6, respectively, overlayed with individual tumor volume. E, FACS analysis of $ALDH^{high}CD44^{high}$ cell proportion in tumors grown with sgRNA-control or sgRNA-IL-6 HDMECs. n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

tocilizumab reduced the migration of $ALDH^{high}CD44^{high}$ cells (Fig. 4.4C and 4.4D; Supplementary Fig. 4.2A). We repeated the migration experiments using a different approach to verify the reproducibility of the data. Here, we used microfluidics device (Fig. 4.4E and 4F) that was previously described (Chen et al., 2015; Burgos-Ojeda et al., 2015). Endothelial cell CM induced strong migration of $ALDH^{high}CD44^{high}$ cells (Fig. 4.4G; Supplementary Fig. 4.2C). We observed a reduction in cancer stem cell migration when the IL-6 pathway was inhibited either

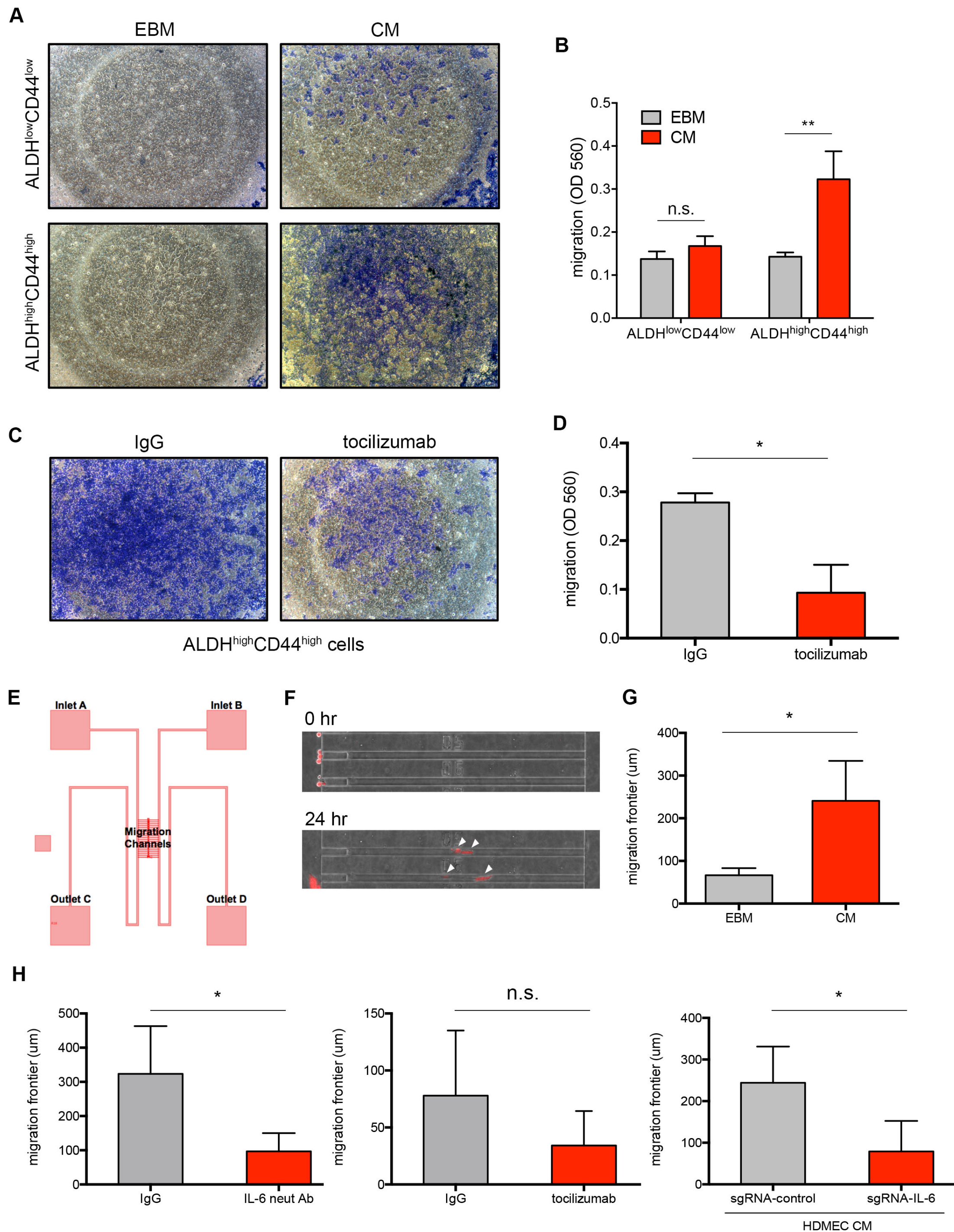


Figure 4.4. Endothelial cell-secreted IL-6 induces cancer stem cell migration. A, representative pictures of migrated UM-SCC-22B $ALDH^{low}CD44^{low}$ or $ALDH^{high}CD44^{high}$ cells stained with crystal violet in transwell insert after 24 hours of incubation in EBM or HDMEC CM. B, bar graph depicting migrated $ALDH^{low}CD44^{low}$ or $ALDH^{high}CD44^{high}$ cells over 24 hour-period in transwell system. C, representative pictures of $ALDH^{high}CD44^{high}$ cells migrated after tocilizumab treatment (2 ug/mL) for 24 hours. D, quantification of cells migrated after tocilizumab treatment.

E, microfluidics migration device layout. F, representative pictures of cells migrated after 24 hours in the presence of HDMEC CM in the microfluidics chip. Tumor cells were marked with mOrange fluorescence protein. G, endothelial cell-secreted factors induce migration of ALDH^{high}CD44^{high} cells in microfluidics device. H, the effect of IL-6 inhibition on ALDH^{high}CD44^{high} cells motility. IL-6 signaling was inhibited by neutralizing IL-6 in HDMEC CM, treating tumor cells with tocilizumab or using CM from sgRNA-IL-6 HDMEC. n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

with an IL-6 neutralizing antibody or with tocilizumab (Fig. 4.4H). To validate the data obtained with antibodies target to the IL-6 pathway, we performed migration studies using as chemotactic stimulus the CM from sgRNA-IL-6 HDMEC. Again, migration of ALDH^{high}CD44^{high} cells was reduced (Fig. 4.4H; Supplementary Fig. 4.2D), suggesting the chemotactic effect of endothelial cell-secreted IL-6 on migratory behavior of cancer stem cells.

Endothelial cell-secreted IL-6 induces EMT marker expression in head and neck cancer stem cells

The results from migration experiments led to our speculation that the enhanced migratory phenotype of cancer stem cells might be associated with induction of EMT. Western blot showed that ALDH^{high}CD44^{high} marker combination successfully isolated cells expressing higher levels of stemness-related proteins, Nanog, Notch 3 and Notch 4 (Fig. 4.5A; Supplementary Fig. 4.3A). In addition, ALDH^{high}CD44^{high} cells expressed higher levels of mesenchymal cell-related proteins, Vimentin and Snail, as compared with ALDH^{low}CD44^{low} cells (Fig. 4.5B). Interestingly, we found that ALDH^{high}CD44^{high} cells expressed higher levels of IL-6R and its co-receptor gp130 than ALDH^{low}CD44^{low} cells (Fig. 4.5C). Then, we tested whether the enhanced migratory ability of ALDH^{high}CD44^{high} cells in the presence of endothelial cell CM correlates with differential EMT level. We observed that HDMEC CM induced Vimentin and Snail expression in ALDH^{high}CD44^{high} cells but not in ALDH^{low}CD44^{low} cells (Fig. 4.5D; Supplementary Fig. 4.3B). Similar results were reproduced in ALDH^{high}CD44^{high} cells treated with recombinant IL-6 (Fig. 4.5E). Notably, IL-6R blockade with tocilizumab inhibited

endothelial cell-induced Snail and Vimentin expressions in ALDH^{high}CD44^{high} cells (Fig. 4.5F; Supplementary Fig. 4.3C). Collectively, these results demonstrate that endothelial cell-secreted IL-6 induces key mediators of EMT specifically in cancer stem cells.

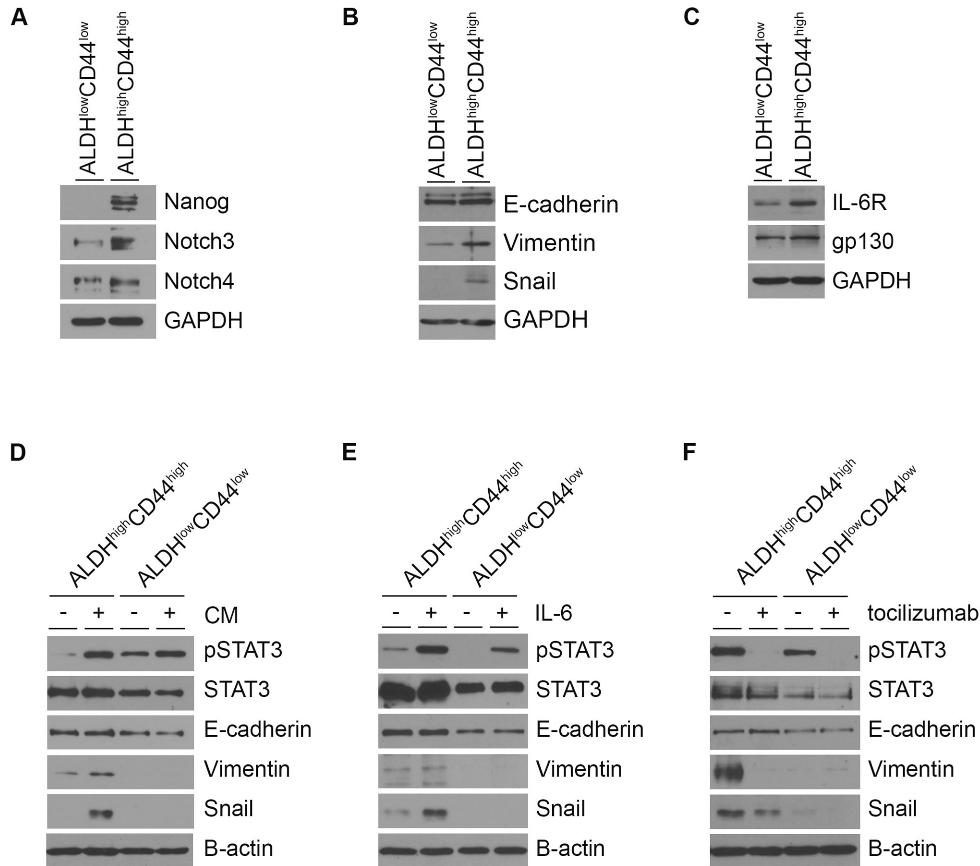


Figure 4.5. Endothelial cell-secreted IL-6 induces EMT marker expression in head and neck cancer stem cells. A, stemness-related markers, Nanog, Notch 3, Notch 4, were detected in ALDH^{high}CD44^{high} and ALDH^{low}CD44^{low} cells. B, IL-6R and gp130 levels in UM-SCC-22B ALDH^{high}CD44^{high} cells compared to ALDH^{low}CD44^{low} cells. C, EMT state of ALDH^{high}CD44^{high} cells. D, E, F, effect of HDMEC CM, IL-6 and tocilizumab on EMT markers in ALDH^{high}CD44^{high} cells and ALDH^{low}CD44^{low} cells.

STAT3 regulates EMT markers in head and neck cancer stem cells

It is well known that STAT3 is a key downstream effector of IL-6 signaling through IL-6R (Zhong et al., 1994). We observed that STAT3 was phosphorylated in ALDH^{high}CD44^{high} cells treated with HDMEC CM or recombinant human IL-6, and that tocilizumab inhibited STAT3

phosphorylation (Fig. 4.5D, E and F). Here, we silenced STAT3 in tumor cells using shRNA constructs (Fig. 4.6A; Supplementary Fig. 4.4A). STAT3 knockdown resulted in decreased orosphere-forming ability of HNSCC cells in ultralow attachment plates, suggesting the importance of STAT3 signaling to the survival and self-renewal of head and neck cancer stem cells (Fig. 4.6B; Supplementary Fig. 4.4B). STAT3 silencing of unsorted HNSCC cells was associated with lower Vimentin and Snail expression (Fig. 4.6C). Further, STAT3 silencing resulted in a significant decrease in the $ALDH^{high}CD44^{high}$ cell population (Fig. 4.6D; Supplementary Fig. 4.4C). Notably, STAT3 silencing inhibited expression of the EMT markers, Snail and Vimentin, in $ALDH^{high}CD44^{high}$ cells (Fig. 4.6E).

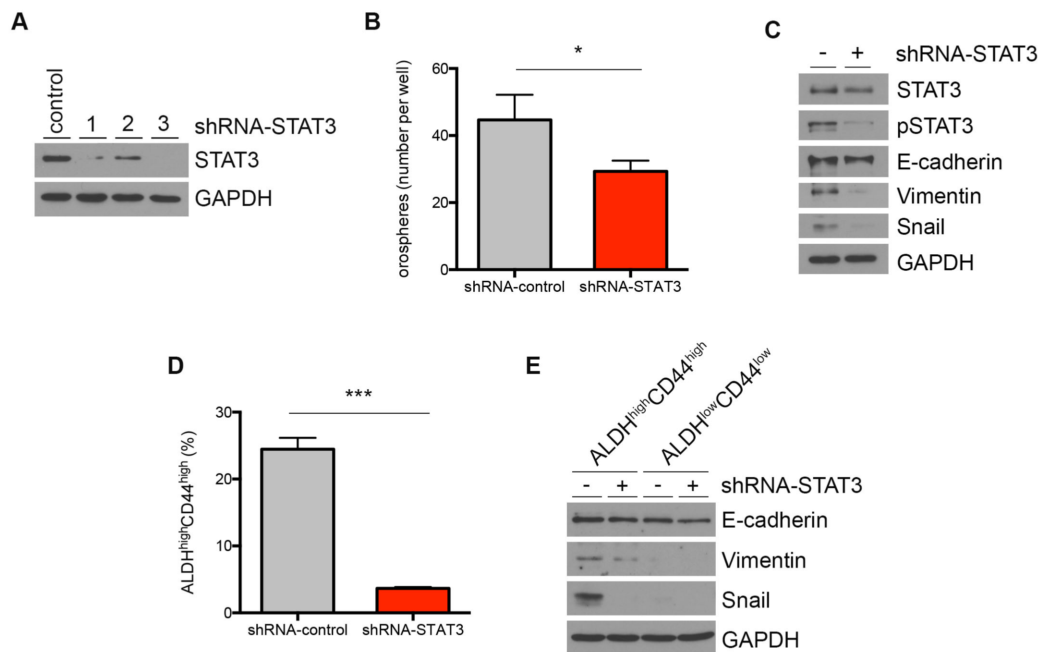
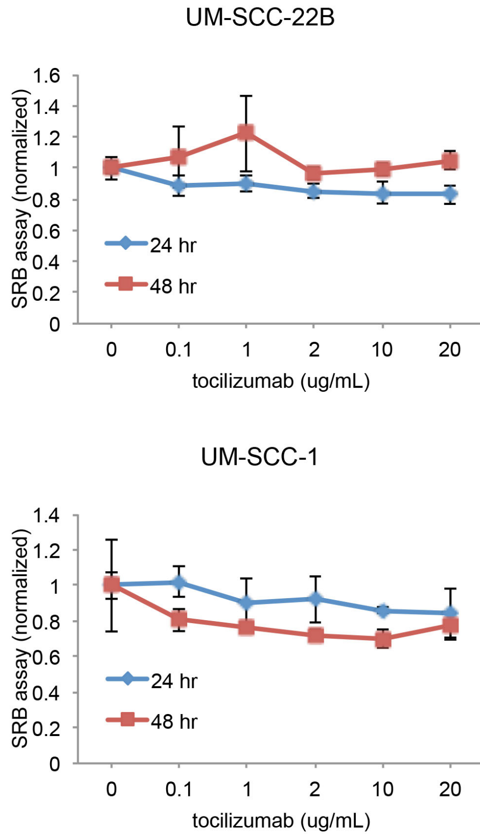


Figure 4.6. STAT3 regulates EMT markers in head and neck cancer stem cells. A, Western blot showing STAT3 knockdown efficiencies of different constructs. B, bar graph depicts average number of orospheres generated from shRNA-control or shRNA-STAT3 UM-SCC-22B. C, Western blot of STAT3 knockdown cells probing for STAT3, pSTAT3, E-cadherin, vimentin, snail and GAPDH. D, FACS analysis of $ALDH^{high}CD44^{high}$ cell percentage in STAT3 knockdown UM-SCC-22B cells. E, EMT marker detection in $ALDH^{high}CD44^{high}$ cells with STAT3 knockdown. n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

A



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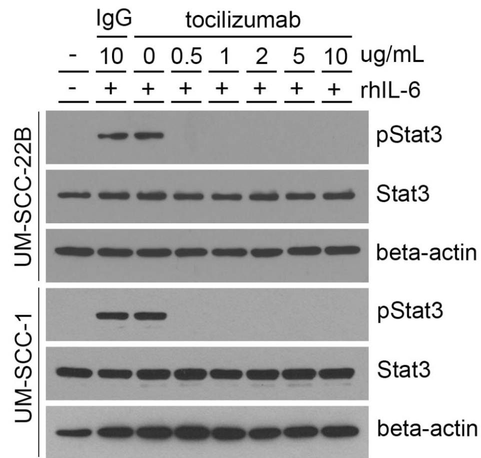
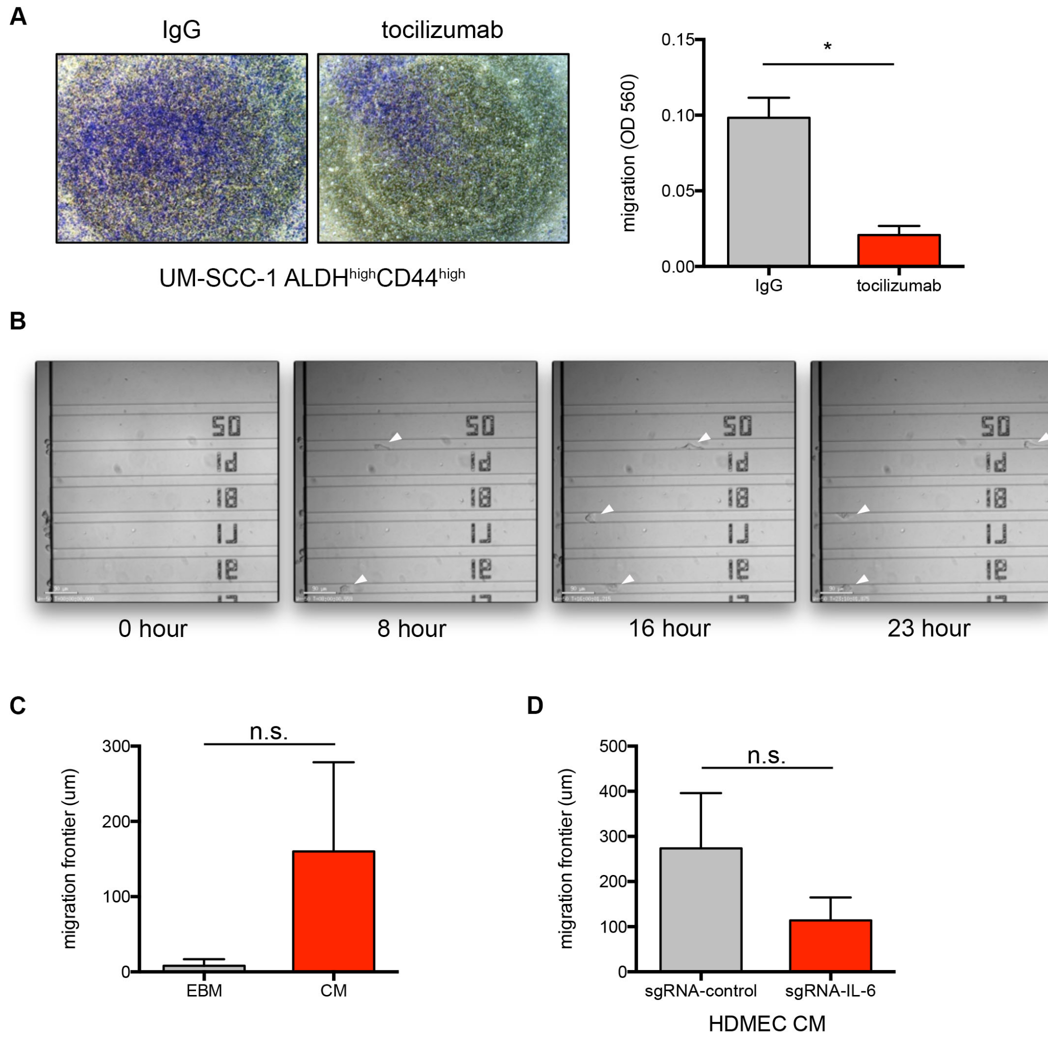
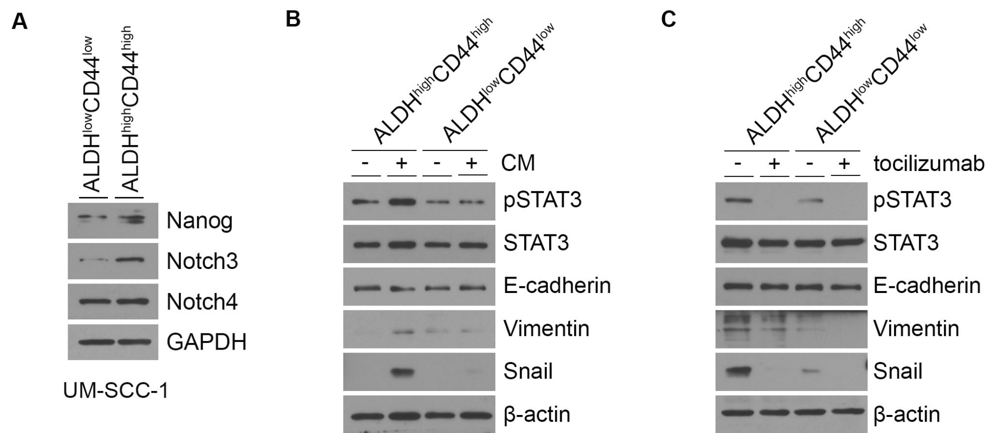


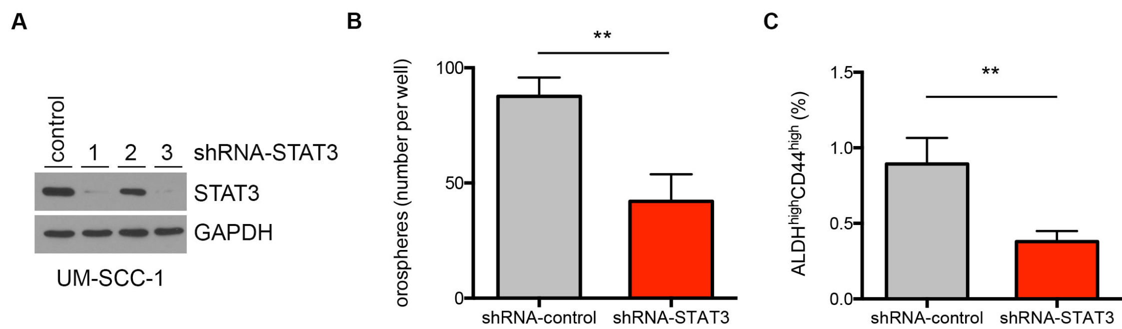
Supplementary Figure 4.1. Tocilizumab concentration used for the experiment did not affect the cell viability. A, sulfarhodamine assay result of UM-SCC-22B and UM-SCC-1 after 24 and 48 hour tocilizumab treatment. B, Western blot of UM-SCC-22B and UM-SCC-1 cells treated with different concentrations of tocilizumab to inhibit phosphorylation of STAT3. We used 2 ug/mL tocilizumab for all in vitro assays.



Supplementary Figure 4.2. Blockage of endothelial cell-initiated IL-6 pathway reduces migration of UM-SCC-1 cancer stem cells. A, representative pictures of migrated cells in transwell 24 hours after tocilizumab treatment. Bar graph depicts the OD560 level of crystal violet stained migrated cells. B, time-lapse images of tumor cells migrating through the migration channel of microfluidics device over 24 hour period in the presence of endothelial cell CM. C,D, migration frontiers of ALDH^{high}CD44^{high} cells in the presence of endothelial cell CM (C) or sgRNA-IL-6 HDMEC CM (D). n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



Supplementary Figure 4.3. Endothelial cell-secreted IL-6 induces EMT in UM-SCC-1 cancer stem cells. A, $ALDH^{high}CD44^{high}$ cells from UM-SCC-1 express more stemness related markers, Nanog and Notch 3, than $ALDH^{low}CD44^{low}$ cells. B, C, EMT protein markers expressed in UM-SCC-1 $ALDH^{high}CD44^{high}$ and $ALDH^{low}CD44^{low}$ cells with endothelial cell CM (B) and tocilizumab treatment (C) for 24 hours.



Supplementary Figure 4.4. Effect of STAT3 knockdown in UM-SCC-1 cancer stem cells. A, STAT3 knockdown efficiency of three shRNA-STAT3 constructs in UM-SCC-1. shRNA-STAT3 construct 3 was used for further study. B, bar graph depicts number of orospheres generated from shRNA-STAT3 UM-SCC-1. C, FACS analysis of the $ALDH^{high}CD44^{high}$ cell population between shRNA-control and shRNA-STAT3 UM-SCC-1. n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Discussion

Tumor dissemination is the major problem in the management of patients with advanced head and neck cancer. Majority of patients with locally advanced head and neck cancer develop tumor metastasis/relapse, and the survival rate for patients with metastasis/relapse is between 5 to 9 months (Bhave et al., 2011). However, our understanding of the mechanisms leading to metastatic spread is still very limited. It has been recently proposed that cancer stem cells play a critical role in metastasis development in several cancer types (Hermann et al., 2007; O'Brien et al., 2007; Balic et al., 2006). Chinn et al. highlighted enhanced potential of cancer stem cells to generate lymph node metastasis in HNSCC (2015). Although the importance of cancer stem cells in the metastatic process is well recognized, it is still unclear how cancer stem cells move away from the tumor nests towards blood vessels. Here, we demonstrated endothelial cell-secreted IL-6 induces cancer stem cell motility and also unveiled the therapeutic potential of IL-6 signaling blockage in HNSCC.

The importance of IL-6 on cancer pathobiology has been well recognized from different types of tumors. Serum level IL-6 has been identified as prognostic marker in many types of cancer, including ovarian cancer (Berek et al., 1991), prostate cancer (Nakashima et al., 2000), breast cancer (Zhang et al., 1999), colon cancer (Galizia et al., 2002), melanoma (Tartour et al., 1994) and HNSCC (Duffy et al., 2008). Here, we observed that high IL-6R or gp130 level in the invasive front of tumors correlated with poor outcome in HNSCC patients. IL-6 signaling through IL-6R/gp130 induces robust activation of STAT3 signaling. It has been shown that STAT3 activation enhances invasion and motility of tumor cells (Kamran et al., 2013), contributing to the aggressiveness of the tumor. To our knowledge, this work is likely the first to

demonstrate that high IL-6R or gp130 in the invasive front of the tumor are predictive markers of HNSCC patient overall survival.

With mounting evidence showing the importance of IL-6 in tumor biology, IL-6 pathway targeting drugs that either block the receptor or binding the ligand have been tested. Tocilizumab is a humanized anti-IL-6R antibody that inhibits both soluble and membrane-bound IL-6R to prevent IL-6 pathway activation. The half-life of the antibody is long enough (11-13 days depending on the concentration) to allow for monthly intravenous injections (Jones and Ding 2010). The drug is well tolerated by the patients with minimal side effects (Jones and Ding 2010). In this study, we tested the effect of tocilizumab on HNSCC tumors and found that two doses of tocilizumab are sufficient to reduce the cancer stem cell fraction. Such result highlights the importance of IL-6 pathway in the survival of head and neck cancer stem cells and the therapeutic potential of IL-6 inhibition in HNSCC. Our result showed that tocilizumab in itself was not toxic to the tumor cells. There are two possible explanations to how tocilizumab decreases cancer stem cell fraction. First, IL-6 pathway induces STAT3 pathway, which is known to be an important regulator of self-renewal (Niwa et al., 1998). With the loss of STAT3 pathway, the cancer stem cells may lose the self-renewal potential to maintain their population pool and result in differentiation. Another explanation could be due to decrease in the microvassel density of the tumor by tocilizumab treatment (Shinriki et al., 2009). Since cancer stem cells reside in perivascular niche, loss of the protective niche environment may result in differentiation of the cancer stem cells to the rest of the tumor population. We have previously shown that cisplatin increases the cancer stem cell fraction in pre-clinical model of HNSCC (Nör et al., 2014), suggesting a possible explanation for why conventional therapies fail to prevent metastasis and recurrence of head and neck cancer. These studies suggest that the combination of

chemotherapy and tocilizumab may reduce the tumor size (*i.e.* debulk the tumor) while at the same time ablate the cancer stem cells, and therefore reducing the chances of tumors to metastasize or relapse.

Our group recently reported that treatment of tocilizumab alone slowed the growth rate of xenograft model of mucoepidermoid carcinoma tumors, another subtype of head and neck cancer (Mochizuki et al., 2015). Importantly, the tumor growth rate and the fraction of cancer stem cells significantly decreased when the tumors were treated with tocilizumab and cisplatin together, suggesting that combination of existing chemotherapy and IL-6 pathway blocking agent may also be beneficial for other head and neck cancer patients.

Here, we observed that IL-6 knockout endothelial cells resulted in slow tumor growth with smaller fraction of cancer stem cells. These results suggest that endothelial cells are a key source of the IL-6 that is required to maintain the cancer stem cell population in the perivascular niche, which perhaps modulates the aggressiveness of the tumor. It has been shown that IL-6 induces EMT in breast cancer models (Sullivan et al., 2009), and that it promotes metastasis to lymph node and lungs in HNSCC (Yadav et al., 2011). Indeed, we and others reported that inhibition of the IL-6 pathway inhibits migration of HNSCC (Yadav et al., 2011; Neiva et al., 2009). Further, we showed that head and neck cancer stem cells express more mesenchymal cell-related proteins and are more motile than non-cancer stem cells in response to endothelial cell-secreted factors. We demonstrated that inhibition of endothelial cell-activated IL-6 pathway prevented EMT in cancer stem cells and inhibited their migration, unveiling the role of IL-6 on cancer stem cell motility. Interestingly, we did not observe induction of migration upon recombinant human IL-6 treatment alone (data not shown). By itself, IL-6 might endow cancer stem cells with migratory phenotype via EMT without necessarily acting as chemokine to induce

cell movement. In contrast, the full endothelial cell-secreted milieu is efficient at inducing cancer stem cells migration, and within this milieu IL-6 plays a critical role as demonstrated in several blocking experiments (Figure 4.4). We conclude that tumor cell migration towards blood vessels might be the result of endothelial cell-secreted factors working together, and that IL-6 primes the cancer stem cells to respond to these factors. We postulate that tumor cells may acquire migratory phenotype through the IL-6/IL-6R/STAT3 axis, and potent chemokines, such as CXCL8, initiate the actual cellular movement to a specific direction.

A major challenge in studying cancer stem cells is the rarity of this cell population. Head and neck cancer stem cell proportion ranges between 0.6-4.5% in primary tumors (Clay et al., 2010) and 1-10% in cell lines (Zhang et al., 2014). Working with such small population of cells makes it difficult to do in-depth analysis with most common research tools, as the unique responses of cancer stem cells may not be reflected in studies with bulk cells. Recent advances in microfluidics technologies enabled cancer biologists to answer questions that were once thought not possible to answer. For example, microfluidics devices allowed isolating circulating tumor cells from blood samples (Nagrath et al., 2007) and looking at RNA levels of single cells after different treatment (Spurgeon et al., 2008). Our group used a novel microfluidics migration platform to assess the migration ability of cancer stem cells in the presence of endothelial cell CM and showed the impact of IL-6 pathway in cancer stem cell motility. Indeed, we showed that the results seen from microfluidics devices were in parallel with transwell migration assays performed with sorted cancer stem cells. In comparison to transwell system, microfluidics platform creates gradient of endothelial cell CM within the migration channel, thus emulating the biological environment more accurately. In addition, the microfluidics devices allow analysis of the movement of single cells during migration process. Further studies with cells that are highly

motile in the presence of endothelial cell CM with single cell capture technology may give us better understanding of cellular mechanisms that lead to metastatic dissemination in HNSCC.

We used lentiviral CRISPR/Cas9 system to generate stable IL-6 knockout endothelial cells. Using this system, we were able to generate endothelial cells that secreted IL-6 80-90% less and still have intact vessel-forming ability. However, integration of lentiviral construct in cell genome can cause genome instability and disrupt normal gene expression (Xiao et al., 2013). For future experiments, integrase-defective CRISPR/Cas9 lentiviral vector should be used to reduce the off-target effect (Wang et al., 2015).

In conclusion, we demonstrated here the impact of endothelial cell-initiated IL-6 signaling to the migratory phenotype of head and neck cancer stem cells, which are the primary mediators of HNSCC tumor dissemination. These results suggest the possibility that a combination therapy involving conventional chemotherapy to debulk the majority of the more differentiated tumor cells together with an IL-6 pathway-inhibiting agent to ablate cancer stem cells might be beneficial for patients with HNSCC.

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CHAPTER V

DISCUSSION AND CONCLUSIONS

Discussion

The idea that stem cells initiate tumor growth first arose 150 years ago. Virchow (1858) first argued that immature cells could give rise to tumors. Approximately 20 years later, Durante (1874) and Cohnhein (1875) suggested that the dormant embryonic stem cells remaining in adult tissue could become tumor cells. The term "tumor stem cells" was first used by Makino with definition of "a small subpopulation of cells that were insensitive to chemotherapy and had chromosomal features different from the bulk of cells" (1959). Twenty years later, Bonnet and Dick first showed that CD34+CD38- cells from acute myeloid leukemia had enhanced tumor-forming ability (Bonnet and Dick, 1997). More importantly, these cells were able to self-renew and give rise to the rest of the tumor population that was similar to that of the original tumor (Bonnet and Dick, 1997). Since these seminal discoveries, cancer stem cells were identified and characterized in multiple solid tumors (Hermann et al., 2007; Beier et al., 2007; Bapat et al., 2005; Dalerba et al., 2007; Prince et al., 2007; Ma et al., 2007).

In this Ph.D. dissertation, we showed that head and neck cancer stem cells highly express several stemness-related proteins, *e.g.* Nanog, Notch3 and Notch4 (Figure 4.5.A). Such observation is in accordance with the previous reports by other groups (Ponti et al., 2005; Yang et al., 2008). However, it is not yet known how stemness-related gene and protein are highly

expressed in cancer stem cells. One possible explanation would be that these cancer stem cells originate from normal stem cells. Stem cells are long-lived, allowing mutations to accumulate with time and resulting in transformation of these cells. Transformed stem cells exhibit deregulated proliferation as a consequence of mutations, as well as the stem cell features of self-renewal ability and multipotency. There is also a possibility that differentiated cells acquire mutations that allow them to have stem-like traits. Nanog, Oct4 and Sox2 are transcription factors that are important in embryonic stem cells. In 2007, Thomson group demonstrated that expression of genes, including *NANOG, OCT4,* and *SOX2*, in human somatic cells generated induced-pluripotent stem cells with embryonic stem cell characteristics (Yu et al., 2007). Notably, these three transcription factors are often overexpressed in head and neck squamous cell carcinomas (Reers et al., 2014; Koo et al., 2015; Habu et al., 2015; Lee et al., 2014).

Another potential contributing factor that enhances stemness of cancer stem cells is the tumor microenvironment. In head and neck squamous cell carcinoma, cancer stem cells reside in perivascular niche (Krishnamurthy et al., 2010). Endothelial cell-secreted factors increase cancer stem cell fraction *in vitro* (Zhang et al., 2014; Krishnamurthy et al., 2014) and the number of orosphere formation from cancer stem cells (Krishnamurthy et al., 2010). Endothelial cell conditioned medium increases Bmi-1, protein representing self-renewal potential, expression in head and neck squamous cell carcinoma cells (Zhang et al., 2014). Brain cancer stem cells have also been shown to be located in perivascular niche (Calabrese et al., 2007). Recent studies revealed that endothelial cell-secreted factors induce differentiated CD133- glioblastoma multiforme cells to give rise to CD133+ cancer stem cells (Fessler et al., 2015). In colon cancer, cancer stem cells express high Wnt expression, which is important in stemness. Interestingly, myofibroblast-secreted factors activate Wnt signaling pathway in colon cancer stem cells

(Vermeulen et al., 2010). The same authors reported that cells expressing high levels of Wnt were located in close proximity to the stromal myofibroblasts (Vermeulen et al., 2010), supporting the idea that microenvironment-initiated signaling induces tumor cells to exhibit stem-like characteristics. Collectively, cancer stem cells may acquire stem-like phenotype by combination of "internal" genetic mutation events and "extrinsic" microenvironment-initiated pathway activations.

The importance of cancer stem cell in tumor growth, recurrence and metastasis emphasizes the need for therapeutic agents targeting cancer stem cells. Recent advances in tumor immunotherapy have made a significant impact on cancer treatment. Several studies have suggested that cancer stem cells can be targeted using immunotherapeutic approach. Tseng and colleagues reported that natural killer cells selectively eradicated undifferentiated head and neck cancer stem cells but not the differentiated cells (2010). Visus and colleagues also showed that ALDH1A1 specific $CD8^+$ T cells were able to kill $ALDH^{bright}$ cells and inhibit tumor growth and metastasis (2011). Ning et al. pulsed dendritic cells with either unsorted tumor cells or cancer stem cells to generate vaccine (Ning et al., 2012). In this study, the authors observed that vaccine against $ALDEFLUOR^+$ cells resulted in significant tumor growth inhibition compared to control vaccine (Ning et al., 2012). Vaccines against cancer stem cells are currently in clinical trials. Initial findings from nasopharyngeal cancer stem cell vaccine showed that sera isolated from patients who received cancer stem cell vaccine contained high level of IgG against cancer stem cells, which in turn resulted in killing of cancer stem cells (Lin et al., 2015).

We showed in this Ph.D. thesis that inhibition of the IL-6 signaling pathway reduces the cancer stem cell fraction *in vitro* and *in vivo* (Figure 4.2.B, C) and highlighted the potential of a monoclonal anti-IL-6R antibody, tocilizumab, as cancer stem cell-targeting agent. Blocking the

IL-6 pathway may be a counterintuitive approach to achieve anti-tumor effect since IL-6 is originally known to be a pro-inflammatory cytokine. It has been shown that head and neck squamous cell carcinomas secrete IL-6 (St John et al., 2004; Thomas et al., 2004), and that high serum IL-6 levels correlate with poor outcome of head and neck cancer patients (Duffy et al., 2008). Constitutive secretion of IL-6 results in high activation of downstream signal transducer, STAT3. When STAT3 activity is inhibited, the head and neck squamous cell carcinoma cells lose proliferation ability and survival potential (Sriuranpong et al., 2003). STAT3 is also known to be a negative regulator of immune response. In tumors with high levels of STAT3 activation, the immune system does not respond to the established tumors due to lack of dendritic cell activity (Fuchs and Matzinger 1996; Pardoll 1998; Steinman and Nussenzweig 2002; Banchereau and Steinman 1998). However, when STAT3 activity is blocked, pro-inflammatory cytokines are secreted and innate and adaptive immunities are activated (Wang et al., 2004). In other words, blocking the STAT3 pathway by inhibiting IL-6 pathway activation can potentially reduce cancer stem cells and the bulk tumor cells by activating the immune system. Further studies need to be carried out to confirm and assess the efficacy of using IL-6 pathway blocking agents, like tocilizumab, in targeting cancer stem cells and re-activating the immune response against head and neck cancer.

Conclusions

Despite the advances in our knowledge on head and neck cancer, the 5-year survival rate has not improved much for last three decades. The cancer stem cell hypothesis postulates that a subpopulation of cells that have self-renewal ability, therapy resistance and high tumorigenic potential plays a critical role in tumor metastasis and recurrence. Indeed, this unique population

of cells has been identified from multiple solid tumors and hematopoietic malignancies. Although different markers/methods are now available to isolate cancer stem cells, our knowledge of the biology of cancer stem cells and the mechanism allowing cancer stem cells to actively contribute to the progression of the disease is still rather limited.

The cancer stem cell hypothesis proposes that current failure in cancer treatment is due to inability of conventional therapies to clear out the uniquely tumorigenic cell population (*i.e.* cancer stem cells). According to this theory, complete remission of tumor can only be achieved if both cancer stem cells and the bulk tumor cells are ablated. Today, intensive efforts directed at the understanding of the molecular pathways that are critical to cancer stem cell survival or function are ongoing with the ultimate goal of developing therapeutic strategies that target and eliminate cancer stem cells. Chapter II is an overview of the progress that has been made in cancer stem cell field and potential drugs that can target head and neck cancer stem cells (Kim et al., 2016a). In head and neck cancer, cells with high aldehyde dehydrogenase (ALDH) activity and high CD44 expression have superior tumor-forming ability and enhanced orosphere-forming ability (Krishnamurthy et al., 2010). Recent work in head and neck cancer showed that increase in stemness-related markers, such as β-catenin, Oct4, and Bmi-1, enhances the self-renewal ability and the cancer stem cell fraction (Koo et al., 2014; Nör et al., 2014; Giudice et al., 2013). In addition, molecules originated in the tumor stroma appear to regulate the cancer stem cell pool and survival (Krishnamurthy et al., 2010).

In order to study the cancer stem cell population, it is critical to establish techniques and tools to successfully isolate and culture cancer stem cells for further analysis. Due to its multipotency, cancer stem cell can repopulate the entire population after it is plated in standard attachment setting within 24 hours (Gupta et al., 2011). Therefore, it is critical to prevent

differentiation of cancer stem cells when assessing the cancer stem cell-targeting agent. Chapter III described the methods we use to isolate cancer stem cells from the primary or xenograft tumors and culture as orospheres (Kim et al., 2016b). The therapeutic potential of anti-tumor drugs can be tested on the sorted cancer stem cells grown as orospheres, as described in this chapter.

In Chapter IV, we studied the role of endothelial cell-activated signaling, specifically IL-6 signaling, in cancer stem cell motility and the therapeutic potential of an IL-6 receptor (IL-6R) inhibitor, tocilzumab. We observed that endothelial cell-secreted factors induced expression of mesenchymal cell-related markers (Vimentin ad Snail) and enhanced the motility of head and neck cancer stem cells. Recombinant human IL-6 alone also had similar effect on cancer stem cells. Upon IL-6 pathway blockage, endothelial cell-induced expression of Vimentin and Snail, and the cancer stem cell motility was decreased. High serum level IL-6 has been associated with high tumor recurrence rate and poor overall survival of head and neck cancer patients (Duffy et al., 2008). Here, we showed that high levels of IL-6R and co-receptor gp130 in the invasive front of head and neck tumors correlated with poor overall survival in a study with up to 12 years of follow-up. Tocilizumab treatment significantly reduced the cancer stem cell fraction in pre-clinical trials in head and neck squamous cell carcinoma xenografts. This work unveiled the impact of endothelial cell-secreted IL-6 on cancer stem cell motility and epithelial-mesenchymal transition. Furthermore, it provided scientific rationalte for the testing of tocilizumab as a novel therapeutic option for head and neck cancer.

In summary, tumor metastasis and recurrence in head and neck cancer may be due to cancer stem cell's ability to survive conventional therapies and invade surrounding tissues. Cancer stem cells acquire migratory phenotype by the interaction they have with the surrounding

environment, such as blood vessels. We demonstrated that endothelial cell-secreted IL-6 enhances the migratory phenotype of cancer stem cells and resulting in tumor dissemination. Collectively, this work suggests the potential benefit of combining an anti-cancer stem cell therapy (*e.g.* tocilizumab) with an anti-tumor differentiated cells agent (*e.g.* cisplatin) for the treatment of patients with head and neck squamous cell carcinoma.

The main conclusions of this dissertation are:

- Expression levels of IL-6R and gp130 in the tumor invasive front correlate with the overall survival of head and neck squamous cell carcinoma patients.
- Cancer stem cells acquire a highly motile phenotype in the presence of endothelial cell-secreted factors.
- Endothelial cell-secreted IL-6 induces mesenchymal cell-related protein expression and enhances cancer stem cell motility.
- IL-6 pathway blocking agent, such as tocilizumab, targets the cancer stem cell population but its effect on tumor metastasis and recurrence remains to be determined.
- Collectively, this work suggests that patients with head and neck squamous cell carcinoma might benefit from an IL-6 targeted therapy.

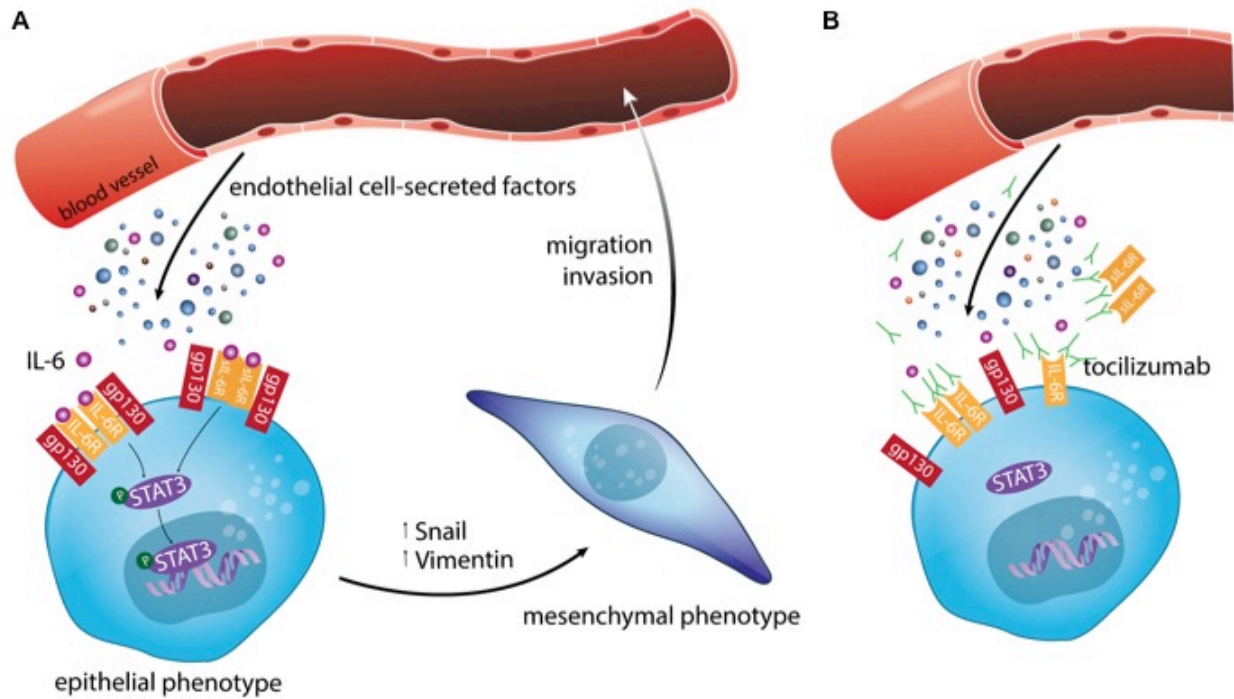

Figure 5.1. Proposed schematic model of the endothelial cell-secreted IL-6 and cancer stem cell mesenchymal cell-related protein expression. A, Endothelial cells secrete multiple factors, including IL-6. IL-6 pathway is activated by either binding to membrane-bound IL-6R and soluble IL-6R (sIL-6R). Downstream signaling pathway, STAT3, is activated and induces transcription of downstream genes. Such activation results in increase in Snail and Vimentin expression, allowing the cancer stem cells to acquire mesenchymal phenotype with increased motility. B, IL-6 pathway inhibition, such as tocilizumab, can prevent IL-6 pathway activation, resulting in cancer stem cells with inactivation of STAT3 and decrease in migration.

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