ENDOTHELIAL CELL - STEM CELL CROSSTALK IN HEAD AND NECK CANCER

by

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DEDICATION

To my parents, and my husband
for believing in me when I didn't….

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ABSTRACT

Head and neck squamous cell carcinomas contain a sub-population of highly tumorigenic cells that exhibit self-renewal and multipotency. These cells can be isolated from primary head and neck squamous cell carcinomas using Aldehyde Dehydrogenase (ALDH) activity and CD44 expression, and have been named head and neck cancer stem cells (HNCSC). It has been proposed that the HNCSC are the “drivers” of head and neck tumor progression, and therefore have to be eliminated to achieve cancer cure. However, little is known about mechanisms underlying the survival and self-renewal of HNCSC. The hypothesis underlying this dissertation is that head and neck cancer stem cells (*i.e.* ALDH+CD44+) are localized in the perivascular niche and depend on endothelial cell-secreted factors for their survival and self-renewal.

Here, we observed that HNCSC are found in close proximity to blood vessels in primary head and neck squamous cell carcinomas. Endothelial cell-secreted factors promoted the proliferation, self-renewal and survival of HNCSC *in vitro,* as evidenced by the increase in number of orospheres (i.e. non-adherent colonies of cells) formed in soft agar or ultra-low attachment plates. *In vivo*, selective ablation of tumor endothelial cells caused a marked decrease in the fraction of HNCSC suggesting that cancer stem cells depend on intact perivascular niches for their survival.

In our search for a possible mechanism to explain the dependency of HNCSC on endothelial cells, we observed that cancer stem cells have a constitutively active IL-6R/STAT3 pathway, while tumor endothelial cells secrete high levels of IL-6. Inhibition of endothelial cell IL-6 caused a decrease in the self-renewal and tumorigenicity of the HNCSC. Notably, a humanized antibody to IL-6R delayed tumor initiation and decreased the survival of HNCSC *in vivo*. Collectively, these results unveil the endothelial cell-secreted factors as critical regulators of HNCSC survival and self-renewal, and suggest that patients with head and neck cancer might benefit from targeted therapies against cancer stem cells.

CHAPTER I

INTRODUCTION

Cancer Cell Biology: Clonal vs Cancer Stem Cell

Tumors are not insular masses of proliferating cells (Hanahan and Weinberg, 2000). Instead, a tumor can be seen as an “organ” composed of transformed cells that interact with stromal cells within the tumor microenvironment (Figure. 1.1). The understanding of cancer as a complex tissue where tumor cells rely on interactions with stromal cells to progress towards malignancy and overcome host defenses has been solidified by extensive research in the last decade (Hanahan and Weinberg, 2011). Collectively, this work suggested that not all tumor cells are equal. Indeed, malignant cells with different tumorigenic potential have been found in many tumor types. The possibility of identifying those cells with higher tumorigenic potential, and selectively eliminating them, is the clinical rationale underlying this work.

According to the stochastic hypothesis, tumor cells are homogeneous, *i.e.* every cancer cell has equal propensity to initiate and propagate tumors and metastasize. In effect, it means that there is no selectivity between the cancer cells in a tissue; each cell has the same potential to initiate tumors. The

heterogeneity in tumors is explained by spontaneous shifts in cell phenotypes (Albers *et al.*, 2011). However, emerging evidence from a variety of tumors suggests an alternative explanation called the “cancer stem cell” hypothesis. It states that the tumor tissue has a distinct hierarchy of cells and only a small sub-population of cells within the tumor is capable of initiating cancers. These are called cancer stem cells or “tumor-initiating cells” (Reya *et al*., 2001). The bulk of the tumor tissue, however, is composed of rapidly proliferating cells called transit-amplifying cells and post-mitotic differentiated cells, which do not contribute to tumor initiation. These cells are derived from the cancer stem cells by differentiation but do not cause tumor initiation by itself.

Following these two alternate models of study, extensive work has been done to understand them in different tumors. Recently, it was demonstrated in melanomas that about 25% of unselected melanoma cells were able to create tumors in immunocompromised mice, suggestive of a more stochastic model (Quintana *et al.,* 2008). However, in recent literature, evidence to the contrary has been seen. CD271positive cells in melanomas were able to create tumors more likely than the negative cells supporting the cancer stem cell hypothesis (Civenni *et al*., 2011). In another independent study, CXCR6+ cells created more aggressive tumors than not (Taghizadeh *et al*., 2010). This suggests the existence of primitive melanoma cells capable of phenotypic plasticity, self-renewal and immune evasion (Girouard and Murphy, 2011). A number of features of HNSCC can also be explained using the stochastic model. The existence of large pre-neoplastic areas beyond the surgical margins results in

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

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

In the late 1970's however, the focus of cancer biology shifted to the concept of clonal evolution as mutations in oncogenes and tumor suppressors were found to cause human cancers. Tumor cell populations were considered genetically unstable and acquisitions of genetic mutations created human

malignancies. The step-wise genetic mutations that certain genes acquired in colon cancer was well documented by the Vogelstein laboratory (Fearon *et al*., 1990). However, in the early nineties, technological advances allowed for studies that again suggested a hierarchy of tumorigenic potential of tumor cells. Such studies were facilitated with the advent of technologies such as the Fluorescence Activated Cell Sorting system (FACS), which enabled researchers to study specific cell surface markers in individual cells and use these markers to sort pre-determined sub-populations of cells. For example, FACS helped in identifying that leukemic engraftment was possible only with CD34+CD38- expressing cells (Bonnet *et al.,* 1997). Xenograft assays further demonstrated that only one in a million cells was capable of initiating tumors in acute myeloid leukemia (Lapidot *et al.,* 1994). Notably, the hierarchical concept and the step-wise concept are not mutually exclusive. Rather, these two theories may have a different impact in different stages of tumor progression.

Cancer Stem Cell Hypothesis

It is well known that most human tissues (*e.g.* brain, skin, and intestine) contain stem cells (Blainpain *et al.,* 2004; Vries *et al*., 2010). The cancer stem cell hypothesis is fundamentally based on the application of stem cell concepts derived from embryogenesis to understanding of the tumorigenic process. The following are key features of the cancer stem cell hypothesis: “(1) only a small fraction of the cancer cells within a tumor have tumorigenic potential when transplanted into immunodeficient mice; (2) the cancer stem cell sub-population can be separated from the other cancer cells by distinctive surface markers; (3)

tumors resulting from the cancer stem cells contain the mixed tumorigenic and non-tumorigenic cells of the original tumor; and (4) the cancer stem cell sub-population can be serially transplanted through multiple generations, indicating that it is a self-renewing population" (Prince and Ailles, 2008). Therefore, cancer stem cells are capable of self-renewal and differentiating into other distinctive cells that make up the tumor mass (Reya *et al.,* 2001).

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

Head and neck squamous cell carcinomas

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

Several risk factors have been associated with the development of HNSCC. The most common among them are carcinogen exposure, tobacco use, oral hygiene and family history. Tobacco, however, is the single most common risk factor for HNSCC (Brennan *et al.,* 1995, Ho *et al.,* 1997, Zhang *et al.,* 2000). Alcohol consumption as a risk factor is most commonly seen to be amplifying the

effects of tobacco in a synergistic manner (Talamini *et al.,* 2002). In recent years, the role of human papillomavirus as a causative agent in HNSCC has increased tremendously (Gillison *et al.,* 2000, Mork *et al.,* 2001, Herroro *et al*., 2003).

The initiation and progression of head and neck cancers has been thought for a long time to be an effect of acquisition of various genetic and epigenetic alterations (Califano *et al.,* 1996). In fact, 40 – 60% of patients with HNSCC present with a mutation of the p53 gene (Hollstein *et al.,* 1991, Poeta *et al.,* 2007). In more recent years, the epidermal growth factor receptor (EGFR) has been found to be highly expressed in more than 95% of HNSCC and has been associated with poorer prognosis (Kalyanakrishna and Grandis, 2006). These genetic alterations led credence to the clonal evolution or stochastic hypothesis in HNSCC until recently.

Cancer stem cells in head and neck tumors

In a landmark publication, Prince and collaborators (2007) unveiled the presence of highly tumorigenic, stem-like cells, in HNSCC. They analyzed HNSCC using FACS sorting for expression of CD44, and were able to generate tumors with as few as 5×10^3 cells of CD44+ cells whereas higher numbers of CD44- cells failed to create tumors. They were also able to show that the resultant xenografts were heterogeneous (Prince *et al.,* 2007). These data lend support to the concept that HNSCC follows the cancer stem cell hypothesis, where sub-populations of cancer cells have significantly higher tumorigenic potential than others.

Identification of cancer stem cells in head and neck tumors

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

Prince *et al*. (2007) demonstrated that CD44 serves as a cancer stem cell marker in HNSCC. CD44, a cell surface glycoprotein functions as receptor for hyaluronic acid, is involved in cell adhesion and migration (Gao *et al.,* 2011). 20 out of 30 implantations of CD44+ cells were able to create tumors in immunodeficient mice, whereas only one of 40 implantations of CD44-cells generated tumors (Prince *et al.,* 2007). Following this work, several independent groups confirmed that CD44 either alone or in combination has the properties of cancer stem cell marker and being a tumor initiator (Baumann *et al.,* 2010; Chikamatsu *et al*., 2011). Emerging literature is revealing a role for CD44 in tumor metastasis. Indeed, it has been described that certain forms of CD44 (*i.e.* v3, v6, v10) are associated with tumor progression and metastatic spread of HNSCC (Wang *et al.*, 2009). It has also been shown that CD44+ cells express high levels of Bmi-1 (Prince *et al*., 2007), a self-renewal protein found in embryonic stem cells (Bracken *et al.,* 2006).

The transmembrane glycoprotein CD133 has also been investigated as a putative marker for cancer stem cells (Wu *et al*., 2009). CD133 or Prominin-1 originally found on neuroepithelial stem cells in mice, was first isolated in humans from the hematopoietic stem cells by a monoclonal antibody recognizing a specific epitope called the AC133 (Shmelkov *et al.,* 2005). In some HNSCC cell lines (*e.g.* hep-2), CD133+ cells were found to have increased clonality, *i.e.* the ability to form clones of cells from a single cell in suspension, when compared to CD133-cells (Zhou *et al*., 2007). Oral cancer-enriched cells from cell lines and primary tumors were found to have an increased expression of CD133 and displayed increased migration, and tumorigenicity (Chiou *et al.,* 2008). In fact, correlation of Oct-4, Nanog and CD133 status showed a poorer prognosis of oral cancer patients with increased CD133 expression (Chiou *et al*., 2008). Recently, CD133+ cells were found to possess increased clonogenicity, invasiveness and tumorigenicity as compared to CD133- cells along with resistance to paclitaxel (Zhang *et al*., 2010).

Aldehyde Dehydrogenase (ALDH) is an intracellular enzyme that is involved in converting retinol to retinoic acid (Sobreira *et al.,* 2011). In cancer, ALDH+ cells were initially identified in breast and brain (Ginestier *et al.,* 2007; Rasper *et al.,* 2010). In these tumors, ALDH+ cells were characterized as highly tumorigenic cells that can self-renew, which are hallmarks of cancer stem cells. In HNSCC, ALDH enriches for cancer stem cells and is involved in epithelial to mesenchymal transition (EMT) (Chen *et al.,* 2009). Interestingly, a recent report

demonstrated that as few as 500 ALDH+ cells were able to create tumors unlike the ALDH- cells (Clay *et al.,* 2010).

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

Stem cell niche

Physiological stem cells and cancer stem cells depend on their immediate microenvironment or niche for their survival and function (Borovski *et al*., 2011). The niche provides cues that regulate proliferative and self-renewal signals thereby helping cancer stem cells maintain their undifferentiated state (Kuhn and Tuan, 2010). The non-epithelial stromal cells, inflammatory cells, and the vasculature have been proposed as key components of the niche that support and sustain cancer stem cells (Fuchs *et al*., 2004). Based on this knowledge, cancer stem cells are being targeted for therapy using their niche. Recently, it was shown that hematopietic stem cell niche could be targeted for metastatic bone tumors (Shiozawa *et al*., 2011).

In HNSCC, studies are required to elucidate the existing niches. Endothelial cells present in the perivascular niche provide critical survival and self-renewal cues for cancer stem cells in glioblastomas (Calabrese *et al*., 2007).

Therapeutic implications and significance of cancer stem cells in HNSCC

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

In Figure 1.2, we propose a hypothetical model for the response of HNSCC to different therapeutic strategies. HNSCC is represented as a complex tissue where the cancer stem cells constitute a relatively small number of cells that are capable of undergoing self-renewal and differentiating into a complex and heterogeneous tumor. Conventional chemotherapeutic drugs are successful in de-bulking the tumor. However, it is proposed that slow-growing cancer stem

cells evade conventional therapies and, with passage of time, these cells are activated and regenerate tumors locally or at distant sites (Figure 1.2A). This might explain the relatively high recurrence rates in patients with HNSCC. On the other hand, targeting of the cancer stem cells either directly (Figure 1.2B) or via their niche (Figure 1.2C) could lead to a more definitive cure, as the cancer stem cells are the putative drivers of recurrence and metastatic spread. An emerging concept is the combined use of conventional chemotherapy and cancer stem cell-targeted therapy. This drug combination is appealing since such strategy could potentially allow for tumor de-bulking (with conventional drugs) and prevention of recurrence/metastases (cancer stem cell-targeted drugs).

Extensive work is being done to understand the molecular mechanisms that might be playing a role in the pathobiology of cancer stem cells but not in normal cells, which would allow specific targeting of pathological cells. One such target could be Bmi-1, present in high levels in cancer stem cells (Hayry *et al.,* 2010). Several other signaling pathways (*e.g.* Wnt, PTEN, Notch, Hedgehog) are also currently being explored as potential therapeutic targets (Pannuti *et al.,* 2010; Takahashi-Yanaga and Kahn 2010; Takazaki *et al*., 2011). Various laboratories are isolating cancer stem cells and performing gene array analyses to understand how cancer stem cells might be differentially regulated compared to the rest of the tissue. Certain early studies suggest the role of micro-RNA like the Let7, Micro-RNA-200 in cancer stem cells to be an important player (Lo *et al*., 2011; Yu *et al*., 2011). The ability to selectively target cancer stem cells, while

sparing normal stem cells, will be critical for the future application of the cancer stem cell therapy in the clinic.

Another important conceptual strategy for targeted elimination of cancer stem cells is through the disruption of their supportive niche. In glioblastoma models, the use of anti-angiogenic therapies correlated with a decrease in cancer stem cell fraction (Calabrese *et al.,* 2007). Collectively, these results demonstrate that by interfering with the cancer stem cell microenvironment (*i.e.* the niche); one can compromise the ability of these cells to survive and/or to behave as a stem cell. These data suggest that therapies like anti-angiogenic agents might have the unexpected, yet most welcome, effect of decreasing the presence of highly tumorigenic cancer stem cells. However, recent evidence demonstrated that anti-angiogenic therapy (especially anti-VEGF based therapy) causes a malignant progression of tumors resulting in increased tumors and metastasis due to increased tumor cell infiltration (Paez-Ribes *et al*., 2009; Keunen *et al.,* 2011). More studies are thereby necessary to understand and develop specific targeting molecules against cancer stem cells.

Challenges facing head and neck cancer stem cell research

One of the biggest challenges in cancer stem cell research has been development of methods for culture, expansion, and analyses of undifferentiated cancer cells *in vitro*. The property of surviving in suspension (anchorage independence) has been used for this purpose (Jensen and Parmar, 2006). The method of enriching for cancer stem cells using sphere generation under low

attachment conditions has been proposed and used in various cancer models like the breast, neural and prostate (Dontu *et al.,* 2003, Pastrana *et al*., 2011). In this case, putative cancer stem cells are cultured either in a matrix-based assay like soft agar or in low attachment plates.

Despite intense research in the area of cancer stem cell biology in recent years, the understanding of the impact of cancer stem cells to the pathobiology of HNSCC is still quite primitive. One of the reasons for this is the need to perform most studies with primary HNSCC specimens, which are difficult to obtain. There is still controversy over the existence of cancer stem cells in cell lines, despite the fact that independent reports have shown cancer stem-like cells in cell lines (Harper *et al.,* 2007; Gammon *et al.,* 2011). In addition, the expansion of cancer stem cells is frequently performed *in vivo*, which is time consuming and expensive. However, existing *in vitro* methods offer limited capacity for expansion of cancer cells in an undifferentiated state. It has become clear that the development of improved methods for isolation and expansion of head and neck cancer stem cells is imperative for the acceleration of the pace of discovery in this area.

Statement of purpose

More than half a million new cases of HNSCC are diagnosed every year world-wide. Despite extensive research, the 5-year survival rate for these patients has been low and has not significantly improved over the last couple of decades. The discovery of a small sub-population of cells that possess

exquisitely high tumorigenic potential, associated with the possibility of identifying these cancer stem cells in clinical settings, provides a new conceptual target for cancer therapy. It is well known that a frequent cause of failure of conventional therapy in HNSCC is the high incidence of local recurrence and distant metastasis. Notably, it has been hypothesized that conventional therapies do not eliminate the slow growing cancer stem cells, which appear to be the “drivers” of tumor recurrence and metastases. Therefore, the recent observation that HNSCC follows the cancer stem cell hypothesis suggests that targeted elimination of these tumor-initiating cells might prevent tumor regrowth and distant disease. However, little is known about the cancer stem cells in HNSCC. The role of the niche and possible mechanisms by which the niche can promote the survival of the cancer stem cells in HNSCC has been completely unexplored. The overall goal of our study was to further characterize the existence of cancer stem cells in HNSCC, and the micro-environments it exists in. Emphasis was laid on trying to understand possible molecular mechanisms which might promote the survival of the cancer stem cells for possible therapeutics.

In this dissertation, we try to establish ALDH+CD44+ cells as cancer stem cells in HNSCC with increased tumorigenicity and self-renewal and develop *in vitro* assays to study them in Chapter II. We tested the tumorigenicity of these cells and explore the possible micro-environments or niches that they reside in HNSCC. The observation that the endothelial cells promoted active proliferation and survival of the cancer stem cells in Chapter III led us to explore the signaling pathway in this interaction. We studied using gene silencing techniques and

transgenic mouse models, the role of the inflammatory cytokine, IL-6 in the interaction between the endothelial cells and the head and neck cancer stem cells. More specifically, we were able to demonstrate that perturbation of the IL-6-IL-6R signaling pathway with a humanized antibody delayed the tumorigenicity of the head and neck cancer stem cells by decreasing their survival in Chapter IV.

Hypothesis

The central hypothesis addressed in this dissertation is that head and neck cancer stem cells exist in vascular niches and that endothelial cell initiated signaling pathways promote the self-renewal and tumorigenicity of the cancer stem cells in head and neck cancers.

Specific Aims

The following specific aims were addressed to answer the central hypothesis:

- **Specific Aim 1:** To develop novel *in vitro* “Orosphere assays” to study and propagate head and neck cancer stem cells i.e., ALDH+CD44+cells (Chapter II).
- **Specific Aim 2:** To identify a peri-vascular niche and to study the effects of endothelial cell-initiated signaling on the survival and self-renewal of head and neck cancer stem cells (Chapter III).

- **Specific Aim 3:** To study the effect of endothelial cell initiated IL-6 signaling on the self-renewal, survival and tumorigenicity of cancer stem cells (Chapter IV)

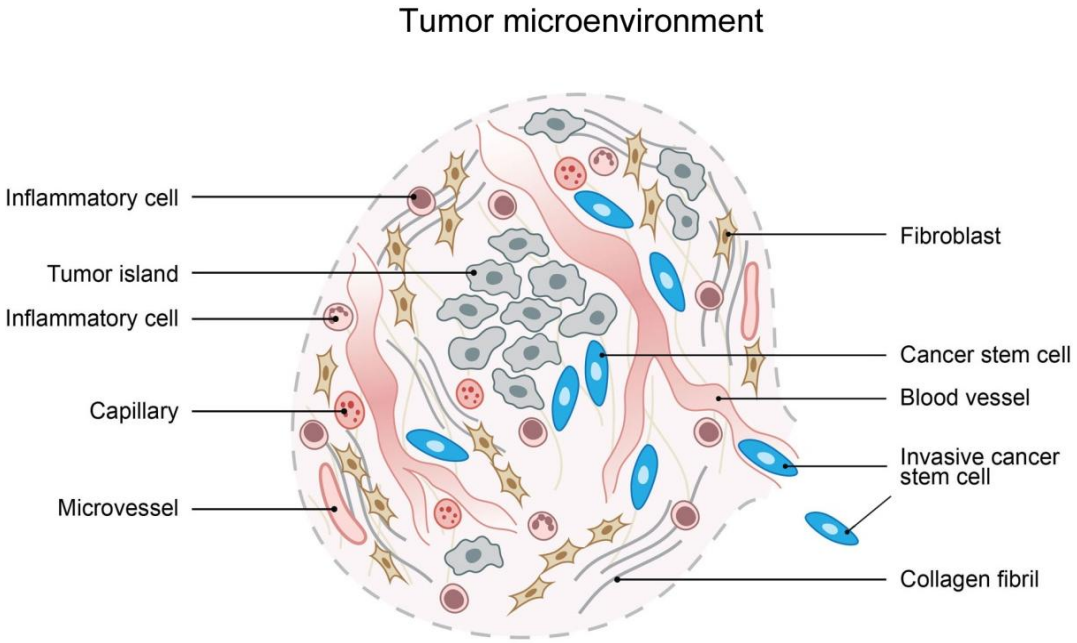

Figure 1.1. Cancer tissue is a complex "organ". The tumor tissue microenvironment is composed of a variety of cells including tumor cells, cancer stem cells, inflammatory cells, cancer-associated fibroblasts along with blood vessels. The cancer stem cells are rare cells found primarily in the invasive edge of tumors in supportive niches.

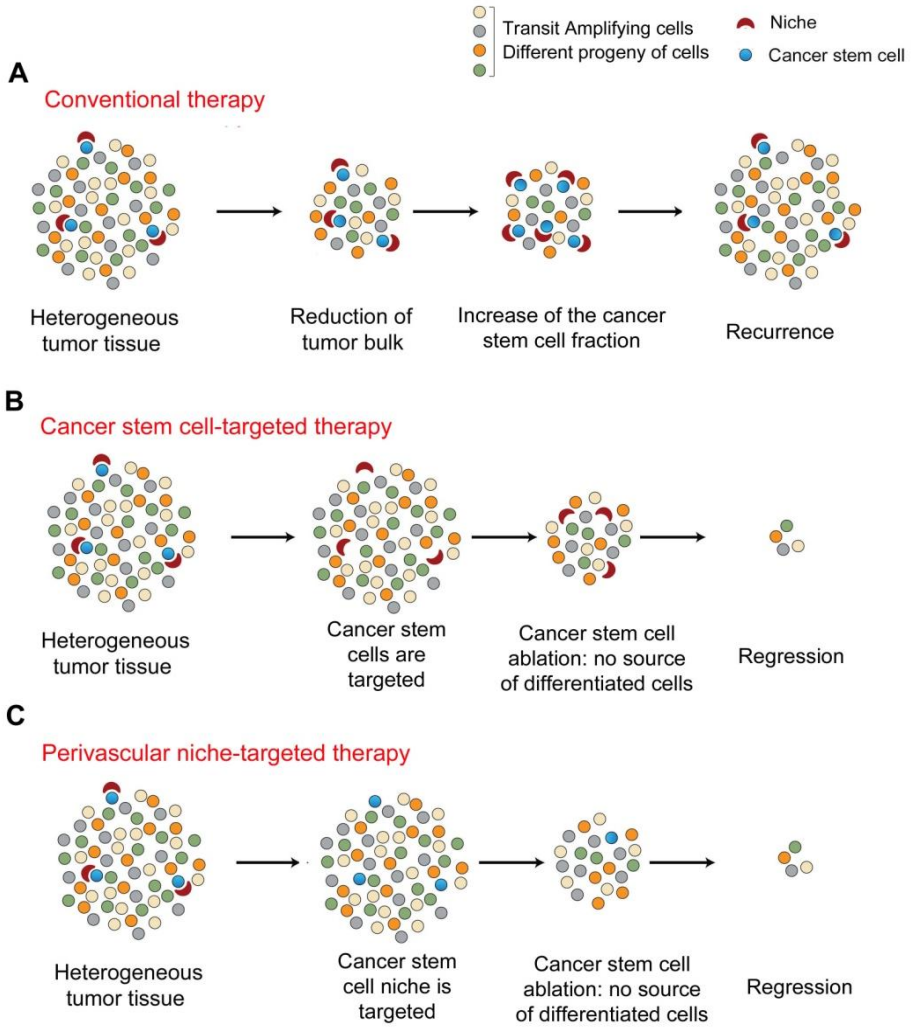

Figure 1.2. Possible implications of the cancer stem cell hypothesis to therapy. **(A),** Conventional chemotherapy targets primarily the highly proliferative cells that constitute the bulk of the tumor. With suitable microenvironments, the cancer stem cells proliferate and the tumor recurs. **(B)**, Direct cancer stem cell targeting or **(C),** indirect cancer stem cell targeting via disruption of (*e.g.*) their perivascular niche can potentially eliminate cancer stem cells. Ablation of the stem cells may inhibit the regeneration of the tumor and ultimately result in tumor regression.

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CHAPTER II

OROSPHERE ASSAY: A METHOD FOR PROPAGATION OF HEAD AND NECK CANCER STEM CELLS

Abstract

Recent evidence suggests that head and neck squamous cell carcinomas (HNSCC) harbor a small sub-population of highly tumorigenic cells, named cancer stem cells. A limiting factor in cancer stem cell research is the intrinsic difficulty of expanding cells in an undifferentiated state *in vitro*. Here, we describe the development of the orosphere assay, a method for the study of putative head and neck cancer stem cells. An orosphere is defined as a non-adherent colony of cells sorted from primary HNSCC or from HNSCC cell lines and cultured in 3-D soft agar or ultra-low attachment plates. Aldehyde dehydrogenase (ALDH) activity and CD44 expression were used here as stem cell markers. This assay allowed for the propagation of head and neck cancer cells that retained stemness and self-renewal. The orosphere assay is well suited for studies designed to understand the pathobiology of head and neck cancer stem cells.

Introduction

The cancer stem cell hypothesis provides a plausible mechanism for tumor recurrence and metastatic spread (Mimeault *et al*., 2010). According to the cancer stem cell hypothesis, a small sub-population of cancer cells is highly tumorigenic, capable of self-renewal and multipotency (Reya *et al*., 2001). Cells with such features may constitute the “drivers” of the tumorigenic process. If this hypothesis were indeed true for head and neck squamous cell carcinomas (HNSCC), selective targeting of these cancer stem cells would be essential to improve patient outcomes. Following the discovery of cancer stem cells in HNSCC (Prince *et al.,* 2007), investigators throughout the world have begun studies to understand the pathobiology of these cells. The development and optimization of a method for *in vitro* expansion of head and neck cancer stem cells in an undifferentiated state would be beneficial for the progress of research in this area, and hopefully will accelerate the process of developing improved treatment modalities for HNSCC.

Two cardinal properties of stem cells allow for their identification and purification: A) Self-renewal, *i.e.* the ability of stem cells to self-perpetuate; and B) Multipotency, *i.e.* the ability of cells to undergo differentiation and generate the complex cellular components observed in a tissue/organ or in cancer (Rudland *et al*., 1998, Weissman 2000, Morrison *et al*., 1997). It is possible to maintain human head and neck cancer stem cells in an undifferentiated state by serially passaging them *in vivo*, in immuno-deficient mice (Prince *et al.,* 2007). However, this strategy is time consuming and expensive. Furthermore, it is difficult to

perform mechanistic studies of signaling pathways involved in the biology of cancer stem cells exclusively in animal models. A third property of stem cells, *i.e.* the ability to form spheres and grow under low attachment conditions (Dontu *et al.,* 2005), inspired the development of *in vitro* assays for the study of normal and cancer stem cells.

Exploiting the fact that stem cells possess anchorage independence, *i.e.* the ability to survive and proliferate in suspension cultures unlike the non-stem cells (Reynolds and Weiss, 1996, Dontu *et al*., 2003), adherent-free culture conditions have been proposed as basis for *in vitro* assays for propagation of cancer stem cells. Suspension cultures have been utilized as a method to study stem cell properties in several tumor types, including those of the breast and brain (Reynolds and Weiss, 1992, Dontu *et al.,* 2003, Hemmati *et al.,* 2003). Most of these suspension cultures are done in 3-dimensional structures, such as soft agar matrices or dishes coated with fibronectin or matrigel (Miskon *et al*., 2010, Stenberg *et al*., 2011, Denning *et al.,* 2006). These strategies allow for stem cell expansion and proliferation making them a valuable assay for self-renewal. However, the setup of these cultures is technically challenging, and the intrinsic difficulty associated with the retrieval of these cells from their matrix makes this method not ideal when mechanistic studies involving serial passaging, flow cytometry or gene expression analyses, are required. In an attempt to address such issues, the culture of cells in low-attachment plates has been proposed as an alternative strategy to deprive cells from anchorage, while

facilitating their retrieval of cells for further analysis (Deleyrolle *et al.,* 2009, Dev *et al.,* 2009, Zhang *et al.,* 2011).

Fluorescence Activated Cell Sorting (FACS) and magnetic bead sorting are common approaches for the identification and isolation of putative stem cells (McLelland *et al.,* 2011, de Wynter *et al*., 1995). Using FACS, we observed that the fraction of putative cancer stem cells in primary HNSCC is small (Chapter III). Here, we describe a method for the propagation of head and neck cancer stem cells named the orosphere assay. The name reflects the fact that this method was optimized for studies of stem cells sorted from tumors or cell lines derived from the oral cavity and head and neck region. This method enables the expansion of cancer stem cells in an undifferentiated state by culturing them in ultra-low attachment plates or in 3-D soft agar matrices. The use of ultra-low attachment plates allowed for serial passaging of cells (*i.e.* demonstration of self-renewal), and for the retrieval of cells for mechanistic studies.

Materials and methods

Sorting and culture of head and neck cancer stem cells

Head and neck squamous cell carcinoma cells (UM-SCC-74A, UM-SCC-74B; gift from Dr. Carey, University of Michigan) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen; Grand Island, NY, USA), 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin. The identity of the tumor cell lines was confirmed by genotyping at the University of Michigan DNA sequencing core facility. Alternatively, putative cancer stem cells were isolated from a primary tumor, as described. Briefly, informed consent was obtained from an 81-year old female patient prior to surgical removal of a squamous cell carcinoma from the floor of the mouth. The specimen was cut into small pieces, minced until they passed through a 25 ml pipette tip, and suspended in a 9:1 solution of DMEM-F12 (Hyclone, Waltham, MA, USA) containing collagenase and hyaluronidase (Stem Cell Technologies; Vancouver, BC, Canada). The mixture was incubated at 37^0C for one hour and passed through a 10-ml pipette every 15 minutes for mechanical dissociation. Cells were filtered through a 40-µm nylon mesh (BD Falcon; Franklin Lakes, NJ, USA), washed with low glucose DMEM (Invitrogen) containing 10% FBS, and centrifuged at 800 rpm for 5 minutes. Single cell suspensions obtained from primary specimens (as well as from HNSCC cell lines) were washed, counted, and re-suspended at 10^6 cells/ml PBS. The Aldefluor kit (Stem Cell Technologies) was used to identify cells with high ALDH activity. Briefly, cells were suspended in activated Aldefluor substrate (BAA) or in DEAB (specific ALDH inhibitor) for 45 minutes at 37^0C. Then, cells

were exposed to anti-CD44 antibody (clone G44-26BD; BD Pharmingen; Franklin Lakes, NJ, USA) and lineage markers eliminated (*i.e.* anti-CD2, CD3, CD10, CD16, CD18; BD Pharmingen). Viable cells are identified with 7-Aminoactinomycin (7-AAD, BD Pharmingen). FACS (Fluorescence Activated Cell Sorting) sorted cells were cultured in low glucose DMEM (Invitrogen), 10% fetal bovine serum, and 100 U/ml Penicillin-streptomycin in low attachment conditions, as described below. Cells were defined as putative head and neck cancer stem cells (ALDH+CD44+) or control cells (ALDH-CD44-). To induce cell differentiation, FACS-sorted cells were cultured in regular tissue culture plates (BD Falcon). Studies were done in triplicate specimens per condition, and experiments were performed at least three independent times to verify reproducibility of the data.

Orospheres in ultra-low attachment plates

FACS-sorted cells ($5x10^3$ cells/well) were seeded in 6-well ultra-low attachment plates (Corning; New York, NY, USA) and cultured in low glucose DMEM, 10% fetal bovine serum and 100 U/ml Penicillin-streptomycin at 37^0C and 5% CO_2. Orospheres were arbitrarily defined as a non-adherent colony of at least 25 cells. Orospheres can be mechanically dissociated into single cell suspensions and then re-seeded in new ultra-low attachment plates to generate secondary and tertiary orospheres (indicative of self-renewal).

Orospheres in soft agar

Alternatively, orospheres can be generated using low melting point agarose (Invitrogen). 6-well regular attachment plates (Fisher) were pre-coated

with a layer of 1.2% agarose mixed with equal volume of 2x DMEM (Invitrogen) to make an inert basal layer. This layer is solidified at room temperature for 30 minutes. Then, 500 FACS-sorted cells/well were resuspended in 2x DMEM, 2.5% FBS, mixed with equal volumes of 0.6% agarose. After the second agarose layer gelifies at room temperature for 45 minutes, 500 µl low glucose DMEM (Invitrogen) is added onto the surface of the 3-dimensional matrix and cells are incubated at 37^0C thereafter. Usually the orospheres in soft agar are visualized after 7 days. Quantification of the number of orospheres/well is done under light microscopy.

Immunocytochemistry

For immunocytochemistry, $2x10^3$ FACS-sorted cells/well were cultured in LabTek II Chamber Slide (Thermo Scientific; Rochester, NY, USA) for up to 7 days. Antigen retrieval was performed using Dako Retrieval solution (S1699; Carpinteria, CA, USA) with gradual warming up from 40^0C to 98^0C within 40 minutes. Slides were incubated in 3% hydrogen peroxidase for 10 minutes. Primary antibodies against Cytokeratin 17 (1:200; Abcam, ab2502; San Francisco, CA, USA) or Involucrin (1:200; Abcam ab27496) were incubated at 4^0C overnight. Following a 20-minute incubation with appropriate secondary antibodies, the Romulin AEC Chromogen Kit (Biocare Medical; Concord, CA, USA) was used to visualize the proteins.

Immunofluorescence and confocal imaging

For confocal imaging, $2x10^3$ FACS-sorted cells/well were seeded in a 24-well ultra-low attachment plate for 3 days (Corning). Orospheres were fixed in

cold 10% buffered formalin (Fisher; Pittsburgh, PA, USA) for 30 minutes. For immunofluorescence, primary antibodies were pre-labeled with Alexafluor 488 or 594 using Zenon labeling kit (Molecular Probes, Z25007, Z25102; Invitrogen). Primary antibodies, *i.e.* anti-ALDH1 (1:50; BD Biosciences, 61195; Franklin Lakes, NJ); CD44 (1:200; Abcam, ab51037) were added directly to the plate and incubated at 4^0C overnight. Orospheres were transferred to LabTek II Chambered Coverglass (Thermo Scientific) and mounted with Prolong Gold anti-fade mounting medium with DAPI (Invitrogen). Confocal imaging was performed using Leica Inverted Confocal SP5X (Leica; Los Angeles, CA, USA). Post-processing was done with NIH Image J software.

Statistical analyses

One-way ANOVA followed by appropriate post-hoc tests was performed using the SigmaStat 2.0 software (SPSS, Chicago, IL). Statistical significance was determined at $P<0.05$.

Results

We show in Chapter III ALDH+CD44+ cells sorted from primary HNSCC exhibit self-renewal and are more tumorigenic than control ALDH-CD44- cells. Such features characterize the ALDH+CD44+ cells as putative head and neck cancer stem cells. Here, we describe the characterization and optimization of a method that was developed to propagate and to evaluate the stem cell properties of cells derived from primary head and neck tumors or from HNSCC cell lines. Single cell suspensions were prepared from freshly dissected human HNSCC, or from HNSCC cell lines. Cells were sorted for high/low ALDH activity (Aldefluor kit) and CD44 expression. A representative flow sorting of the head and neck cancer stem cells from a primary human HNSCC is shown in Figure 2.1, wherein the percentage of lineage-negative viable cancer stem cells (ALDH+CD44+) is 0.97%, while the percentage of lineage-negative viable non-cancer stem cells (ALDH-CD44-) is 3.09%. After flow sorting, cells were cultured under low attachment conditions to form non-adherent spheres named orospheres. Orospheres were arbitrarily defined as non-adherent colonies of at least 25 cells. To generate these orospheres, we optimized conditions for HNSCC cells cultured either in ultra-low attachment plates or in soft agar 3-D matrices. While orospheres can be readily seen within 3 days in ultra-low attachment plates, it takes approximately 7 days to generate orospheres in soft agar (Figure 2.1B, 2.1C). Notably, the orospheres shown here were generated either from cells sorted from a single primary human HNSCC (Figure 2.1B), or from a head and neck cancer cell line, *i.e.* UM-SCC-74A (Figure 2.1C).

To begin to understand the biology of the cells forming the orospheres, we cultured them for 3 days in ultra-low attachment plates and visualized the expression of the two stem cell markers used to sort the cells initially (ALDH1 and CD44) by confocal microscopy (Figure 2.2A). To determine if the culture of putative cancer stem cells in low attachment represents a self-renewal method resulting in stem cell expansion, and not just an aggregation of stem-like cells, we seeded a single ALDH+CD44+ cell/well in 96-well ultra-low attachment plate and monitored its clonal expansion for 5 days (Figure 2.2B). We observed that a higher number of individual clones were formed by the putative cancer stem cells (ALDH+CD44+) when compared to control ALDH-CD44- cells ($P<0.05$). A clone was defined as a colony of at least 10 cells, starting from a single cell.

To evaluate if the orosphere assay is a valid method for testing self-renewal of head and neck cancer stem cells, we cultured orospheres generated from ALDH+CD44+ cells or control cells for 3 days under low attachment conditions. Then, the orospheres were mechanically dissociated and re-seeded as single cell suspension in new ultra-low attachment plates. This process was repeated serially to generate secondary and tertiary orospheres (Figure 2.2C). This experiment revealed two general trends: A) More orospheres were generated from the ALDH+CD44+ than from the control cells over time, demonstrating the self-renewal of the putative cancer stem cells. And, B) A progressive decrease in the overall number of orospheres was observed between the primary and the tertiary passage.

We wanted to ascertain that suspension culture in low attachment plates was the reason for the continued enrichment of cancer stem cells, and that ALDH+CD44+ cells do retain their stemness over time. We therefore cultured ALDH+CD44+ cells in regular attachment conditions or in ultra-low attachment plates. FACS analysis revealed the maintenance of an increased percentage of ALDH+CD44+ cells when culturing in ultra-low attachment conditions as compared to regular attachment plates (Figure 2.2D). The reverse experiment was performed to evaluate if the same putative head and neck cancer stem cells (ALDH+CD44+) would lose their stemness and differentiate when cultured in regular attachment plates. This analysis was performed by immunostaining for Cytokeratin 17 (an epithelial cell marker) and Involucrin (a differentiated cell marker) (Aragaki *et al.,* 2010, Balasubramanian *et al*., 2007). We observed that on Day 0, the ALDH+CD44+ cells were more spherical and expressed high levels of Cytokeratin 17 and low levels of Involucrin (Figure 2.3A, 2.3B). By Day 7, the ALDH+CD44+ cells became more elongated and reversed the expression levels of Cytokeratin 17 and Involucrin.

Discussion

The orosphere assay is conceptually derived from suspension cultures developed to study normal or cancer stem cells from tissues such as the brain, breast, or prostate (Reynolds and Weiss, 1992, Dontu *et al.,* 2003, Reynolds *et al.,* 2006, Guzman-Ramirez *et al.,* 2009). Pioneer work from Reynolds and Weiss demonstrated that cells dissected from the striatum of the adult mouse brain could be cultured as free-floating spheres and exhibited stem cell properties (Reynolds and Weiss, 1992, Reynolds *et al*., 2009). The Wicha laboratory characterized human mammary stem/progenitor cells from reduction mammoplasties based on their anchorage independence and survival in low attachment plates (Dontu *et al.,* 2003). These seminal findings provided the conceptual framework for the development of sphere-based assays as a means to propagate cancer stem cells in an undifferentiated state *in vitro.* Here, we describe a method in which putative cancer stem cells are sorted from heterogeneous HNSCC primary tumors or from established HNSCC cell lines. These putative cancer stem cells differentiate under regular attachment conditions and generate heterogeneous tumor cell monolayers within a few days. On the other hand, the same cells cultured in low attachment conditions are capable of retaining stem-like cell properties (Figure 2.4). Notably, the method described here is clearly inspired by the existing protocols from other tumor types, but was optimized for use in head and neck tumor models.

One of the critical challenges facing stem cell studies is the definition of markers that discriminate highly tumorigenic cells (cancer stem cells) from cells

that possess low tumorigenic potential. Mounting evidence suggests that stem cell markers are tumor-specific, and that CD44, CD133, and ALDH are emerging as useful markers in HNSCC. Seminal work from the Prince laboratory used CD44 expression as a marker for the identification of a sub-population of highly tumorigenic stem cells in primary HNSCC (Prince *et al.,* 2007). More recently, it was shown that CD44+ cells sorted from a HNSCC cell line cultured in uncoated dishes formed tumor spheres that were resistant to chemotherapeutic drugs (Okomato *et al.,* 2009). CD133, a transmembrane glycoprotein, is considered a putative marker for cancer stem cells in head and neck tumors. CD133 positive cells sorted from HNSCC cell lines or primary tumors showed enhanced clonality and tumorigenicity when compared to control cells (Zhou *et al.,* 2007, Chiou *et al.*, 2008, Zhang *et al.,* 2010). Alternatively, ALDH activity, which was initially characterized as a useful stem cell marker in breast cancer (Ginestier *et al*., 2007), was also validated in head and neck tumor models (Chen *et al*., 2009, Clay *et al.*, 2010). Of note, since most markers are expressed in both normal and pathologic stem cells, it is plausible that the combination of markers may enhance one's ability to identify cancer stem cells from complex primary tumor tissues. Indeed, it has been recently observed that the combination of ALDH activity and CD44 expression further discriminates a small sub-population (<3%) of cells in primary HNSCC that exhibit stem-like properties and are highly tumorigenic.

As with most methods, the orosphere assay has its inherent limitations, as follows: A) The overall number of orospheres decreases upon serial passaging;

and B) The percentage of ALDH+CD44+ cells is higher in ultra-low attachment plates than in regular culture plates, but it decreases over time. Collectively, these findings suggest that there might be a certain degree of cell differentiation even in low attachment conditions *in vitro.* Although these limitations can be overcome by expanding cancer stem cells *in vivo*, (Prince *et al*., 2007) such strategy makes the process of propagating cells in an undifferentiated state labor and animal intensive, and expensive. While the “orosphere” assay has the advantages of being technically simple, reproducible, and relatively inexpensive, one must remain mindful of the limitations of the assay and interpret the results with caution. Ideally, the orosphere assay should be used for cell and molecular biology studies that are verified in appropriate animal models.

We described here the protocols for generating orospheres in either soft agar 3-D matrices or in ultra-low attachment plates. Careful consideration should be given to the advantages and disadvantages of each method, before selecting the best approach for a specific experimental question. The soft agar method is more time consuming. One has to pre-coat the plate with a layer of agarose, wait for its gelification, apply a second layer containing both agarose and cells, wait again, and finally cover the 3-D gel with culture medium. Along the same lines, it takes about 7 days to generate orospheres in soft agar, while it takes only 3 days in ultra-low attachment plates. In addition, the soft agar approach does not allow for retrieval of the cells for mechanistic studies (*e.g.* flow cytometry, gene expression analyses) or for serial passage studies (*e.g.* to evaluate self-renewal properties). As a potential advantage though, the soft agar assay tends to be a

more rigorous testing of stem cell properties. We observed that non-cancer stem cells do not survive well under these conditions and do not readily form orospheres. On the other hand, the culture of undifferentiated cells in ultra-low attachment plates is simpler, since there is no need for coating and gelification steps. This culture condition is highly suitable for the retrieval of cells for serial passage or for mechanistic studies. Knowing the pros and cons of both strategies should direct the decision process towards selecting the soft agar or the ultra-low attachment approach.

The field of cancer stem cell biology has attracted much attention in recent years due to the discovery that these cells may drive the progression of certain tumor types, including HNSCC. As such, the emergence of targeted therapy against cancer stem cells could have a significant impact on the survival of head and neck cancer patients. We believe that the development and characterization of methods to propagate and study the behavior of cancer stem cells *in vitro* may ultimately contribute to the discovery of mechanism-based therapies for head and neck squamous cell carcinoma.

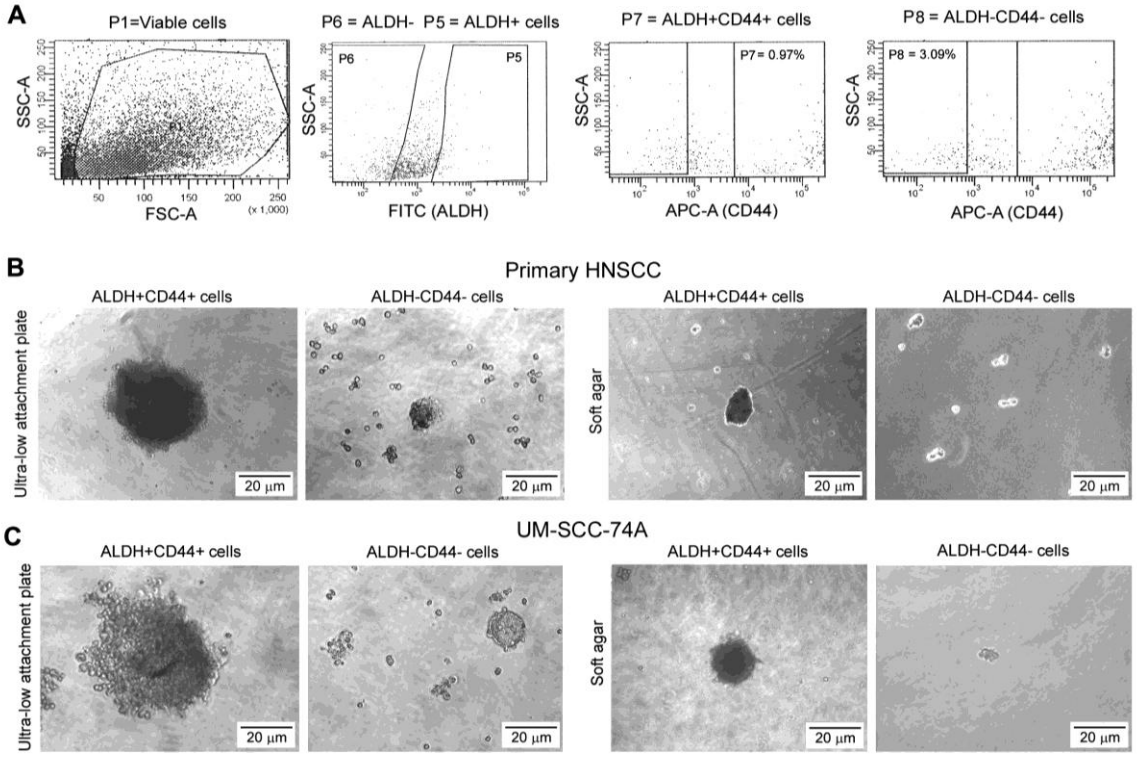

Figure 2.1. ***In vitro*** **propagation of putative head and neck cancer stem cells in orospheres. (A)** Representative flow cytometry sorting of putative cancer stem cells from a primary human head and neck squamous cell carcinoma. Shortly after surgery, single cell suspensions were prepared by digestion of the tumor specimen with collagenase and hyaluronidase. Viable cells (P1) were isolated using 7AAD and are gated for positivity (after eliminating lineage cells) to ALDH (P5), using DEAB (ALDH inhibitor) as reference. ALDH-negative cells are found in P6. The cells were then gated against CD44 in sequence to select ALDH+CD44+Lin- (P7=0.97%) and ALDH-CD44-Lin- (P8=3.09%). **(B,C)** Representative photomicrographs of orospheres arising from ALDH+CD44+ and ALDH-CD44- cells sorted from a primary HNSCC and UM-SCC-74A and cultured either in ultra-low attachment plates (**B**) or in 3-D soft agar matrices (**C**).

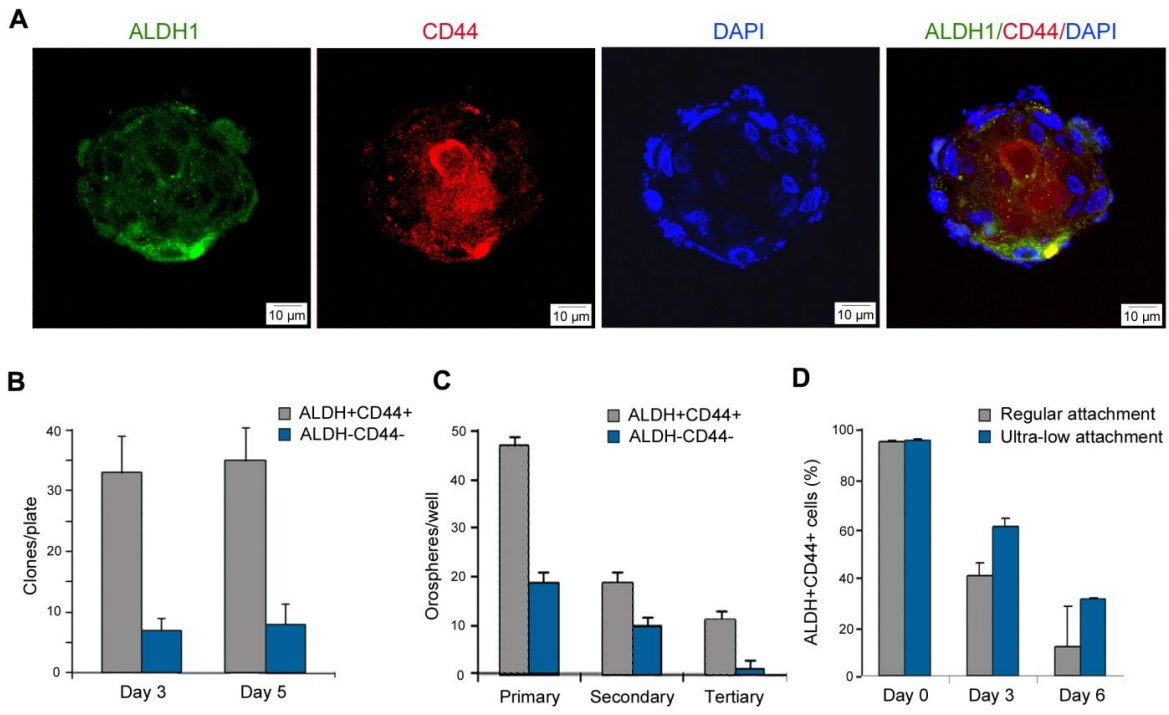

Figure 2.2. **Characterization of stem cell properties of orospheres. (A)** Confocal microscopy of an orosphere generated from the UM-SCC-74B cell line and stained for the stem cell markers ALDH1 (green) and CD44 (red), along with nuclei staining with DAPI (blue). **(B)** Graph depicting the number of clones arising from one individual cancer stem cell (ALDH+CD44+) or non-cancer stem cell (ALDH-CD44-) per well of a 96-well ultra low attachment plate. **(C)** Graph depicting the number of orospheres generated from serial passage assays that evaluate self-renewal of putative cancer stem cells (ALDH+CD44+) or control cells (ALDH-CD44-). **(D)** Graph depicting the percentage of ALDH+CD44+ cells (FACS) over time when cultured in regular attachment or ultra-low attachment conditions.

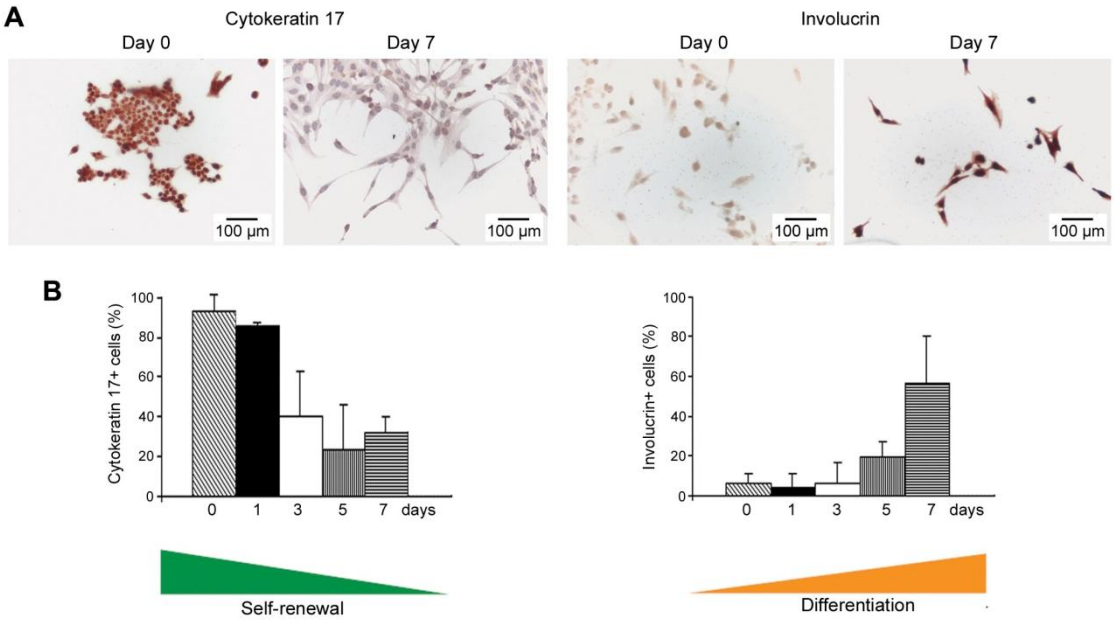

Figure 2.3. **Characterization of the differentiation of ALDH+CD44+ cells cultured in regular attachment plates. (A)** Representative photomicrographs of Cytokeratin 17 and Involucrin immunostaining of ALDH+CD44+ cells cultured under regular attachment conditions for one week. **(B)** Graph depicting the percentage of cells cultured in regular attachment plates and that stained positive for Cytokeratin 17 or Involucrin over time.

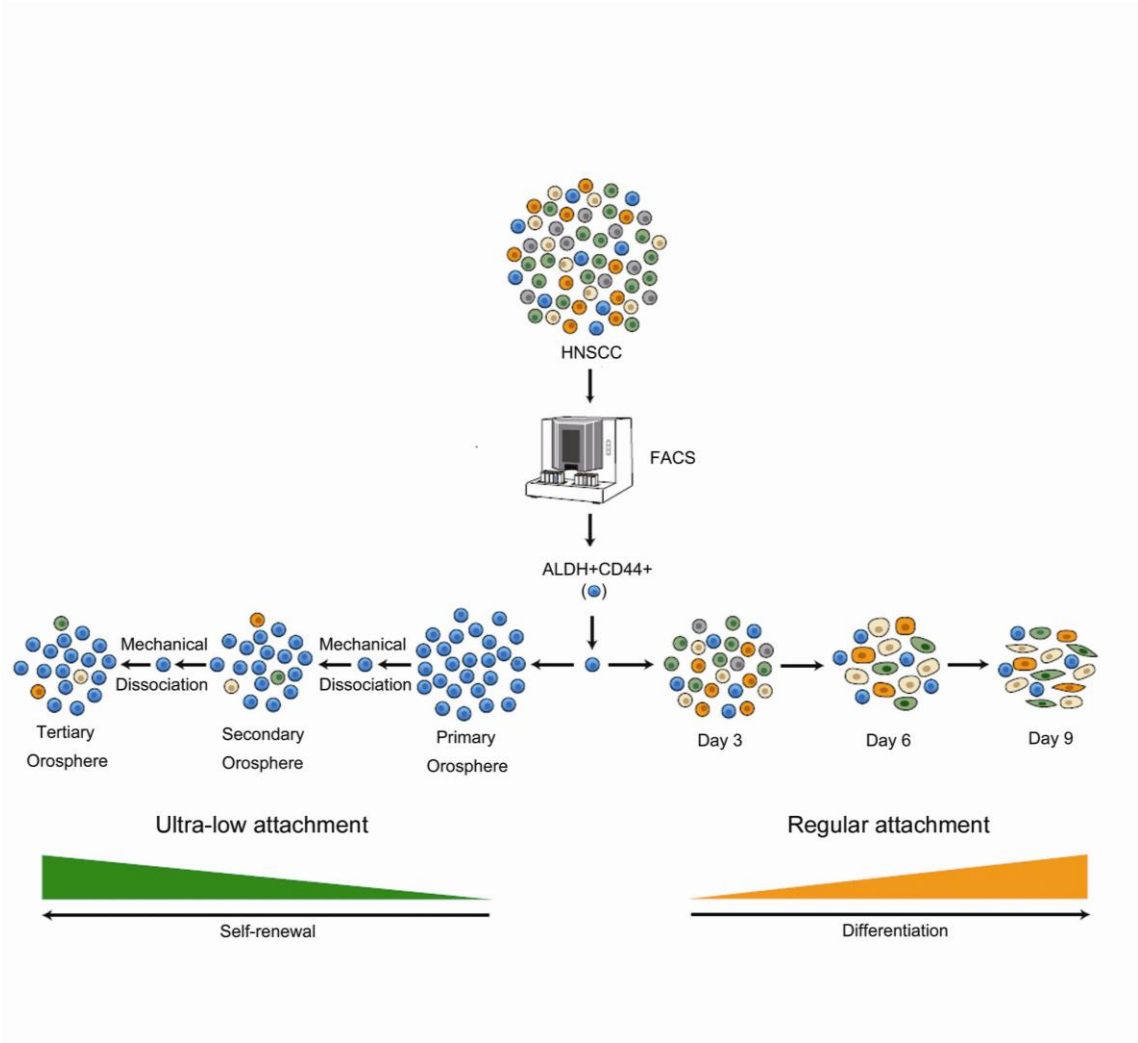

Figure 2.4. Diagram illustrating the *in vitro* propagation of putative head and neck cancer stem cells using the orosphere assay. Single cell suspensions are prepared from head and neck squamous cell carcinomas and sorted for stem cell markers, such as ALDH and CD44. The putative cancer stem cells can be serially passaged and expanded in ultra-low attachment conditions using the orosphere assay. Alternatively, these cells can be differentiated when cultured in regular attachment conditions generating a heterogeneous cancer cell line.

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CHAPTER III

ENDOTHELIAL CELL-INITIATED SIGNALING PROMOTES THE SURVIVAL AND SELF-RENEWAL OF CANCER STEM CELLS

Abstract

Recent studies have demonstrated that cancer stem cells play an important role in the pathobiology of head and neck squamous cell carcinomas (HNSCC). However, little is known about functional interactions between head and neck cancer stem-like cells (CSC) and surrounding stromal cells. Here, we used Aldehyde Dehydrogenase activity and CD44 expression to sort putative stem cells from primary human HNSCC. Implantation of 1,000 CSC (ALDH+CD44+Lin-) led to tumors in 13 (out of 15) mice, while 10,000 non-cancer stem cells (NCSC; ALDH-CD44-Lin-) resulted in 2 tumors in 15 mice. These data demonstrated that ALDH and CD44 select a sub-population of cells that are highly tumorigenic. The ability to self-renew was confirmed by the observation that ALDH+CD44+Lin- cells sorted from human HNSCC formed more spheroids (orospheres) in 3-D agarose matrices or ultra-low attachment plates than controls and were serially passaged *in vivo*. We observed that approximately 80% of the CSC was located in close proximity (within 100-μm radius) of blood vessels in human tumors, suggesting the existence of perivascular niches in HNSCC. *In*

vitro studies demonstrated that endothelial cell-secreted factors promoted self-renewal of CSC, as demonstrated by the up-regulation of Bmi-1 expression and the increase in the number of orospheres as compared to controls. Notably, selective ablation of tumor-associated endothelial cells stably transduced with a caspase-based artificial death switch (iCaspase-9) caused a marked reduction in the fraction of CSC in xenograft tumors. Collectively, these data demonstrated that endothelial cell-initiated signaling enhances the survival and self-renewal of head and neck cancer stem cells.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer type, accounting for more than 500,000 new cases each year in the world (Chin *et al*., 2006). The integration of platinum-based chemotherapy to the curative management of HNSCC resulted in an improvement in the control of local-regional disease and enhanced organ preservation (Forastiere, 2008). However, as the control of local-regional disease improved, the incidence of distant metastatic disease has risen (Gendel *et al*., 2003, Sano *et al*., 2007). As a result, the overall survival rate for patients with HNSCC has not improved significantly over the last 30 years and continues to be one of the lowest among the major cancer types. This clinical observation suggests that by creating a non-favorable local environment for head and neck tumor cells with current therapies, these cells acquire a more aggressive phenotype leading to distant metastasis. Better understanding of the pathobiology of HNSCC is urgently needed for the development of more effective therapies.

Cancer stem cells (CSC) constitute a sub-population of cells that are multi-potent, self-renewing, and capable of generating the entire heterogeneous population seen in tumors (Reya *et al.,* 2001, Tu *et al*., 2009, Setoguchi *et al.,* 2004, Gupta *et al*., 2009). Cancer stem cells are believed to “drive” tumorigenesis of some cancer types, including breast and head and neck tumors (Prince *et al*., 2007, Cho *et al*., 2008, Shackleton *et al*., 2009). This implies that the successful growth of a metastasis of tumors that follow the cancer stem cell model requires that at least one cancer stem cell resists to therapy (Hill *et al*.,

2009). Notably, cancer stem cells are slow-dividing cells that are capable of resisting to current therapies for cancer (Hermann *et al.,* 2010).

Stem cells and cancer stem cells are frequently found in unique microenvironments called the “niche” (Fuchs *et al.,* 2004, Moore *et al.,* 2006). Cell-to-cell interactions through direct contact or secreted factors support the survival and maintain the stemness of stem cells in cancer and in normal tissues (Lobo *et al*., 2007). Perivascular niches have been identified in neural stem cells (Shen *et al.,* 2004, Sugiyama *et al.,* 2006, Veeravagu *et al.,* 2008) and neural tumors (Calabrese *et al.,* 2007). However, it is not known if the stem cells of head and neck tumors are localized in close proximity to blood vessels and depend on interactions with the cellular components of vascular niches for their survival and stemness.

Head and neck cancer stem cells were first identified using CD44 (Prince *et al*., 2007), a marker of stem cells in epithelial tumors (Al-Hajj *et al.,* 2003, Li *et al.,* 2007). Aldehyde Dehydrogenase (ALDH), an enzyme found to be highly active in stem cells of various origins (Corti *et al.,* 2006, Ginestier *et al.,* 2007, Huang *et al.,* 2009), was recently used to identify stem cells in HNSCC (Chen *et al.,* 2009). Here, we utilized ALDH1 and CD44 to identify a sub-population of cells that exhibit several properties of cancer stem cells, including self-renewal and capacity to regenerate heterogeneous tumors. Analysis of human HNSCC demonstrated that the majority of the cancer stem cells are located in close proximity to blood vessels. Using 3-D models *in vitro,* we showed that endothelial cell-secreted factors promote proliferation and self-renewal of HNCSC along with

increased expression levels of Bmi-1. Notably, selective ablation of tumor-associated endothelial cells with a caspase-based artificial death switch resulted in a significant decrease in the number of cancer stem cells *in vivo*. Collectively, these data unveil the functional interdependency of cancer stem cells and vascular endothelial cells in head and neck tumors, and show proof-of-principle evidence that therapeutic targeting of tumor blood vessels reduces the number of CSC.

Materials and Methods

Cell Culture

Head and neck squamous cell carcinoma cell lines (UM-SCC-1, UM-SCC-74A, UM-SCC-74B, UM-SCC-17A, UM-SCC-17B, UM-SCC-11B; gift from Dr. Carey, University of Michigan) were cultured in Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen; Grand Island, NY, USA) and human dermal microvascular endothelial cells (HDMEC; Cambrex, Walkersville, MD) in endothelial cell growth medium-2 (EGM2-MV; Lonza, Walkersville, MD, USA). FACS sorted cells were cultured in low glucose DMEM, 10% fetal bovine serum (Invitrogen) and 100 U/ml Penicillin-streptomycin (Invitrogen) or 100 U/ml Antibiotic Antimycotic Solution (AAA) (Sigma; St. Louis, MO, USA) in ultra-low attachment plates (Corning; New York, NY, USA). Conditioned Medium (CM) from HDMEC was collected in serum free DMEM from 24-hour cultures. HDMEC stably transduced with iCaspase-9 (HDMEC-iCaspase-9) were generated as described (Nör *et al.*, 2002). The identity of all tumor cell lines was confirmed by genotyping at the University of Michigan DNA sequencing core facility.

Head and neck cancer stem cell sorting

Informed consent was obtained from patients undergoing surgery for removal of HNSCC. The tumors were obtained within 30 minutes post-surgery and transported in DMEM Low Glucose, 10%FBS, AAA to 4^0C immediately. Within 12 hours, the tumors were cut into small pieces and minced with a sterile

scalpel until they could pass through a 25 ml pipette tip. They were suspended in a 9:1 solution of DMEM-F12 (Hyclone, Waltham, MA) containing collagenase and hyaluronidase (Stem Cell Technologies; Vancouver, BC, Canada). The mixture was incubated at 37^0C for one hour and passed through a 10-ml pipette every 15 minutes for mechanical dissociation. Cells were filtered through a 40-μm nylon mesh (BD Falcon; Franklin Lakes, NJ, USA), washed with low glucose DMEM containing 10% FBS, and centrifuged at 800 rpm for 5 minutes. Single cell suspensions obtained from primary specimens (as well as from cell lines or xenografts) were washed, counted, and re-suspended at 1×10^6cells/ml PBS. The Aldefluor kit (Stem Cell Technologies) was used to identify cells with high ALDH activity. Briefly, cells were suspended with activated Aldefluor substrate (BAA) or the negative control (DEAB, a specific ALDH inhibitor) for 45 minutes at 37^0C. Then, cells were exposed to anti-CD44 (clone G44-26BD; BD Pharmingen; Franklin Lakes, NJ, USA) and lineage markers (*i.e.* anti-CD2, CD3, CD10, CD16, CD18; BD Pharmingen). The sorted cells were cultured overnight and either implanted in mice or set up for *in vitro* experiments. Mouse cells were identified using anti-H2Kd antibody (BD Biosciences; Franklin Lakes, NJ, USA) and eliminated. Anti-CD31 (Biolegend; San Diego, CA, USA), anti-HA (Sigma) and 7-Aminoactinomycin (7-AAD, BD Pharmingen) were used in selected experiments. Propidium iodide staining followed by flow cytometry was used for the identification of apoptotic cells (*i.e.* sub-G_0/G_1 cells). Here and throughout this manuscript, studies were done in triplicate specimens per condition and time point, and three experiments were performed to verify reproducibility of the data.

Confocal Microscopy

Frozen sections were treated with peroxidase (Dako, CA) and the antigen retrieval solution (Dako) was used for 45 minutes at 90˚C. The anti-ALDH1 (BD Biosciences), CD44 (Abcam, Cambridge, MA, USA) and Factor VIII (Neomarkers; Fremont, CA, USA) were pre-labeled with Alexafluor 488 or 594 as described using the Zenon labeling kit (Invitrogen) and confocal imaging was performed using Zeiss confocal (Carl Zeiss; Thornwood, NY, USA). Z–stacked images of 8 individual images were generated. The deconvolution was done using Autoquant (Media Cybernetics; Bethesda, MD, USA) and the 3-D reconstruction completed using the Imaris Software (Bitplane AG; Zurich, Switzerland).

Colony formation assay and orospheres

Colony formation assays were performed in 3-D suspension cultures, as described (Ginestier *et al.,* 2007, Dontu *et al.,* 2003). Orospheres (*i.e.* spheroids of HNSCC-derived cells) were generated from $5x10^3$ cells cultured in triplicate in ultra-low attachment plates (Corning). Alternatively, cells were mixed with 0.3% agarose and layered on plates that were pre-coated with a layer of 0.6% agarose. Cells were maintained in low glucose DMEM containing or not conditioned medium from HDMEC at a ratio of 3:1. Orospheres generated in ultra-low attachment plates were mechanically dissociated into single cell suspensions and replated to generate secondary and tertiary cultures.

SCID mouse model of human tumor angiogenesis

Xenograft tumors vascularized with functional human microvessels were generated in severe combined immunodeficient (SCID) mice (CB 17 SCID, Taconic; Germantown, NY, USA), as described (Nör *et al.,* 2001). Briefly, 1,000 CSC (ALDH+CD44+Lin-) or 10,000 NCSC (ALDH-CD44-Lin-) were seeded with HDMEC for a total of $1x10^6$ cells in poly-(L-lactic) acid (PLLA; Medisorb, Nicosia, Cyprus) biodegradable scaffolds. Bilateral scaffolds were implanted subcutaneously in the dorsum of each mouse. Mice were monitored daily for tumor growth for 6 months or until the volume of the tumor reached 0.85 cm^3. Alternatively, mice received scaffolds containing $9.99x10^5$ HDMEC-iCaspase-9 and $1x10^3$ CSC, or controls. 24 days after transplantation of the scaffolds, mice received daily intra-peritoneal injections of 2 mg/kg AP20187 (ARIAD; Cambridge, MA, USA) for 4 days to activate iCaspase-9 and selectively ablate tumor blood vessels, as described (Nor *et al.,* 2002).

Immunohistochemistry

Immunostaining for ALDH1 (BD Biosciences, 61195, 1:100), Factor VIII (Neomarkers, 1:500), pancytokeratin (Dako, clone A E1/E3 3515; 1:500) was performed, and DAB or AEC substrate was used to develop the color. Factor VIII and ALDH1 quantification was done in 6 random areas of 6 sections in 8 individual tumors in a 400X magnification. The ALDH1-positive cells of all sections were added and expressed as a percentage for each patient and the

average of the 8 patients was calculated. Isotype matched immunoglobulins were used as negative controls.

Proliferation Assays

For proliferation experiments, $2x10^3$ cells (ALDH+CD44+ and ALDH-CD44-) were cultured in 24-well ultra-low attachment plates and treated with control or endothelial cell (HDMEC) conditioned medium every 2 days for a week. WST-1 (Roche) reagent was added, incubated for 2 hours and read off the spectrophotometer (Genious; Tecan). Results were normalized against initial plating density. Experiments were done in triplicate wells per condition, and each graph is representative of three independent experiments.

Western Blots

40,000 ALDH+CD44+ or ALDH-CD44- cells were plated in 6-well ultra-low attachment plates, serum starved overnight, and treated with endothelial cell conditioned medium (CM) for 0-24 hours. Western blots were performed with rabbit anti-human Bmi-1 (Millipore, 05-637) or mouse anti-human caspase-9 (Cayman Laboratory).

Generation of HDMEC-icaspase9-HA cells

These cells were generated, as described (Nör *et al.,* 2002). Briefly, HDMEC was transduced with either iCaspase-9 (HA tagged) or LXSN (empty retroviral vector control) and selected for G418 for a minimum of 2 weeks. Expression of iCaspase-9 was checked with Western Blots as described above.

Statistical Analyses

T-test or one-way ANOVA followed by post-hoc analyses was performed using the SigmaStat 2.0 software (SPSS, Chicago, IL). Statistical significance was determined at $P < 0.001$ (unless otherwise specified).

Results

ALDH+CD44+Lin- cells retrieved from primary head and neck squamous cell carcinomas are highly tumorigenic. CD44 was used as the marker for stem cells in the study that demonstrated the existence of stem cells in head and neck squamous cell carcinomas (Prince *et al.,* 2007). However, a relatively large proportion of cells were found to be positive for CD44 in that study. Here, we tested the combined use of ALDH activity and CD44 expression in different combinations to identify cells that have a cancer stem-like phenotype (Figure 3.1A) and ALDH+CD44+ cells were more stem like. Confocal microscopy of ALDH-positive cells (green) was seen to co-localize with some of the CD44 (red) cells in a primary HNSCC patient (Figure 3.1 B). The efficacy of tumor take was compared when putative CSC cells (ALDH+CD44+Lin-) or non-CSC cells (ALDH-CD44-Lin-) were implanted in immuno-deficient mice (Figure 3.2A). Single cell suspensions were prepared from 4 different patients with primary human head and neck squamous cell carcinomas immediately after surgical resection (Table 3.1). Viable cells were selected with 7-AAD (P1) and then gated in sequence for ALDH activity and CD44 expression, after elimination of lineage (Lin) cells (Figure. 3.2A). We observed that 1.78% of the cells were ALDH+CD44+Lin- (putative CSC) and 8.7% were ALDH-CD44-Lin- (NCSC) in a representative tumor (HN 10) (Figure. 3.2A, Table 3.1). To evaluate the tumorigenicity of these cells, 1,000 ALDH+CD44+Lin- or 10,000 ALDH-CD44-Lin- (10-fold more cells) were co-implanted with human endothelial cells to generate human xenograft tumors vascularized with human blood vessels in

immuno-deficient mice, as described (Nor *et al.,* 2001). 13/15 implants generated palpable tumors (tumors that are bigger than the scaffold volume), in the ALDH+CD44+Lin- group as compared to 2/15 of the ALDH-CD44-Lin- group, demonstrating that the combination of ALDH and CD44 allowed for selection of a highly tumorigenic sub-population of cells (Figure 3.2B). To evaluate their capacity of self-renewal, viable tumor xenografts were retrieved at six months or when their volume reached 850 mm^3, processed into single cell suspensions, and serially transplanted to other mice. All implants containing ALDH+CD44+Lin- cells generated secondary tumors, whereas none of the implants containing ALDH-CD44-Lin- cells generated tumors (Figure 3.2B). The tumor volumes of the primary and secondary xenografts generated with ALDH+CD44+Lin- cells was higher than with the ALDH-CD44-Lin- cells ($p < 0.001$) at the end of the experimental period (Figure 3.2C). Notably, the fraction of the putative CSC (ALDH+CD44+Lin-) in the primary and secondary xenografts remained low and comparable to the fraction of these cells in the primary human tumors (Figure 3.2D). This observation along with the fact that the primary xenografts on digestion yielded both ALDH+CD44+ cells and ALDH-CD44- cells as well as the heterogeneity of tumors and being capable of being serially implanted into generating secondary xenografts indicated that the ALDH+CD44+Lin- cells exhibit features of multipotency.

Xenografts generated with ALDH+CD44+Lin- cells resemble the primary tumors. The histological organization of the primary and secondary xenograft tumors generated from the ALDH+CD44+Lin- cells was comparable to the

primary tumor from which these cells were retrieved (Figure 3.3A). In contrast, most of the implants seeded with ALDH-CD44-Lin- did not generate tumors. And, in the few instances (2 out of 15 implants) that tumors were generated from these cells, they were structurally disorganized and had smaller tumor islands as compared to xenografts generated from ALDH+CD44+Lin- cells (Figure 3.3A). The epithelial origin of the tumor xenografts was confirmed by positive immunostaining for pancytokeratin (Figure 3.3B). Analysis of the localization of the stem-like cells (ALDH-positive; arrows) within these xenografts revealed that the majority of these cells were found within 100-µm of blood vessels (Figure 3.3C,D). This observation led us to a more in depth analysis of the localization of stem-like cells in primary tumors.

Head and neck cancer stem-like cells exhibit perivascular localization. In oral mucosa, the small sub-population of ALDH-positive cells was found primarily in the basal layer of the squamous epithelium (Figure 3.4A), the expected localization of stem cells in this tissue. In HNSCC, the ALDH-positive cells were seen in tumor islands, in close proximity to blood vessels (Figure 3.4A). To assess the relative percentage of potential stem cells in oral mucosa and HNSCC, we prepared single cell suspensions and sorted them for ALDH and CD44. The average proportion of ALDH+CD44+ was 0.2% (0.2%, 0.43%, 0.316%) average in oral mucosa and 2.62% in HNSCC (2.86%, 3.15%, 1.85%) (from 3 different patients). Confocal microscopy and 3-D image reconstruction were used to evaluate the spatial relationship between ALDH-positive cells and blood vessels in 8 patients with HNSCC (Figure 3.4B-D). An area with 100-µm

radius around each blood vessel was selected as representative of the "perivascular" area, since this is the approximate area of diffusion of oxygen and nutrients around vessels (Janssen *et al.,* 2002). We observed that the majority of the CSC (*i.e.* approximately 80%) was found in the perivascular area in human HNSCC (Figure 3.4D).

Endothelial cell-derived growth factor milieu promotes proliferation, survival, and self-renewal of HNCSC. To understand whether endothelial cell-secreted factors have a direct functional effect on HNCSC, we studied the effect of endothelial cell conditioned medium on proliferation, survival and self-renewal of ALDH+CD44+ selected from a panel of established head and neck tumor cell lines *in vitro*. The proliferation of both, ALDH+CD44+ and ALDH-CD44- cells cultured in low attachment conditions was enhanced by exposure to endothelial cell conditioned medium (Figure 3.5A). The increase in cell numbers may also be attributed to an enhancement in survival mediated by the endothelial cell-derived factors (Figure 3.5B).

Due to the difficulty in obtaining primary HNSCC, we performed a series of *in vitro* experiments using an established panel of cell lines (Table 3.2). To evaluate the self-renewal potential of CSC, we plated them in agarose and observed the formation of colonies in a three-dimensional culture condition (Figure 3.8). We observed the formation of sphere-like colonies developed from single cells (Figure 3.6A and B), using a method inspired by the work on "mammospheres" (Dontu *et al.,* 2003). These colonies derived from head and neck tumor stem-like cells were named "orospheres". The number of colonies

generated from ALDH+CD44+ cells was greater than ALDH-CD44- cells ($P < 0.001$), using cells sorted from three established HNSCC cell lines, *i.e.* UM-SCC-17A, UM-SCC-1 and UM-SCC-74A (Figure 3.6A). To evaluate the behavior of these cells over time, the orospheres were dissociated and passed twice. While the overall number of orospheres decreased over time, the ALDH+CD44+ group persistently presented higher number of orospheres than the control group over three serial passages *in vitro* (Figure 3.6B). Notably, ALDH+CD44+ cells strongly express the marker of self-renewal Bmi-1, as compared to control ALDH-CD44- cells (Figure 3.6C). In the same experiment, we observed that endothelial-cell secreted factors enhances expression of Bmi-1 in ALDH+CD44+ cells over time, indicating an inductive effect of these factors on the self-renewal properties of the CSC (Figure 3.6C).

To understand the effect of endothelial cells on self-renewal of stem cells, and to ensure comparability of these established cell lines with primary samples, we isolated ALDH+CD44+Lin- cells from primary HNSCC, and performed the orosphere assay with primary cells. A 3-fold increase in the number of orospheres was observed in the group treated with endothelial cell conditioned medium ($P < 0.001$), as compared to untreated controls (Figure 3.7A). These results were verified in experiments performed with 6 additional head and neck tumor cell lines (Figure 3.7B; Figure 3.8). Notably, the inductive effect of endothelial cell-secreted factors on the number of orospheres generated from ALDH+CD44+ cells was maintained during 3 serial passages *in vitro* (Figure 3.7C).

Selective ablation of blood vessels reduces the proportion of cancer stem cells. A critical question that remained unanswered was if the effects of endothelial cells on the survival and self-renewal of head and neck tumor stem cells was also observable *in vivo*. To address this question, we utilized two complementary approaches. Firstly, serial dilutions (*i.e.* 1000, 100, 10 and 1 ALDH+CD44+Lin- cells;) from a primary HNSCC, were prepared, seeded in the scaffolds with or without HDMEC, and implanted in the subcutaneous space of immunodeficient mice. Co-implantation of endothelial cells and CSC resulted in larger tumors than implantation of CSC by themselves (Figure 3.10). Secondly, endothelial cells were stably transduced with a caspase-based artificial death switch (iCaspase-9) (Figure 3.9A). This unique approach allows for selective elimination of endothelial cells transduced with iCaspase-9 upon activation by the dimerizer drug AP20187 and ablation of tumor vasculature *in vivo* (Nor *et al.,* 2002, Dong *et al*., 2007). Here, we showed that treatment with AP20187 induces apoptosis of HDMEC-iCaspase-9, but not untransduced cells (*i.e.* ALDH+CD44+ cells) (Figure 3.9B). As expected, AP20187-induced apoptosis of the iCaspase-9-transduced endothelial cells resulted in a significant reduction in the microvessel density of tumors retrieved from mice injected with AP20187, as compared to vehicle-treated controls (Figure 3.9C). Notably, the proportion of ALDH+CD44+Lin- cells within the xenograft tumors was significantly reduced when endothelial cells were selectively ablated by activation of iCaspase-9, as compared to vehicle-treated controls (Figure 3.9D).

Discussion

The poor survival and high recurrence rates in patients with HNSCC demand a re-assessment of the pathobiology of these cancers. Here, we showed that head and neck cancer stem cells reside in perivascular niches. Notably, we observed that endothelial cell-secreted factors have a major impact on the self-renewal and survival of cancer stem cells. These data suggest that therapeutic targeting of the tumor endothelium may reduce the rate of head and neck tumor recurrence and metastasis by decreasing the proportion of cancer stem cells.

Prince and colleagues (2007) reported that CD44+ cells from primary HNSCC exhibit a cancer stem-cell phenotype, and are capable of initiating tumors at low numbers (Prince *et al.,* 2007). ALDH1 has been recently described as a putative marker for CSC in head and neck tumors (Chen *et al.,* 2009). Here, we demonstrate that the combination of ALDH1 and CD44 selects a sub-population of cells with properties of cancer stem cells than if used as single markers. One thousand ALDH+CD44+ cells were capable of initiating tumors much more efficiently than 10,000 ALDH-CD44- cells. ALDH+CD44+ cells could also be transplanted serially and generated secondary xenografts, evidencing the self-renewal nature of these cells. It is noteworthy that although control ALDH-CD44- cells formed a few primary xenografts (2 out of 15), we did not observe any tumor being formed from the serial transplantation experiments with these cells. The two tumors generated from ALDH-CD44- cells could be due to the existence of a few progenitor cells with the capability of tumor initiation or possible inaccuracy of FACS sorting. Moreover, the histology of the xenografts

obtained from the ALDH+CD44+ cells resembled that of primary tumors. These findings confirmed that the xenografts were of human epithelial origin and supported the hypothesis that ALDH+CD44+ cells have a behavior that is consistent with the behavior of cancer stem cells.

We observed that ALDH-positive cells are found primarily in the basal layer of the oral epithelium, where stem cells of the skin have been traditionally found (Fuchs *et al.,* 2004, Blanpain *et al.,* 2004). In contrast, in the HNSCC the ALDH-positive cells have a more disperse localization within the tumor microenvironment. Of note, the ALDH-positive cells were consistently localized within close proximity of blood vessels. The close association of cancer stem cells and blood vessels has earlier been documented in the nervous system and that these vascular niches helped in the maintenance of stem cells and cancer stem cells (Shen *et al*., 2004, Calabrese *et al.,* 2007, Shen *et al.,* 2008). However, such association has not been reported yet for head and neck tumors.

The cancer stem cells are believed to escape current therapies like radiation and chemotherapy and possibly lead to recurrences in various cancers. Thereby, identifying and targeting cancer stem cells or their niches might be a novel therapeutic strategy in the clinic (Okamoto *et al.,* 2009). However, to be able to target the cancer stem cells or their niches, we have to understand its pathobiology, identify their niches and their effects on the cancer stem cells. Endothelial cells have been implicated in the self-renewal and survival of neural cancer stem cells (Calabrese *et al.,* 2007). Studies in hematopoietic stem cells suggest that the vascular niche can promote cell survival signals (Sugiyama *et*

al., 2006), which could make them resistant to chemotherapies. Other groups have studied the effects of endothelial cell survival and self-renewal on cancer stem cells in neural tumors (Calabrese *et al.,* 2007, Folkins *et al.,* 2007). Anti-angiogenic agents (e.g. bevacizumab) have been shown to mediate depletion in the cancer stem cells in models of gliomas and medulloblastomas. Here, we used a unique experimental approach to selectively eliminate tumor-associated endothelial cells and evaluate the effect on the stem cell compartment. Unlike previous experimental strategies that were based on anti-angiogenic drugs, the approach used here eliminates the risk of a direct effect of the drug on the viability or stemness of the tumor stem cells. In our strategy, in 4 days, we saw a significant reduction in the CSCs%, suggesting that the endothelial cells play a direct role in the survival of the cancer stem cells. Our experiments revealed that selective ablation of tumor-associated blood vessels are sufficient to decrease the proportion of head and neck tumor stem cells *in vivo*.

Our work demonstrates that endothelial cells initiate signaling events that enhance the survival and self-renewal of stem cells in head and neck tumors. In addition, the data presented here supports the concept that head and neck cancer indeed follows the cancer stem cell hypothesis, since implantation of few cells consistently gives rise to tumors that can be serially passaged *in vivo*. Collectively, these data suggest that therapeutic strategies that include anti-angiogenic agents might have the benefit of reducing the proportion of cancer stem cells in head and neck tumors. These results might translate into lower recurrence rates and better survival of head and neck cancer patients.

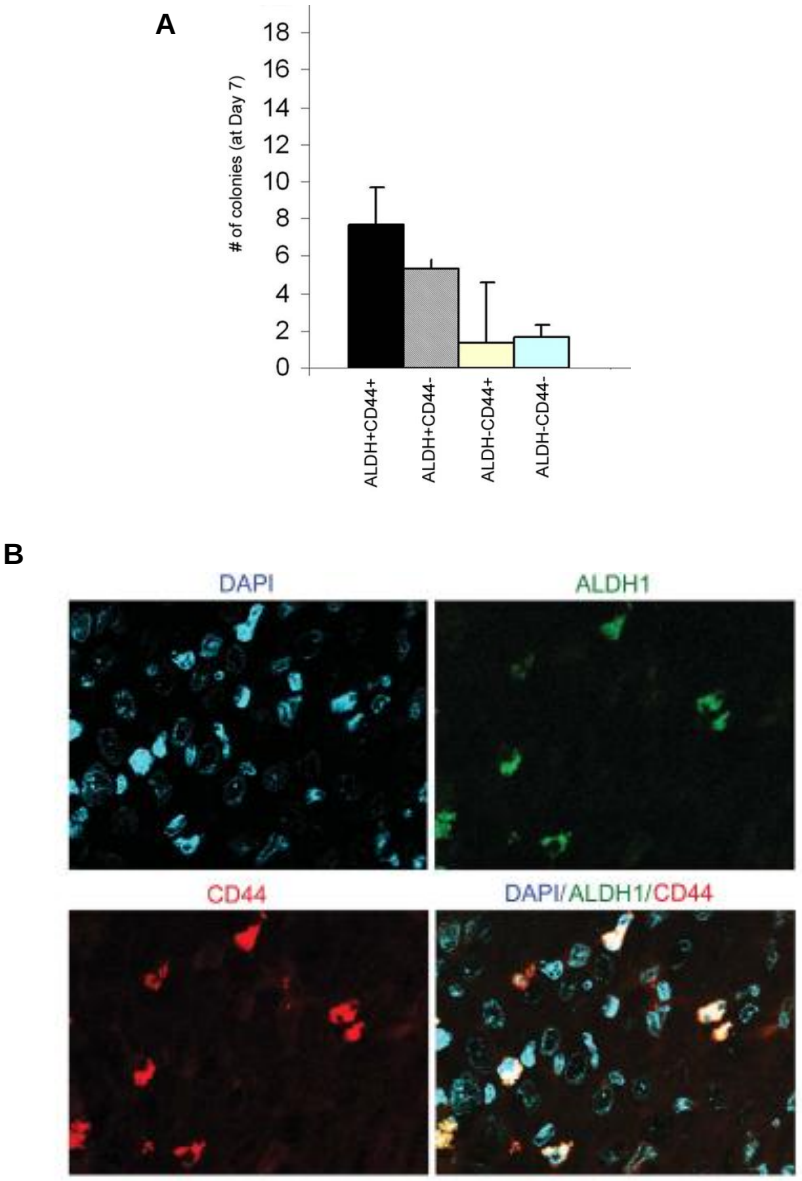

Figure 3.1. ALDH+CD44+ cells have a purer sub-population of cells exhibiting cancer stem like cells. (**A)**, Graph depicting the quantification of the relative number of colonies/well arising from ALDH+CD44+, ALDH+CD44-, ALDH-CD44+ and ALDH-CD44- cells treated with endothelial cell conditioned medium (CM) or unconditioned medium for one week. **(B),** Confocal microscopy of human HNSCC immunostained for ALDH1 (green), CD44 (red) and DAPI for the nuclei. The overlay image shows the predominant co-localization of the ALDH positive (green) and CD44-positive cells

A.

Primary Tumor	Age/ Sex	Ethnicity	Site of the tumor	CSC (ALDH+CD44+) (%)	NCSC (ALDH+CD44-) (%)	Viable Xenografts/ Total implants 1,000 cells of ALDH+CD44+	Viable Xenografts/ Total Implants 10,000 cells of ALDH-CD44-
HN 04	57/F	Caucasian	Buccal mucosa	1.76	06.24	1/1 Primary 1/1 Secondary	0/1 Primary 0/1 Secondary
HN 08	64/F	Caucasian	Right retromolar trigone	3.34	12.77	1/2 Primary N/A Secondary	0/2 Primary N/A Secondary
HN 09	56/F	Afro-american	Left maxillary ridge	0.97	09.37	1/2 Primary N/A Secondary	0/2 Primary N/A Secondary
HN 10	63/F	Afro-american	Tongue and floor of the mouth	1.78	08.70	10/10 Primary 4/4 Secondary	2/10 Primary 0/ 4 Secondary

Table 3.1. Table depicting the characteristics of the HNSCC. The patients in Figure 1 and their relative CSC% (ALDH+CD44+) and NCSC% (ALDH-CD44-). The table also illustrates the individual primary and secondary xenografts generated out of the total number of implants from each of the HNSCC by implanting 1,000 CSC or 10, 000 NCSC. N/A indicates no implantation was done due to lack of cells post-sorting.

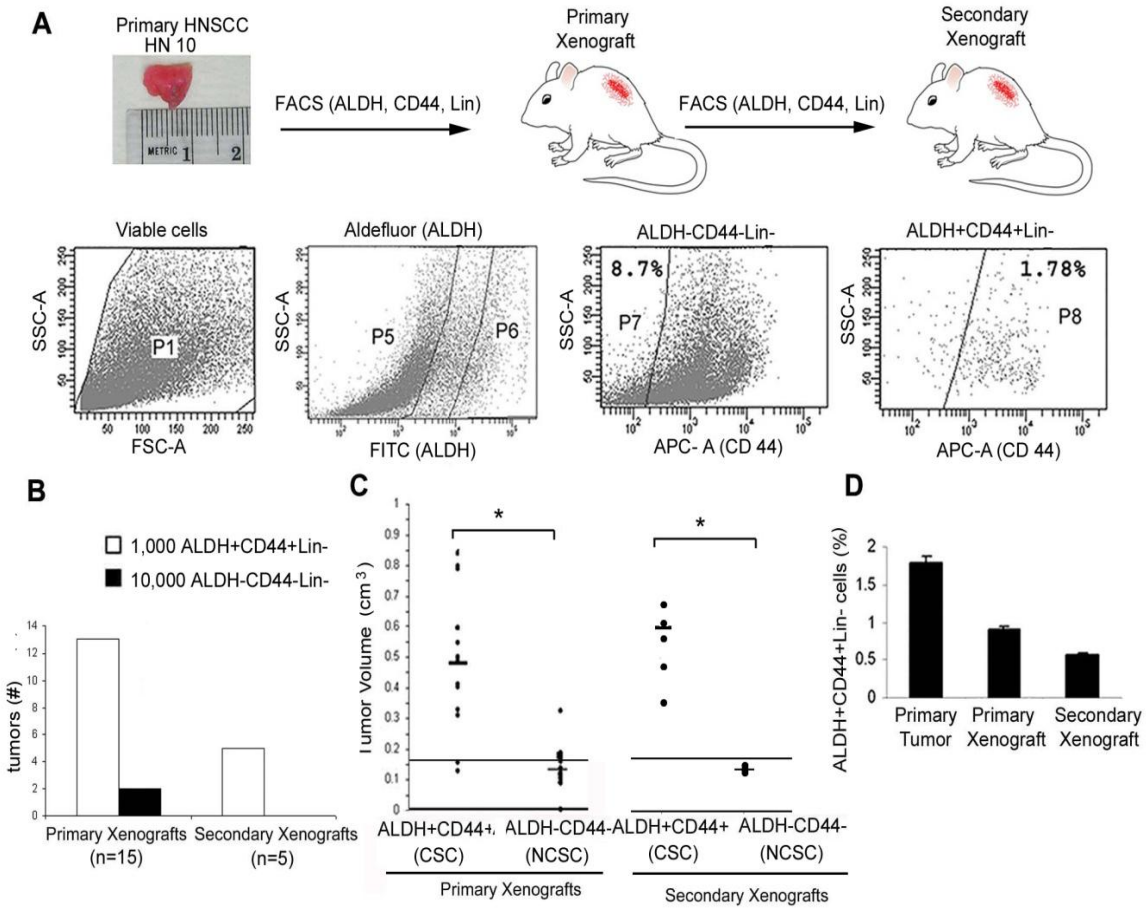

Figure 3.2. Combination of ALDH and CD44 selects highly tumorigenic cells. **(A),** Schematic representation of the approach for testing tumorigenic potential of cells. ALDH+CD44+Lin- cells were isolated from HNSCC and serially transplanted into immunodeficient mice to generate primary and secondary xenografts. Representative flow cytometry: Viable cells (P1) are isolated from a head and neck squamous cell patient (HN 10) using 7AAD and are gated for positivity to ALDH (P6), using DEAB (ALDH inhibitor). ALDH-negative cells in P5. ALDH+CD44+Lin- (P8=1.78%) and ALDH-CD44-Lin- (P7=8.7%). **(B),** Graph depicting the volume of primary and secondary xenografts. **(C),** Graph depicting the xenograft tumors (primary and secondary) obtained by implantation of 1,000 ALDH+CD44+Lin- or 10,000 ALDH-CD44-Lin- cells selected from human HNSCC (n=15). **(D)**, Graph depicting the percentage of putative head and neck cancer stem cells (ALDH+CD44+Lin-) in primary tumors, primary and secondary xenografts

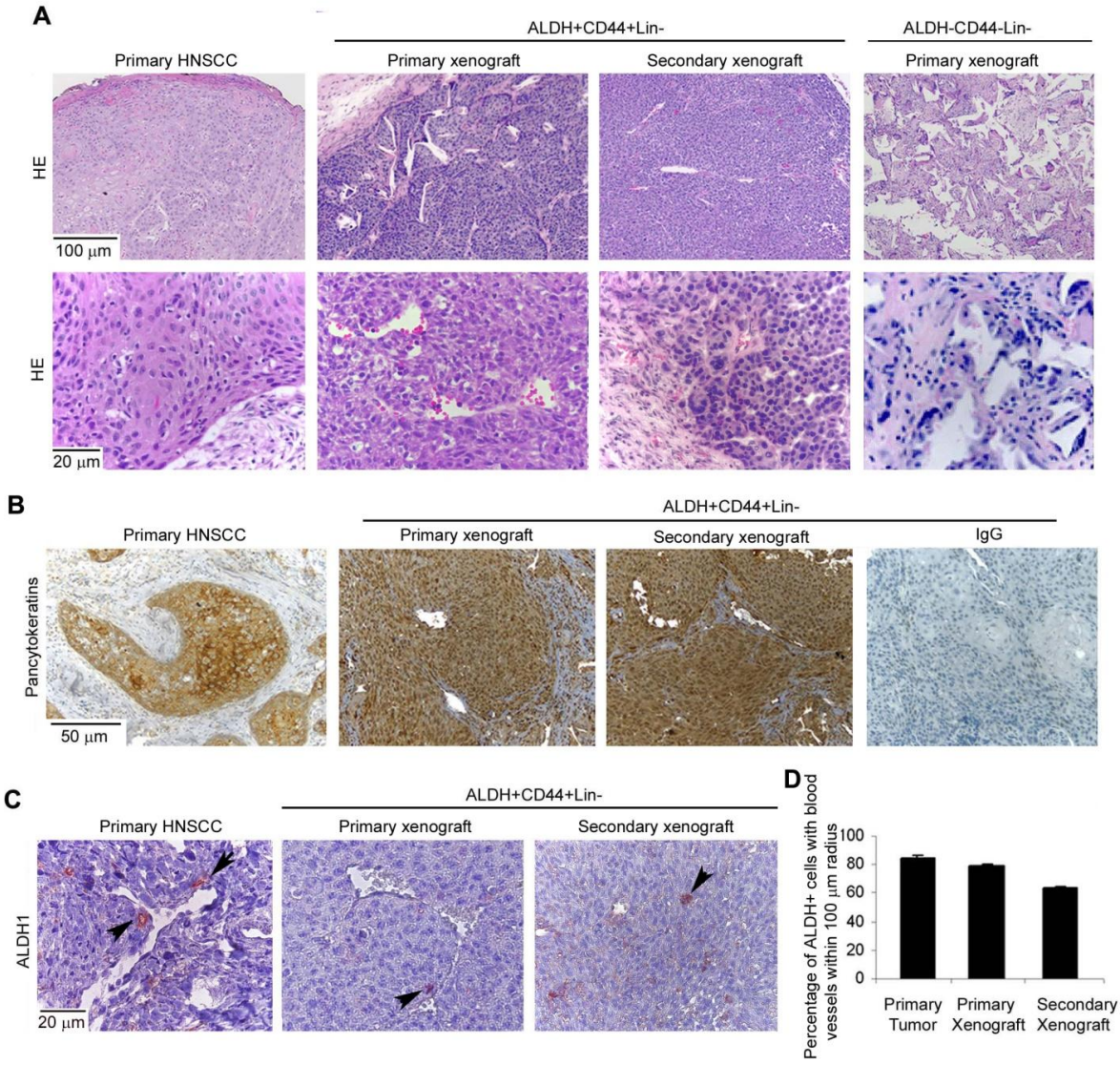

Figure 3.3. Head and neck cancer stem-like cells generate xenografts with histological features that closely resemble those primary tumors. (**A**), Photomicrographs of a representative primary tumor (HN10), and respective primary and secondary xenograft originated by the transplantation of ALDH+CD44+Lin- cells (HE staining). Control tissues generated by the implantation of ALDH-CD44-Lin- cells. **(B),** Expression of pancytokeratin in the primary tumor, respective primary and secondary xenograft, and IgG control group. **(C),** Representative photomicrographs of ALDH1 immunostaining of primary tumors and respective xenografts (Arrows depict ALDH1 positive cells present in spatial relationship to blood vessels. **(D),** Graph depicting the percentage of ALDH1 positive cells found within a 100-μm radius of blood vessels in the primary tumors and xenografts generated by the transplantation of ALDH+CD44+Lin- cells.

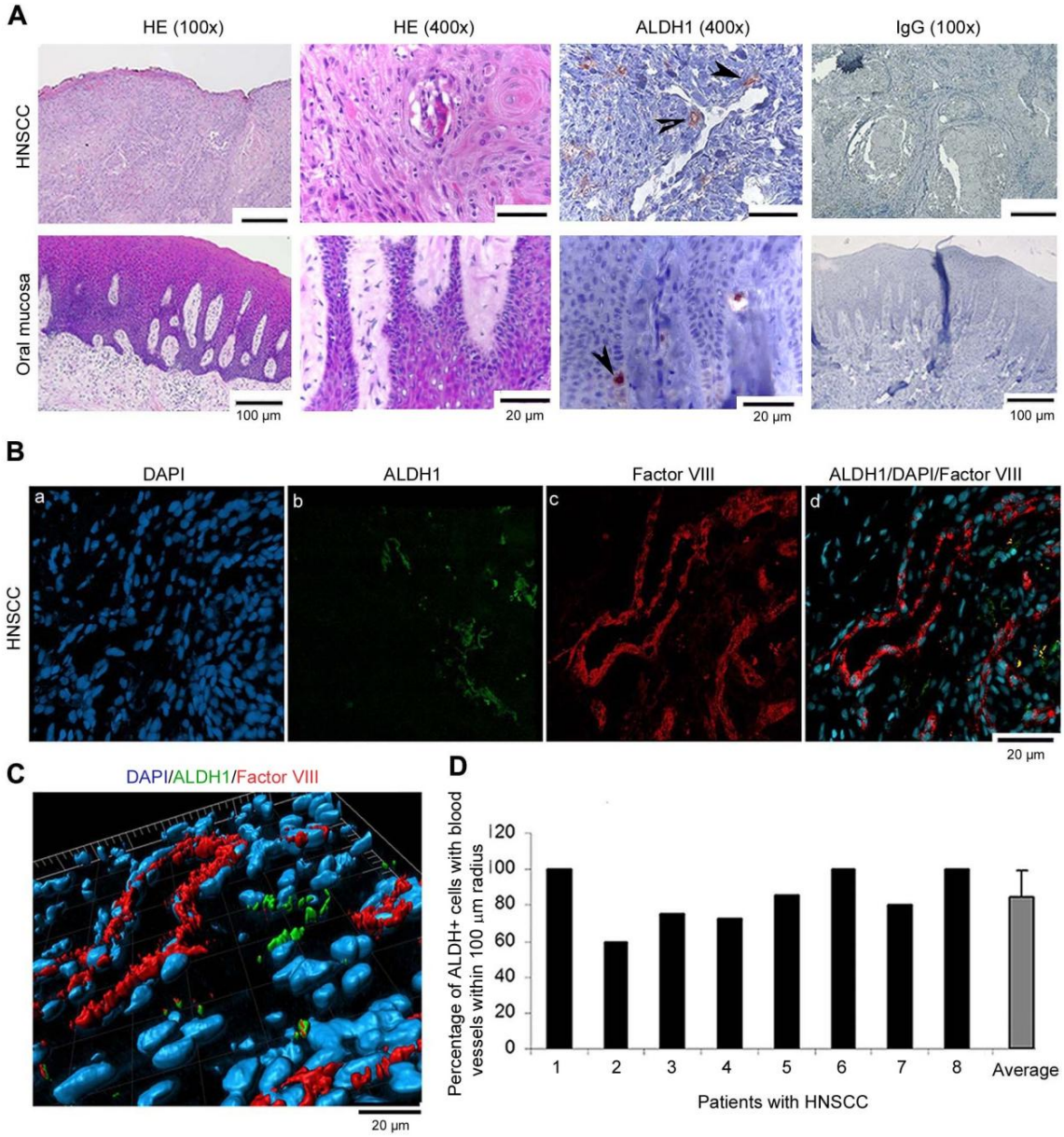

Figure 3.4. Head and neck cancer stem-like cells are localized in close proximity to blood vessels. (**A**), Representative photomicrographs of tissue sections stained with HE or immunostained for ALDH1 in human HNSCC and control oral mucosa (identified by arrows). **(B),** Confocal microscopy of human HNSCC immunostained for ALDH1 (green) and Factor VIII (red) for localization of blood vessels. The overlay image shows the perivascular localization of the ALDH-positive cells. **(C),** 3-D reconstruction of the image in B,d showing the proximity between ALDH-positive cells (green) and blood vessels (red). **(D),** Graph depicting ALDH-positive cells found within 100-µm radius of blood vessels in 6 random areas of 6 different sections taken together and expressed as a percentage for each individual patient and the average of 8 individual HNSCC patients are shown.

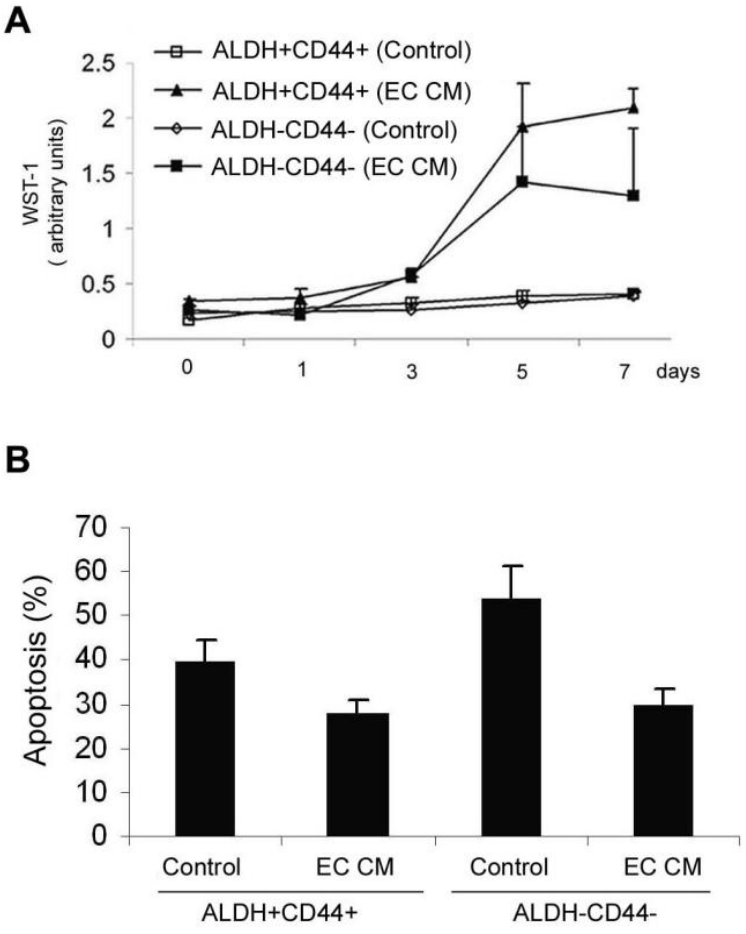

Figure 3.5. Endothelial cell Conditioned Medium promotes proliferation and prevents apoptosis of head and neck cancer stem cells. (A), Graph depicting the effect of endothelial cell conditioned medium (EC CM) on the proliferation of ALDH+CD44+ and ALDH-CD44- cells. **(B),** Graph depicting the percentage of apoptotic cells when ALDH+CD44+ or ALDH-CD44- cells are exposed to the conditioned medium from endothelial cells (EC CM) for one week.

Cell Line	Site of Ocurrence	CSC (ALDH+CD44+) (%)	NCSC (ALDH-CD44-) (%)
UMSCC-1	Retromolar trigone	5.44 (3.90 - 7.70)	17.60 (12.61 – 23.34)
UMSCC-11B	Larynx - post chemotherapy	3.85 (2.81 - 5.59)	21.74 (13.99 – 28.60)
UMSCC-17A	Larynx	2.87 (2.01 - 4.32)	16.00 (12.37 – 20.14)
UMSCC-17B	Soft tissue metastasis From laryngeal cancer	3.59 (2.90 - 4.34)	14.67 (12.65 – 16.13)
UMSCC-74A	Base of tongue	2.45 (1.70 - 3.44)	14.24 (08.26 – 16.12)
UMSCC-74B	Tongue recurrence	2.47 (1.62 – 4.36)	38.60 (18.20 – 50.00)

Table 3.2 Table showing the various cell lines used in the study, their origin and their relative cancer stem cells. CSC% (ALDH+CD44+) and NCSC% (ALDH-CD44-).

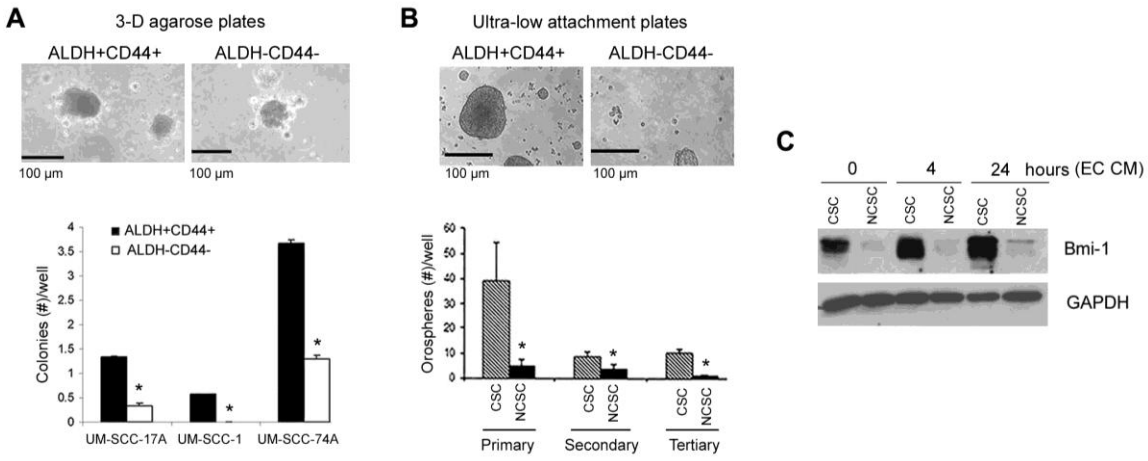

Figure 3.6. ALDH and CD44 select for a sub-population of cells that exhibit self-renewal properties *in vitro*. (A), Representative photomicrographs of colonies (orospheres) arising from ALDH+CD44+ and ALDH-CD44- cells sorted from UM-SCC-74A and grown in 3-D agarose matrices. Graph depicts the quantification of the number of colonies arising from cancer stem-like cells (CSC; ALDH+CD44+) or non-cancer stem cells (NCSC; ALDH-CD44-) sorted from three HNSCC cell lines (UM-SCC-17A, UM-SCC-1, UM-SCC-74A) and cultured in 3-D agarose matrices. **(B),** Representative photomicrographs of orospheres arising from ALDH+CD44+ and ALDH-CD44- cells sorted from UM-SCC-74A and grown in ultra-low attachment plates. Graph depicting the number of orospheres from serial passage assays that evaluate self-renewal of CSC (ALDH+CD44+) and NCSC (ALDH-CD44-) cells *in vitro*. Asterisk depicts $P < 0.001$. **(C),** Western blot depicting the expression of Bmi-1 in CSC (ALDH+CD44+) and NCSC (ALDH-CD44-) cells treated with endothelial cell conditioned medium (EC CM).

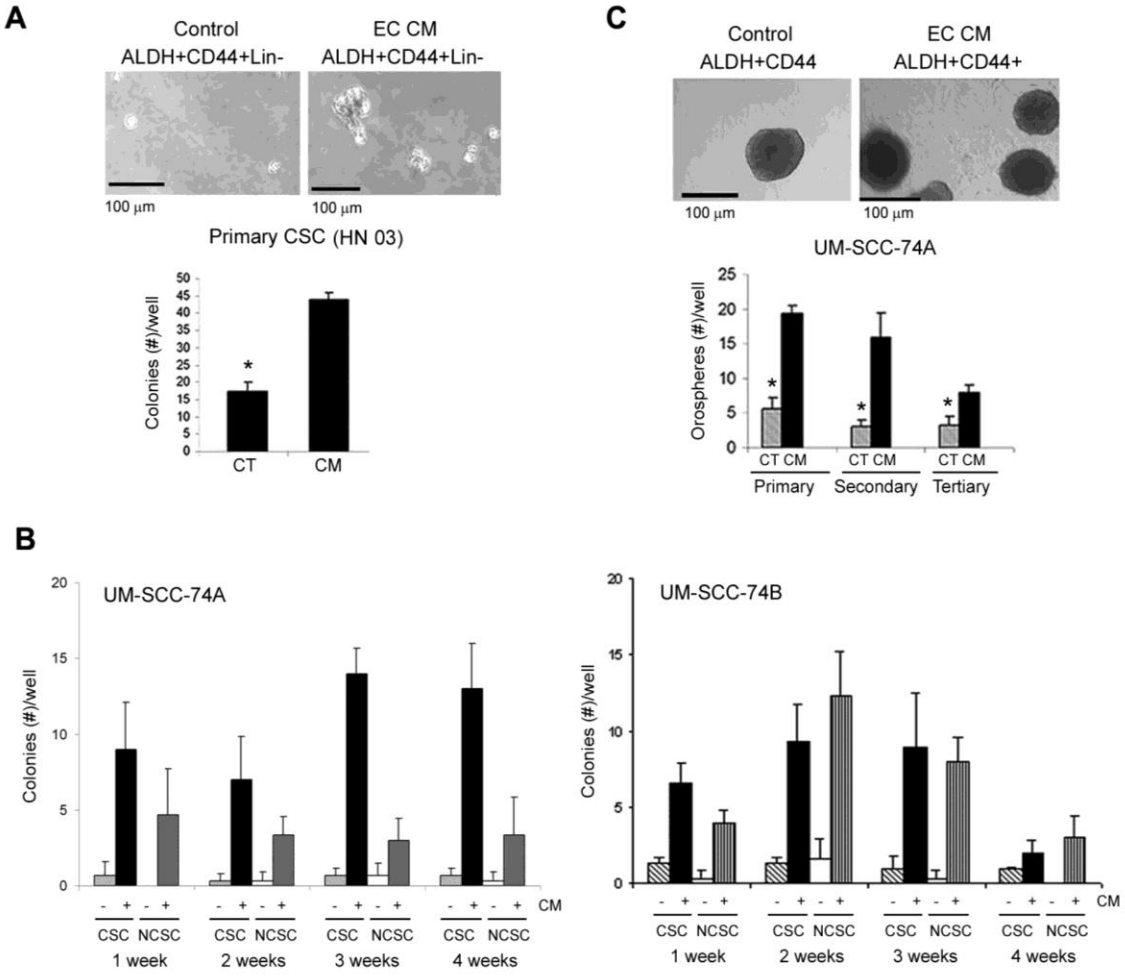

Figure 3.7. Endothelial cell-derived factors promote proliferation and self-renewal of head and neck cancer stem-like cells. **(A),** Photomicrographs of representative colonies arising from ALDH+CD44+Lin- cells sorted from a human HNSCC and grown in 3-D Agarose matrices. Cells were treated with endothelial cell conditioned medium (CM) or unconditioned control medium (CT) for one week. Asterisk depicts $P < 0.001$. **(B),** Time course experiment depicting the number of orospheres arising from CSC (ALDH+CD44+) and NCSC (ALDH-CD44-) cells treated or not with endothelial cell conditioned medium (CM) over a period of 4 weeks. **(C),** Photomicrographs of representative colonies arising from ALDH+CD44+Lin- cells sorted from UM-SCC-74A cells and cultured in ultra-low attachment plates. Cells were treated with endothelial cell conditioned medium (CM) or unconditioned control medium (CT). Graph depicting primary, secondary and tertiary orospheres arising from ALDH+CD44+ treated with endothelial cell conditioned medium (CM) or control medium (CT). Asterisk depicts $P < 0.05$.

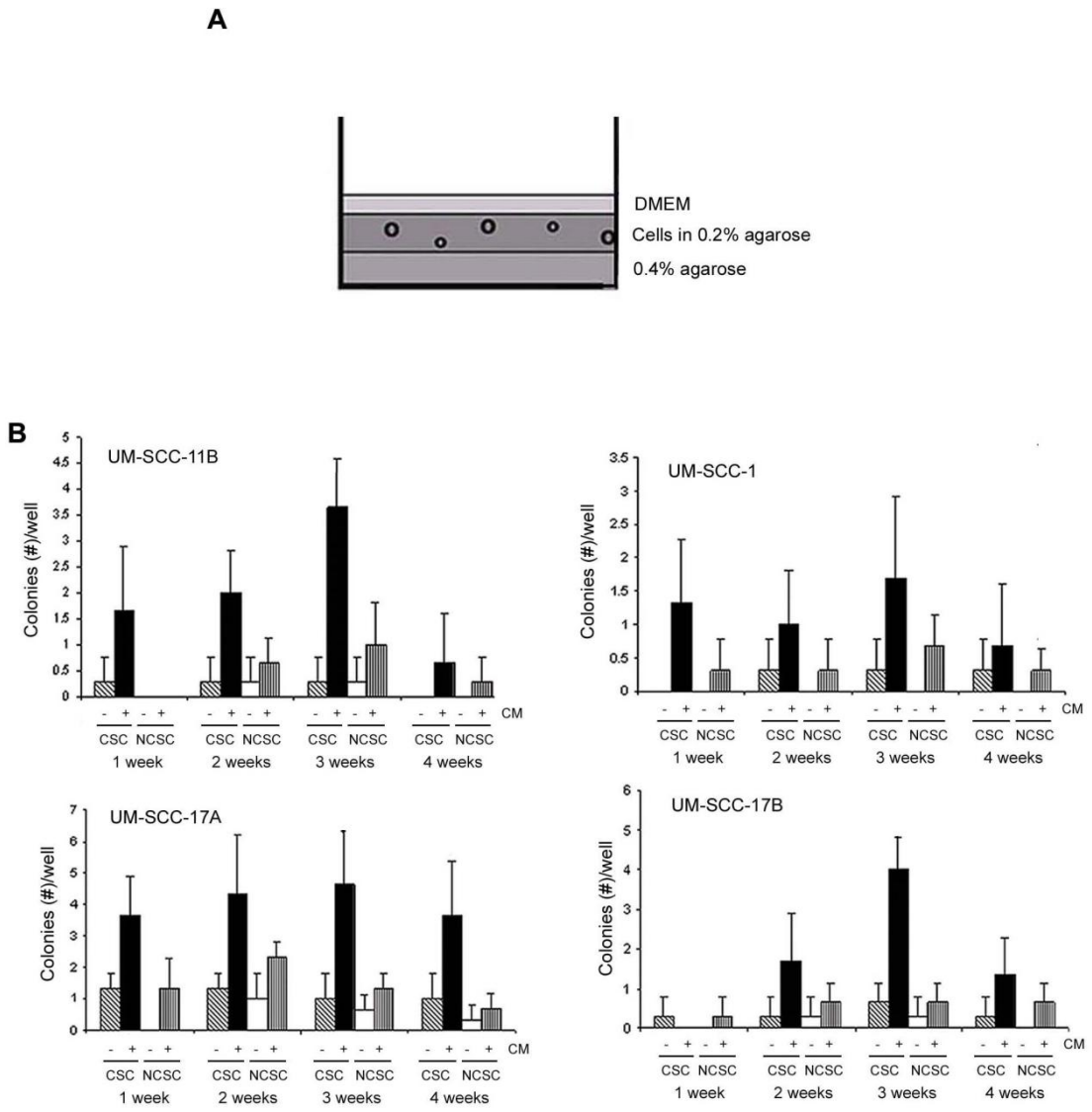

Figure 3.8. Endothelial Cell Conditioned Medium promotes self-renewal of head and neck cancer stem-like cells. (A), Schematic drawing of the colony formation assay in 3-D agarose matrices**. (B),** Graphs depicting the quantification of the relative number of colonies/well arising from ALDH+CD44+ (CSC) and ALDH-CD44- (NCSC) cells treated with endothelial cell conditioned medium (CM) or unconditioned medium for four weeks. Four head and neck squamous cell carcinoma cell lines were used to verify the reproducibility of results, *i.e.* UM-SCC-11B, UM-SCC-1, UM-SCC-17A and UM-SCC-17B.

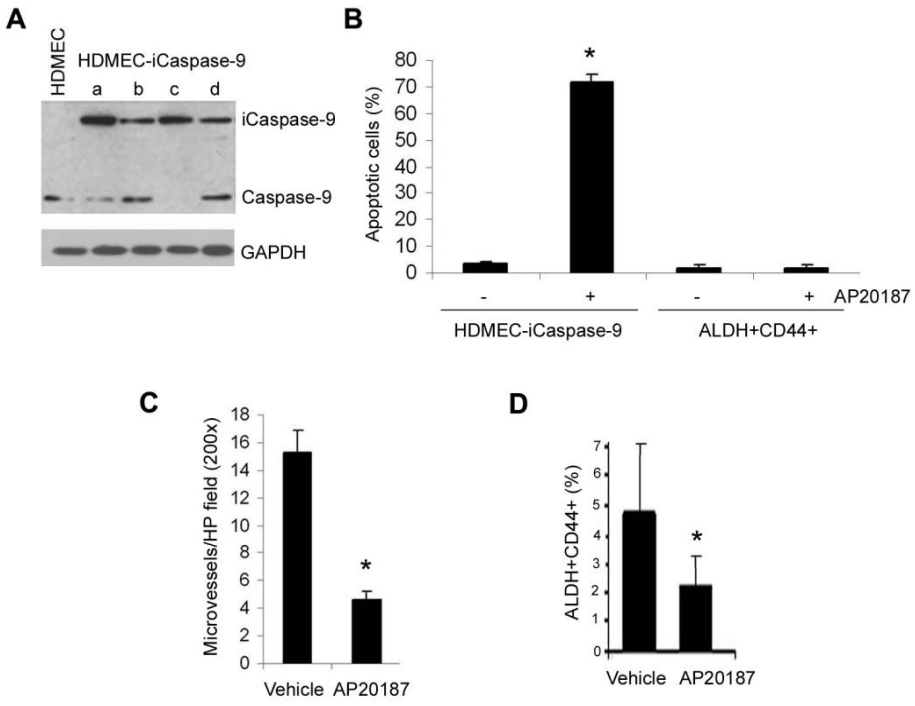

Figure 3.9. Selective ablation of tumor-associated endothelial cells decreases the number of head and neck cancer stem-like cells. **(A),** Western blot showing 4 clones of HDMEC stably transduced with the caspase-based artificial death switch iCaspase9. Clone "d" was selected for remaining experiments depicted in this figure. **(B),** Graph depicting the proportion of apoptotic cells when HDMEC-iCaspase-9 or ALDH+CD44+ cells are exposed to the dimerizer drug AP20187. **(C),** Graph depicting the tumor microvessel density of xenografts generated by the co-transplantation of HDMEC-iCaspase-9 and ALDH+CD44+ cells in immunodeficient mice. Mice were injected with AP20187 to activate Caspase-9 and selectively eliminate the xenograft tumor blood vessels. **(D),** Graph depicting the percentage of ALDH+CD44+ cells in the AP20187-treated tumors compared to vehicle treated controls. Asterisk depicts $P < 0.005$.

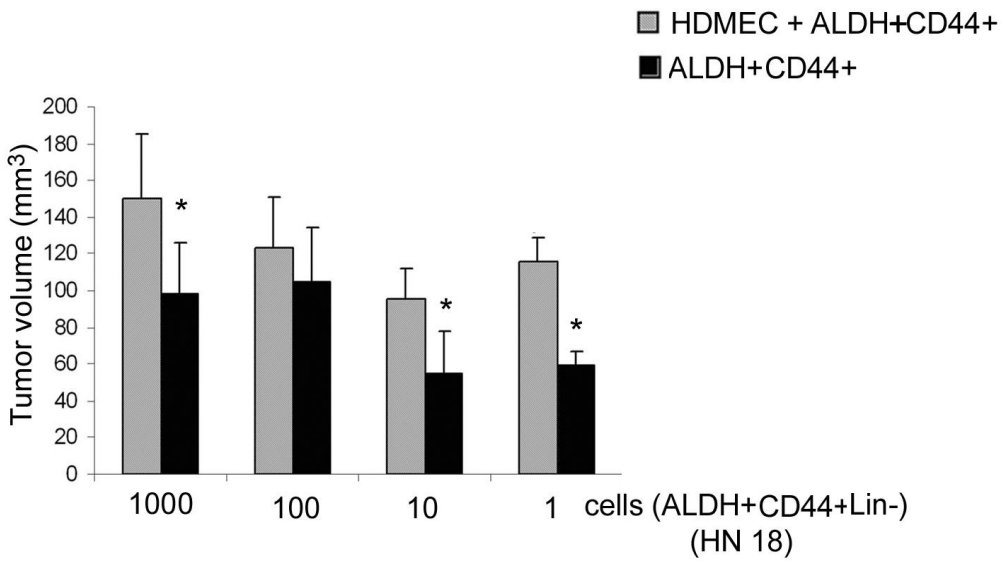

Figure 3.10. Endothelial cells enhance the tumorigenic potential of head and neck cancer stem cells. Graph depicting the volume of tumor generated by the transplantation of 1-1,000 head and neck cancer stem cells (ALDH+CD44+Lin-) from a primary HNSCC (HN 18) with or without endothelial cells (HDMEC). Asterisk depicts $P < 0.001$.

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CHAPTER IV

ENDOTHELIAL CELL-INITIATED IL-6 SIGNALING PROMOTES SELF-RENEWAL, SURVIVAL AND TUMOR INITIATION OF CANCER STEM CELLS

Abstract

Head and neck squamous cell carcinomas display cellular heterogeneity with ALDH+CD44+ cells exhibiting cancer stem-like properties. ALDH+CD44+ cells were recently found to exist in vascular niches and that endothelial cells promoted their self-renewal and survival. However, molecular mechanisms underlying the endothelial cell-cancer stem interaction have been largely unknown. Here, we report that endothelial cells expressed higher IL-6 levels than the adjacent tumor cells in primary head and neck squamous cell carcinomas (HNSCC), and observed that the cancer stem cells preferentially express IL-6R and phosphorylated-STAT3. Targeting endothelial cell secreted IL-6 by either neutralizing antibody or gene silencing (using shRNA) strategies decreased the sphere forming capacity and the invasive potential of the ALDH+CD44+ cells. Endothelial cells stably expressing shRNA-IL-6 decreased tumorigenicity and tumor growth *in vivo*. Tumor initiation and growth was significantly decreased in the absence of IL-6 in the tumor micro-environment as seen in transgenic mouse models. Interrupting IL-6 signaling by means of a humanized IL-6R antibody

resulted in delayed tumor initiation and decreased the survival of head and neck cancer stem cells *in vivo*. Collectively, these data indicate that IL-6/IL-6R represents a potential therapeutic target for head and neck cancer stem cells.

Introduction

Head and neck squamous cell carcinomas, like many other human carcinomas may be driven by a population of cells called the cancer stem cells; possessing stem cell properties of self-renewal and differentiation (Prince *et al*., 2007, Chen *et al*., 2009, Hermann *et al*., 2010). There is evidence in recent years that these cancer stem cells could possibly participate in the processes that lead to resistance to therapy and the establishment of distant metastases (Kakarala *et al*., 2008, Chen *et al*., 2010) resulting in poor patient survival (Al-Swiahb *et al.,* 2010). Recently, CD44+ cells were shown to have increased tumorigenicity and self-renewal properties in HNSCC (Prince *et al*., 2007). Aldehyde Dehydrogenase I, a cancer stem cell marker identified in various tissues of origin like the brain, breast and colon (Corti *et al*., 2006, Ginestier *et al*., 2007, Huang *et al.,* 2009), was recently used to identify stem cells in HNSCC (Chen *et al*., 2009, Krishnamurthy *et al*., 2010). These ALDH+CD44+ cells expressed the stem cell properties of self-renewal and increased tumorigenicity (Krishnamurthy *et al*., 2010) and were found to be radio-resistant (Chen *et al*., 2010).

Stem cells and cancer stem cells of various tissues have been found to reside in the basal layer of the tissues (Tumbar *et al*., 2004, Kopan *et al*., 1989) and have been found to be dependent on the tumor microenvironment for their growth and survival (Lobo *et al*., 2007, Polyak *et al*., 2009, Parmar *et al*., 2011). Recently, peri-vascular niches have been identified in neural stem cells (Shen *et al*., 2004, Sugiyama *et al*., 2006, Veeravagu *et al*., 2008) and neural tumors (Calabrese *et al*., 2007). In HNSCC, we identified a peri-vascular niche and

demonstrated that endothelial cell secreted factors promote survival and self-renewal of head and neck cancer stem cells *in vitro* and *in vivo* (Krishnamurthy *et al*., 2010). Anti-angiogenic therapy thus was an attractive cancer stem-cell based therapy for head and neck cancers. However, recent evidence suggested that anti-angiogenic therapy (especially anti-VEGF based therapy) could lead to higher numbers of tumor cell infiltration thereby causing a malignant progression of tumors and increased metastasis, (Paez-Ribes *et al*., 2009; Keunen *et al.,* 2011). This therefore, suggested a more targeted study needs to be done on possible molecular pathways contributing to cancer stem cell survival and self-renewal.

The inflammatory cytokine, Interleukin-6 (IL-6) has been found to play a pivotal role in the mediation of interaction in cancer stem cells and their micro-environment in numerous tissues including the breast and brain (Sansone *et al.,* 2007, Wang *et al.,* 2009, Liu *et al.,* 2011, Marotta *et al.,* 2011). The IL-6 ligand receptors, IL-6R and gp130, have been found to have aberrant expression levels in glial cancer stem cells (Wang *et al*., 2009). In tumors like lung adenocarcinomas, glioblastomas and head and neck cancers, IL-6 expression levels and its downstream activation target of signal transducer and activator of transcription 3 (STAT3) has been found to be highly expressed (Gao *et al*., 2007, Seethala *et al.,* 2008). Moreover, increased serum IL-6 levels have been co-related with tumor recurrences and poorer patient prognosis in HNSCC in a longitudinal cohort study (Duffy *et al*., 2008). Additionally, STAT3 has been found to be constitutively active in HNSCC and inhibition of STAT3 by various inhibitors

like Erlotinib or Cucurbitacin I has been found to decrease tumorigenesis in squamous cell carcinomas (Leeman-Neill *et al.,* 2011, Chen *et al.,* 2010). However, the specific role of IL-6 signaling is unknown in head and neck cancer stem cells.

In this study, we unveil a paracrine pathway (IL-6-IL6R-STAT3 pathway) for the head and neck cancer stem cells via their niche (the perivascular niche) which promotes self-renewal and tumor initiation in HNSCC. Using Laser capture microdissection technique, we establish the relative IL-6 levels in the tumor endothelial cells and tumor cells in HNSCC primary patients. We demonstrate using neutralizing antibodies and shRNA strategies that endothelial cell-initiated IL-6 promotes self-renewal and invasion *in vitro* and tumor initiation *in vivo* and corroborate this using IL6-/- mice studies. Finally, using a humanized anti-IL6R antibody, we propose a novel molecular targeting therapy for head and neck cancer stem cells as an adjunct with current therapy to potentially delay or prevent recurrences in HNSCC.

Materials and Methods

Cell Culture

Head and neck squamous cell carcinoma cell lines (UM-SCC-74A, UM-SCC-74B, UM-SCC-17B; gift from Dr. Carey, University of Michigan) were cultured in Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen; Grand Island, NY, USA) and human dermal micro vascular endothelial cells (HDMEC; Cambrex, Walkersville, MD) in endothelial cell growth medium-2 (EGM2-MV; Cambrex). The identity of tumor cell lines was confirmed by genotyping at the University of Michigan DNA sequencing core facility. FACS sorted cells were cultured in low glucose DMEM, 10% fetal bovine serum (Gibco, Invitrogen) and 100 U/ml Penicillin-streptomycin (Invitrogen) or 100 U/ml Antibiotic Antimycotic Solution (Sigma) in ultra-low attachment plates (Corning Inc; New York, NY, USA). Conditioned Medium (CM) from HDMEC was collected in serum free DMEM from 24-hour cultures.

Head and neck cancer stem cell sorting

Informed consent was obtained from patients undergoing surgery for removal of HNSCC. All tumors were collected in accordance to the IRB protocols. The tumors obtained were cut and minced with a sterile scalpel until they could pass through a 25 ml pipette tip. They were suspended in a 9:1 solution of DMEM-F12 (Hyclone, Waltham, MA) and Collagenase/Hyaluronidase (Stem Cell Technologies, Vancouver, BC, Canada). This mixture was then incubated at 37^0C for maximum of one hour and passed through a 10-ml pipette

every 15 minutes for mechanical dissociation. Cells were filtered through a 40-μm nylon sieve (BD Falcon, NJ, USA), washed with low glucose complete DMEM and centrifuged at 800 rpm for 3 minutes. Single cell suspensions obtained from primary specimens (as well as from cell lines or xenografts) were washed, counted, and re-suspended at 1×10^6cells/ml PBS. The Aldefluor kit (Stem Cell Technologies) was used to identify cells with high ALDH activity. Briefly, cells were suspended with activated Aldefluor substrate (BAA) or the negative control (DEAB, a specific ALDH inhibitor) for 45 minutes at 37^0C. Then, cells were exposed to anti-CD44 (clone G44-26BD; Pharmingen, NJ, USA) and lineage markers (*i.e.* anti-CD2, CD3, CD10, CD16, CD18, CD31; BD Pharmingen). Mouse cells are identified using anti-$H2K^d$ antibody (BD Biosciences, NJ, USA) and 7-Aminoactinomycin (7-AAD, BD Pharmingen) identified viable cells. Here and throughout this manuscript, studies were done in triplicate specimens per condition and three experiments were performed to verify reproducibility of the data.

Stable Short Hairpin RNA Transduction

Lentivirus expressing a short hairpin RNA (shRNA) construct for silencing IL-6 (Vector Core, University of Michigan) was generated in human 293T cells (human embryonic kidney cells) transfected using the Calcium Phosphate method, as described (Kaneko *et al.,* 2006). Scrambled oligonucleotide sequence was used as control (shRNA-C). Supernatents were collected 48 hours after transfection and HDMEC cells were infected with it in a 1:1 dilution medium containing 4 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO). Cells were selected

for 2 weeks and maintained in EGM2-MV supplemented with 1 µg/ml puromycin (InvivoGen, San Diego, CA). Down-regulation of IL-6 was confirmed by ELISA.

Confocal Microscopy

Paraffin tumor sections were deparaffinized in xylene and rehydrated in decreasing degrees of alcohols. Antigen retrieval was done using Dako Retrieval solution (Dako, CA) for 45 minutes at 98^0C. The anti-ALDH1 (BD Biosciences), anti-P-STAT3 (Santa Cruz 79993-R, CA), anti-IL6R and gp130 (abcam) were pre-labeled with Alexafluor 488 or 594 using the Zenon labeling kit (Invitrogen) and Confocal imaging was performed using Leica Inverted Confocal SP5X (Leica version 2.3.5; Los Angeles, CA).

Immunohistochemistry

Paraffin embedded sections were de-paraffinized in xylene and Antigen Retrieval was done using Dako antigen retrieval solution (Dako, CA). Immunostaining for ALDH1 (BD Biosciences, 61195, 1:100), IL-6 (abcam, 1:100), P-STAT3 (Santa Cruz, CA, 1:200), chromogenicity. Isotype matched immunoglobulins were used as negative controls.

Orospheres

Orospheres (*i.e.* spheroids of head and neck cancer-derived cells) were generated from FACS-sorted cells ($5x10^3$ cells/well) cultured in triplicate in low attachment plates (Corning Inc, NY,USA) (Krishnamurthy *et al*., 2010) and maintained in low glucose DMEM containing or not conditioned medium from HDMEC at a ratio of 3:1. Alternatively, cells were treated with HDMEC CM containing 0.4 µg/ml of anti-IL-6 (R&D Systems, Minneapolis, MN) or 10 µg/ml of

anti-IL-6R (Chugai Pharmaceuticals, Tokyo) or IgG isotype control or 20 ng/ml of rhIL-6. After 3 days, orospheres are visualized and quantified.

SCID mouse model of human tumor angiogenesis

Xenograft tumors vascularized with functional human microvessels were generated in severe combined immunodeficient (SCID) mice (CB17 SCID, Haslett, MI), as described (Nör *et al.,* 2001). Briefly, 1,000 head and neck cancer stem cells (HNCSC- i.e., ALDH+CD44+Lin-) or 1,000 non-cancer stem cells (NCSC-ALDH-CD44-Lin-) were seeded with 5×10^5 cells of either HDMEC-shRNA-C or HDMEC-shRNA-IL-6 in poly-(L-lactic) acid (PLLA; Medisorb) biodegradable scaffolds. Bilateral scaffolds were implanted subcutaneously in the dorsum of each mouse. Mice were monitored daily for tumor growth for the specified time periods in individual experiments. Alternatively, mice received scaffolds containing 5×10^5 HDMEC cells and 1×10^3 HNCSC or NCSCs. From Day 1, mice were treated with weekly intra-peritoneal injections of either 10 mg/kg of humanized anti-IL-6R as described in Tsunenari *et al*., 1997, Shinriki *et al*., 2009 (Chugai Pharmaceuticals, Chuo-ku, Tokyo, Japan) or IgG. After 30 days, mice were euthanized and tumors were retrieved, measured, weighed and processed. For the tumor evaluation of IL-6-/- immunodeficient mice, (a gift from Dr. Laurie McCauley, University of Michigan), WT and IL-6-/- mice were generated by standard homologous recombination techniques as described (Dalrymple *et al*., 1995, Dai *et al*., 2000).

Western Blots

40,000 ALDH+CD44+ or ALDH-CD44- cells from UM-SCC-74A, UM-SCC-74B or primary head and neck cancer stem cells (HN-28) cells were plated in 6-well ultra-low attachment plates, serum starved overnight, and treated with endothelial cell conditioned medium (CM) or DMEM for the indicated time points. Alternatively, HNCSC and NCSC were exposed to HDMEC CM containing 0.4 µg/ml of anti-IL-6 (R&D Systems, Minneapolis, MN) or 10 µg/ml of anti-IL-6R (Chugai Pharmaceuticals, Tokyo) or IgG isotype control. CM collected from HDMEC-shRNA-C or HDMEC-shRNA-IL-6 was used to treat the cells and western blots were performed. Primary antibodies were as follows: mouse anti-human phospho-STAT3, rabbit anti-human STAT3, rabbit anti-human phospho-ERK1/2, mouse anti-human ERK1/2 (Cell Signaling, Danvers, MA); rabbit anti-human IL-6R (Santa Cruz), mouse anti-human gp130 (abcam, San Francisco, CA) and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (Chemicon, Millipore, Billerca, MA).

Enzyme-Linked Immunosorbent Assay

Supernatents of triplicates of 24-hour endothelial or tumor cell cultures were collected and centrifuged to eliminate debris. IL-6 expression was determined using ELISA kits (Quantikine; R&D systems) according to the manufacturer's instructions. Data was normalized by cell number.

Invasion Assays

HDMEC CM was pre-incubated with 0.4 µg/ml anti-IL-6 (R&D systems), or IgG control for 1 hour. 24-well companion plates (inserts) are pre-coated with 40

µl of 1:1 dilution of matrigel and serum free DMEM overnight. 400µl of HDMEC CM or CM from HDMEC-shRNA-C or HDMEC-shRNA-IL-6 was added to the 24-well plates. ALDH+CD44+ and ALDH-CD44- cells (25×10^3 cells) were serum starved overnight, loaded onto the 8-µm pore sized cell culture inserts (Becton Dickinson, Franklin Lakes, NJ) having the matrigel layer. The cells are allowed to invade for 24 hours toward the stimulus like HDMEC CM. Unconditioned DMEM was used as control. Invaded cells were trysinized, collected and stained with 2 µM Cell Tracker Green (Invitrogen) for one hour. Fluorescence was read at 485/535 nm in a micro plate reader (Tecan, Salzburg, Austria).

Laser Capture Microdissection

A three step dissection procedure was done using a LCM microscope (Leica AS LMD; Leica Microsystems) with a pulsed 337 nm UV laser as described (Kaneko *et al.,* 2007. Kaneko *et al.,* 2009). Blood cells in the endothelial cells were excluded first. Then the endothelial cells lining the blood vessels were collected separately in a tube filled with TRIzol (Invitrogen) and immediately placed on ice. Then tumor cells adjacent to the blood vessels were collected in a different tube and quickly transported on ice to -80^0C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the cells obtained by LCM or from the tumor cell line using TRIzol reagent (Invitrogen) and RNA was purified using RNAeasy Micro kit (Qiagen) as per the manufacturer's instructions. The purity of the RNA was determined photometrically (Ultraspec 2000, Pharmacia Biotech, Germany). cDNA synthesis and PCR amplification were done in single tubes with

SuperScript one-step reverse transcription PCR (RT-PCR) and Platinum Taq kit (Invitrogen) using simultaneously a human IL-6 primer set and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set. The sequence of the primers used here were as follows:

IL-6, sense 5'-AAAGAGGCACTGGCAGAAAA-3' and

anti-sense 5'CAGGGGTGGTTATTGCATCT; and

GAPDH, sense 5'-GACCCCTTCATTGACCTCAACT-3', and

antisense 5'-CACCACCTTCTTGATGTCATC-3'.

The PCR products were detected by standard agarose gels with 1.5% ethidium bromide.

Statistical Analyses

One-way ANOVA was performed using the SigmaStat 2.0 software (SPSS, Chicago, IL). Statistical significance was determined at *$p<0.001$ (unless otherwise specified). Statistics for the *in vivo* studies was performed by a Biostatistician with appropriate software as indicated.

Results

A higher level of IL-6 mRNA is present in tumor associated endothelial cells than tumor cells. The identification of cell signaling pathways that promote cancer stem cell survival and self-renewal may lead to novel therapeutic targets for this specialized sub-population of cancer cells. The overall hypothesis underlying this study was to identify possible signaling molecules that might be integral to the interaction between head and neck cancer stem cells (HNCSC) and tumor associated endothelial cells.

In a recent study, we identified that ALDH+CD44+Lin- cells had the properties of self-renewal, and tumorigenicity and were found spatially related to the tumor vasculature (Krishnamurthy *et al*., 2010). Selective ablation of the tumor associated endothelial cells using an inducible caspase system caused a decrease in the survival of ALDH+CD44+ cells *in vivo*. In recent years, there has been evidence of IL-6 promoting the self-renewal and survival of stem cells in breast and glial tissues (Sansone *et al*., 2007, Wang *et al.,* 2009). However, the origin of the IL-6 in the IL-6-STAT3 pathway has been varied. In our study, we hypothesized that there might be a paracrine signaling of IL-6-STAT3 pathway initiated by the endothelial cells apart from a possible autocrine pathway which might be in existence. To test this, we performed laser capture microdissection as described (Kaneko *et al*., 2007, Kaneko *et al*., 2011) from 4 different primary HNSCC. The tumor cells and endothelial cells were collected separately and RT-PCR analysis of these cells revealed a higher expression level of IL-6 mRNA in the tumor associated endothelial cells than the adjacent tumor cells (Figure

4.1A). We corroborated these findings in a head and neck cancer cell line, UM-SCC-74A with primary endothelial cells (HDMEC) as well (Figure 4.1A). We also found statistically significant higher protein expression levels of IL-6 in primary endothelial cells compared to a panel of head and neck cancer cell lines, (UM-SCC-74A, UM-SCC-74B, UM-SCC-17B) by ELISA (Figure 4.1B).

Head and neck cancer stem cells express IL-6 receptors, IL-6R and gp130.

To demonstrate the expression of IL-6R in cancer stem cells in primary HNSCC, we performed confocal imaging for co-localization of ALDH1 with IL-6R in 4 different patients and a representative image is shown (Figure 4.1C). Co-localization of IL-6R (red) was seen only with ALDH1+ cells (green) demonstrating the relative expression of IL-6R in the ALDH1+ cells. The gp130+ cells were found both in ALDH1+ cells and ALDH1- cells in the primary HNSCC (Figure 4.1C). We also evaluated the expression of IL-6 receptors, IL-6R and gp130 in head and neck cancer cell lines, UM-SCC-74A and UM-SCC-74B (Figure 4.1D). Head and neck cancer stem cells, HNCSC; (*i.e.,* ALDH+CD44+cells) expressed elevated levels of IL-6R than the parental cell line (unsorted cells) or the non-cancer stem cells (ALDH-CD44-). There was no significant difference in the gp130 levels between the cancer stem cells and non cancer stem cells.

Endothelial cell-derived IL-6 promotes tumor initiation and growth in HNCSC. To evaluate specifically the role of endothelial cell initiated IL-6 events, we silenced the expression of IL-6 in primary endothelial cells (HDMEC) and confirmed the effectiveness of silencing IL-6 expression by ELISA (Figure 4.S.1).

To examine the role of endothelial cell derived IL-6 on tumor initiation of head and neck cancer stem cells *in vivo*, we isolated HNCSC (ALDH+CD44+Lin- cells) from 3 different patients of primary HNSCC (Patients 19, 20, 21) and implanted in immune-deficient mice with either 1,000 cells of HNCSC and 500,000 cells of HDMEC-shRNA-C or 1,000 cells of HNCSC and 500,000 of HDMEC-shRNA-IL-6 (n=13) (Figure 4.2A). The tumor initiation graph demonstrated a significantly early initiation of tumors in the ALDH+CD44+Lin- +HDMEC-shRNA-C group than the ALDH+CD44+Lin- + HDMEC-shRNA-IL-6 group (Figure 4.2B). Tumor initiation was evaluated by both tumor palpability and the size of the scaffold to be greater than 75 mm^3. The tumor volumes of the individual mice demonstrate the earlier initiation and greater tumor volumes in the HDMEC-shRNA-C group compared to the xenografts generated from the HDMEC-shRNA-IL-6 group (Figure 4.2C). The assessment of the tumor volumes of the xenografts demonstrated that the tumor xenografts from the ALDH+CD44+Lin- + HDMEC-shRNA-C group tumors initiated earlier and tumor volumes were greater than the HNCSC+HDMEC-shRNA-IL-6 group significantly until about 50 days of tumor implantation, after which the effect was decreased (Figure 4.2C). The average tumor volumes of the xenografts from the three different primary HNSCC in this study demonstrated a similar trend with the control group being significantly greater than the shRNA-IL-6 group at day 45 (Figure 4.2D). These studies suggested that endothelial cell secreted IL-6 actively promotes tumor initiation and tumor growth of head and neck cancer stem cells *in vivo*.

The frequency or density of cancer stem cells in a tissue has recently been suggested as a possible risk factor for poorer prognosis in patients (Neumeister *et al*., 2010). As a result, we wanted to study the effect of endothelial cell IL-6 on tumor initiation of different frequencies or numbers of cancer stem cells in the same genetic background (immunodeficient mice). We therefore implanted a serial dilution of 1000, 100 and 10 ALDH+CD44+Lin- cells from the same primary HNSCC (Patient 26) with either HDMEC-shRNA-C or HDMEC-shRNA-IL-6 cells. We also implanted scaffolds with no ALDH+CD44+ cells (0 cells) as negative controls. Tumor initiation or time to palpability of the individual groups (n=3) showed that greater the number of ALDH+CD44+cells, earlier is the initiation of tumors (Figure 4.3A). Nevertheless, tumors in the ALDH+CD44+cells + HDMEC-shRNA-C initiated tumors earlier than the HDMEC-shRNA-IL-6 group at the same density of cancer stem cells as seen in Figure 4.2 and no tumors were created in the 0 group as expected (Figure 4.3A). The overall trend for time to palpability was analyzed by eliminating the 0 group (as no tumors were initiated), and we found a significant delay in tumor initiation in the ALDH+CD44+Lin- + HDMEC-shRNA-IL-6 group (Figure 4.3B). The tumor volume over time demonstrates that ALDH+CD44+Lin- + HDMEC-shRNA-C group initiated tumors earlier and were greater in volume but the ALDH+CD44+Lin- + HDMEC-shRNA-IL-6 tumor xenografts gradually reached the control group by the end of the study (Figure 4.3C). The pair-wise statistical comparisons between different ALDH+CD44+ cell densities suggested greater

the initial numbers of cancer stem cells, greater the effect of IL-6 in promoting tumors (Figure 4.3D).

IL-6 promotes survival of head and neck cancer stem cells *in vivo.* Our earlier studies demonstrated that endothelial cells in the vascular niche of the head and neck cancer stem cells promotes self-renewal and tumorigenicity of the ALDH+CD44+cells. Similar studies demonstrated that anti-angiogenic agents caused a decrease in the cancer stem cell survival in neural tissues (Calabrese *et al*., 2007). In our own work, we demonstrated that selective ablation of tumor endothelial cells caused a decrease in the cancer stem cell fraction (Krishnamurthy *et al*., 2010). To study if IL-6 might be a possible factor promoting survival of cancer stem cells in this interaction, we used our earlier strategy and implanted ALDH+CD44+Lin-cells from a primary HNSCC (Patient 22) with either HDMEC-shRNA-C or HDMEC-shRNA-IL-6 in immunodeficient mice for 30 days; n=3 (Figure 4.4A). The mice were euthanized by day 30 before host IL-6 could significantly contribute to the growth of the xenografts. The analysis of the cancer stem cell fraction revealed a significant decrease in the xenografts with the shRNA-IL-6 group (Figure 4.4B). Notably, there was an increase seen in the ALDH+CD44+ fraction of cells in the tumor xenografts arising from the control group suggesting there might be induction of self-renewal of the cancer stem cells in the presence of IL-6. We repeated this analysis with a head and neck cancer cell line, UM-SCC-74A, (n=6), wherein we observed that the control group initiated tumors earlier as expected (Figure 4.4C) and there was again a

significant decrease in the cancer stem cell fraction in the tumor xenografts of the HDMEC-shRNA-IL-6 group (Figure 4.4D).

Our studies demonstrated that endothelial cell derived IL-6 promotes self-renewal, survival and tumorigenicity of the cancer stem cells *in vivo*. However, the host-derived IL-6 was an important factor in this interaction. To address this key question, we generated tumor xenografts from ALDH+CD44+ and ALDH-CD44- cells in WT type and IL-6-/- immunodeficient mice; a gift from Dr. McCauley, University of Michigan (Figure 4.4E). We observed that tumors from the ALDH+CD44+cells in the WT type mice were significantly greater in volume than the IL-6-/-mice at Day 120. On FACS analysis of the percent of the cancer stems, we observed a significantly decreased percent of ALDH+CD44+cells in the tumor xenografts from the IL-6-/- mice (Figure 4.4F). Taken together, our data suggests that IL-6 is an important molecule in the interaction of head and neck cancer stem cells with their micro-environment contributing to tumor initiation and growth.

Head and neck cancer stem cells (ALDH+CD44+Lin- cells) co-relate with activated STAT3 pathway. The canonical IL-6 signal transduction pathway involves IL-6 ligand binding to the receptor IL-6R and to the common signal transducing receptor gp130. This activation propagates intracellular signaling of Janus kinases leading to the activation of STAT3. Activation of STAT3 has been related to self-renewal and stemness in various tissues including embryonic stem cells, intestinal stem cells and glioblastoma stem cells (Gao *et al.,* 2007, Niwa *et al*., 1998, Matthews *et al.,* 2011, Guryanova *et al*., 2011). To investigate the

possible relationship of activated STAT3 (p-STAT3) and head and neck cancer stem-like cells (ALDH+CD44+ cells) we analyzed by confocal imaging the spatial localization of ALDH1+ cells and phosphorylation of STAT3 in primary head and neck squamous cell carcinomas (HNSCC) (Figure 4.5A). DAPI was used to identify the nuclei and ALDH1+ cells were found to co-localize with p-STAT3 in primary HNSCC. Immunohistological examination of the tumor xenograft micro-environment showed expression of ALDH1 and p-STAT3 around the tumor vasculature (Figure 4.5B, arrow indicates positive cells). We further compared the relative protein expression of p-STAT3, we isolated ALDH+CD44+Lin-cells and ALDH-CD44-Lin-cells from a primary HNSCC and evaluated the phosphorylation of STAT3 by Western Blot (Figure 4.5C). ALDH+CD44+ cells have an increased phosphorylation status compared to the ALDH-CD44- cells. Activation of the ERK pathway showed the reverse expression trend as expected since the ERK-MAPK pathway has been implicated in the differentiation of stem cells (Gu *et al*., 2011, Kim *et al*., 2011, Lin *et al*., 2010, Sakai *et al.,* 2010). Similar phosphorylation pattern was seen in ALDH+CD44+ and ALDH-CD44- from the head and neck cancer cell line, UM-SCC-74A (Figure 4.5C). To test whether endothelial cell secreted factors enhanced activation of STAT3; we exposed ALDH+CD44+ cells to serum-free endothelial cell CM (HDMEC) and analyzed the phosphorylation levels of STAT3 and ERK over time. We found that endothelial cell CM induced phosphorylation in ALDH+CD44+ cells suggesting that head and neck cancer stem cells have an activated STAT3 pathway which could be further induced by endothelial cell CM (Figure 4.5D).

Endothelial cell-derived IL-6 promotes self-renewal and invasion of HNCSC *in vitro*. To assess the functional role of endothelial cell secreted IL-6 on ALDH+CD44+ cells *in vitro*; we exposed the unsorted parental cancer cell line, UM-SCC-74A to endothelial cell CM either pre-treated with neutralizing antibody to IL-6 or IgG and measured the ALDH+CD44+ percent by flow cytometry (Figure 4.6A). The quantification revealed that endothelial cell CM increased the cancer stem cell fraction which was reduced to parental levels in the presence of neutralizing antibody to IL-6 (Figure 4.6B). Treatment with rhIL-6 (20 ng/ml) also had an inductive effect on the percent of cancer stem cells, though to a lesser degree than endothelial cell CM (Figure 4.6B). This could possibly be due to additional factors in endothelial cell CM contributing to self-renewal or the possibility of CM containing more amounts of IL-6 than the rhIL-6 (20 ng/ml). The induction of phosphorylation of STAT3 by endothelial cell CM was decreased in the presence of neutralizing antibody to IL-6 (0.4 µg/ml) in the ALDH+CD44+ cells as seen by Western blot (Figure 4.6C). On generating orospheres from ALDH+CD44+ cells and ALDH-CD44- cells treated with endothelial cell CM with or without neutralizing antibody to IL-6 (Figure 4.6D), we observed greater number of orospheres arising from ALDH+CD44+ cells than from ALDH-CD44- cells which was further induced on treatment with endothelial cell CM. Neutralizing IL-6 reversed this induction as seen in the graph (Figure 4.6E).

The role of IL-6 on the self-renewal of ALDH+CD44+ cells was also tested using the Conditioned medium from the HDMEC-shRNA-IL-6 cells generated and used in the *in vivo* experiments. We also found an induction of the cancer stem

cell fraction of the parental cell line on treatment with the HDMEC-shRNA-C and HDMEC-shRNA-IL-6 CM (Figure 4.7A, 4.7B). The phosphorylation of STAT3 was also decreased on treatment with HDMEC-shRNA-IL-6 CM compared to HDMEC-shRNA-C CM (Figure 4.7C). We also analyzed the number of orospheres from the ALDH+CD44+ and ALDH-CD44- cells on treatment with the CM from the shRNA cells and observed similar trends of decreased self-renewal in the presence of shRNA-IL-6 CM group (Figure 4.7D). Taken together, these findings suggest that endothelial cell secreted IL-6 promotes self-renewal of head and neck cancer stem cells *in vitro*.

Cancer stem cells being invasive and migratory are believed to cause secondary tumors (Biddle *et al.,* 2011), and so we tested the invasive potential of ALDH+CD44+cells in the presence of HDMEC CM containing IL-6 and its absence in a transwell system coated with matrigel. The invasion assays revealed that the invasive potential of the ALDH+CD44+cells was inhibited in the presence of the neutralizing antibody to IL-6 or on treatment with HDMEC-shRNA-IL-6 (Figure 4.S.2).

Humanized IL-6R antibody decreases cancer stem cell survival and delays tumor initiation. Serum IL-6 levels have been shown to be an independent predictor of poor survival in head and neck squamous cell carcinoma patients (Duffy *et al.,* 2008, Trikha *et al.,* 2003, Hong *et al.,* 2007, Chen *et al.,* 1999). However, molecular targeting of IL-6 becomes challenging as individual patients have varying amounts of IL-6 and individualized therapy becomes necessary in the clinic. An alternative strategy to inhibit the IL-6-ligand initiated signaling would

be to target the IL-6R receptor and thereby prevent activation of STAT3. Tocilizumab is an FDA approved, humanized anti-human IL-6R antibody engineered by grafting the complementarily determining regions of a mouse anti-human IL-6R into human IgG (Shinriki *et al*., 2009).

The anti-IL-6R (10µg/ml) inhibited the induction of orospheres by the endothelial cell CM of HNCSC (Figure 4.8A) and decreased the phosphorylation of STAT3 *in vitro* (Figure 4.8B). To test the efficacy of inhibition of IL-6-IL-6R signaling on tumor initiation, we implanted ALDH+CD44+Lin- and ALDH-CD44-Lin-cells from a primary HNSCC (Patient 34) with primary endothelial cells (n=6); and treated with IL-6R antibody (or IgG controls) from Day 1 post-implantation every week for four weeks intra-peritoneally at 10 mg/kg body weight (Figure 4.8C). No significant weight reduction was observed in the mice during the course of this experiment (Figure 4.S.3). A hundred percent tumor initiation was observed in the IgG group (n=6), whereas only one tumor was created in the treatment group (IL-6R Ab) (Figure 4.8D). Tumor volumes at retrieval showed a greater volume in the IgG group compared to the treatment group (Figure 4.8E). Notably, there was a significant reduction in the cancer stem cell fraction (ALDH+CD44+Lin- cells) in the anti-IL-6R treated xenografts compared to the IgG control group (Figure 4.8F).

Discussion

The poor survival and high recurrences in head and neck squamous cell carcinomas can be possibly explained by the cancer stem cell hypothesis. It was recently found in a prospective study of about 444 patients of HNSCC that high serum IL-6 levels was related with increased recurrences and poorer prognosis (Duffy *et al.,* 2008). STAT3, a transcriptional factor activated by IL-6 has been found to be over-expressed in various tumors including HNSCC. Targeting cancer stem cells via the IL6/STAT3 pathway has been done in mammary tumors, lung adenocarcinomas and neural tumors (Sansone *et al*., 2007, Gao *et al.,* 2007, Wang *et al.,* 2009). The concept of IL-6 (an inflammatory stimulus), triggering a physiological mechanism of repair in the cancer tissue by stimulating proliferation and self-renewal of the cancer stem cells has been suggested (Sansone *et al*., 2007).

In our earlier study, we demonstrated the sub-population of ALDH+CD44+ cells to be cancer stem-like in HNSCC (Krishnamurthy *et al*., 2010). We found that these cells exist in vascular niches and that tumor endothelial cell actively promoted self-renewal and survival of the cancer stem cells. In this study, we observed tumor endothelial cells synthesize greater amounts of IL-6 mRNA than the tumor cells (Figure 4.1). The protein expression of IL-6 is also greater in primary endothelial cells in comparison to a panel of head and neck squamous cell carcinoma cell lines. In fact, it was recently shown in Burkitt's lymphoma, IL-6 is secreted from thymic blood vessels in response to therapy and promotes the survival of Doxorubicin-resistant lymphoma cells (Gilbert and Hemann, 2010).

Work from lung carcinomas recently showed that endothelial cell secreted factors could regulate different aspects of cancer cell function such as invasion, proliferation and tumor growth *in vivo* (Franses *et al.,* 2011).

The activation of phosphorylation of STAT3 along with the presence of the IL-6R in the primary cancer stem cells demonstrated an active IL-6/IL-6R/STAT3 pathway in the head and neck cancer stem cells. We observed active induction of self-renewal in the ALDH+CD44+cells *via* IL-6 from the endothelial cells *in vitro* which was significantly reduced in the presence of neutralizing antibody to IL-6. Though we observed an induction in the number of orospheres on treatment with rhIL-6, it was to a lesser degree than the induction by the endothelial cell conditioned medium. This suggests the possibility of other endothelial cell secreted factors that might contribute to the self-renewal of cancer stem cells. In fact, Interleukin-8 (CXCL-8) is a chemokine found in the cancer stem cells or its micro-environment and has been postulated as a therapeutic target for cancer stem cells in various tissues (Razmkhah *et al*., 2010, Ginestier *et al.,* 2010, Hwang *et al*., 2011).

In our study, we demonstrated that silencing the IL-6 gene in the endothelial cells alone resulted in a delay in the tumor initiation and growth of tumor xenografts arising from the ALDH+CD44+ cells (Figure 4.2). However, this effect was depreciated after 50 days which could possibly be explained by the host IL-6 contributing to the tumor environment. It is also a distinct possibility that the endothelial cells stably expressing shRNA-IL-6 clone had been lost and replaced by normal cells. Nonetheless, our studies demonstrated an important

role of IL-6 cells and its micro-environment composed of tumor endothelial cells. In our work, although we demonstrated the increased expression of IL-6 in the endothelial cells relative to the tumor cells (Figure 4.1); this does not preclude the possibility of an existing autocrine IL-6 pathway in the head and neck cancer stem cells. Moreover, we did not examine the relative IL-6 levels in the cancer stem cells compared to the non-cancer stem cells. In glioblastoma stem cells, the non cancer stem cells were found to have increased IL-6 expression levels than the cancer stem cells and were found to be the source of IL-6 signaling (Wang *et al*., 2009).

The importance of IL-6 on tumor initiation, irrespective of its source was demonstrated in our work on the xenografts generated from the IL-6-/- immuno-deficient mice (Figure 4.4). We observed the tumor growth over time for four months and still found a significant difference in tumor volumes between the WT type and the IL-6-/- mice unlike using the shRNA strategies wherein the effect is lost by 80 days. This demonstrates that IL-6 derived from different sources can potentially promote tumor initiation of cancer stem cells. Thereby, targeting IL-6 signaling pathway could be beneficial to patients with squamous cell carcinomas. In fact, CNTO328, an anti-IL-6 chimeric monoclonal antibody is in clinical trials for refractory multiple myelomas and B-cell Non-Hodgkin's lymphoma (van Zaanen *et al*., 1996, Voorhees *et al.*, 2009). The fact that serum IL-6 levels has been related to increased tumor recurrences and poorer prognosis (Duffy et al., 2008) and in our work we see an increased tumor initiation in the presence of IL-

6, suggests that Interleukin-6 could also be a potential prognostic marker in patients with head and neck squamouc cell carcinomas.

Tocilizumab (Actemra) is a humanized monoclonal antibody against IL-6R, recently approved by the FDA for use in autoimmune disorders. The use of anti-IL-6R in our model delayed the tumor initiation by preventing the activation of STAT3 (Figure 4.8). Moreover, the analysis of the cancer stem cells in our xenografts showed a significant decrease in the Tocilizumab treatment group suggesting that inhibition of IL-6 signaling decreases the survival of the head and neck cancer stem cells. The *in vitro* invasion data (Figure 4.S.2) suggested that the invasive potential of the head and neck cancer stem cells is increased in the presence of IL-6 which could translate into metastasis *in vivo*. In fact, in a recent study, targeting STAT3 with Cucurbitacin I inhibited tumorigenicity and distant metastasis of head and neck cancer stem cells (Chen *et al.,* 2010). This might suggest a role of IL-6/STAT3 signaling in EMT transition of cells as seen in breast cancers (Sullivan *et al.,* 2009). Although the complete mechanism of ALDH+CD44+ cells is still unclear, our data suggests that IL-6/STAT3 paracrine signaling with the micro-environment might possibly be involved with the tumor initiation and even progression of cancer stem cells

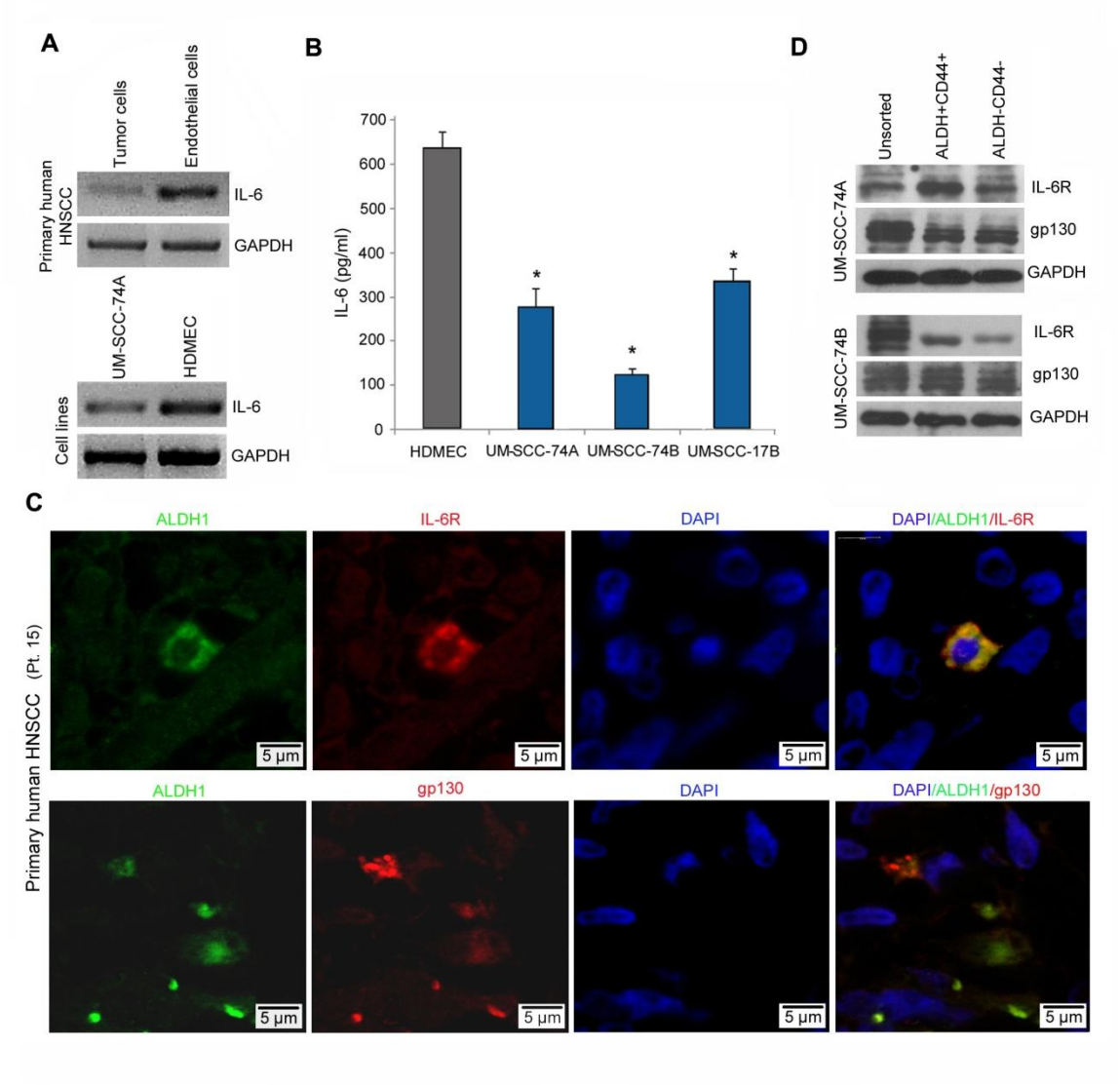


Figure 4.1. Analysis of IL-6 expression in tumor endothelial cells and IL-6R in head and neck cancer stem cells. (**A**) RT-PCR showing the mRNA expression of IL-6 in tumor cells compared to the tumor endothelial cells obtained by Laser Capture micro-dissection from primary HNSCC. Similar trend is observed in the expression of IL-6 in a head and neck cancer cell line, UM-SCC-74A to primary endothelial cells (HDMEC). (**B**) Graph showing Interleukin-6 levels in primary endothelial cells (HDMEC) compared to three different head and neck squamous cell carcinoma cell lines, UM-SCC-74A, UM-SCC-74B, UM-SCC-17B obtained by ELISA. Data was normalized by cell number (*$p<0.001$). (**C**) Confocal microscopy of primary human HNSCC (patient 15) immunostained for ALDH1 (green) and IL-6R (red) or gp130 (red) and DAPI to identify nuclei. The overlay image shows the co-localization of the ALDH-positive cells with the IL-6R-positive cells. (**D**) Western Blot for IL-6R and gp130 in head and neck cancer stem-like cells (ALDH+CD44+cells) and non-cancer stem cells (ALDH-CD44-cells) in HNSCC cell lines, UMSCC-74A and UM-SCC-74B.

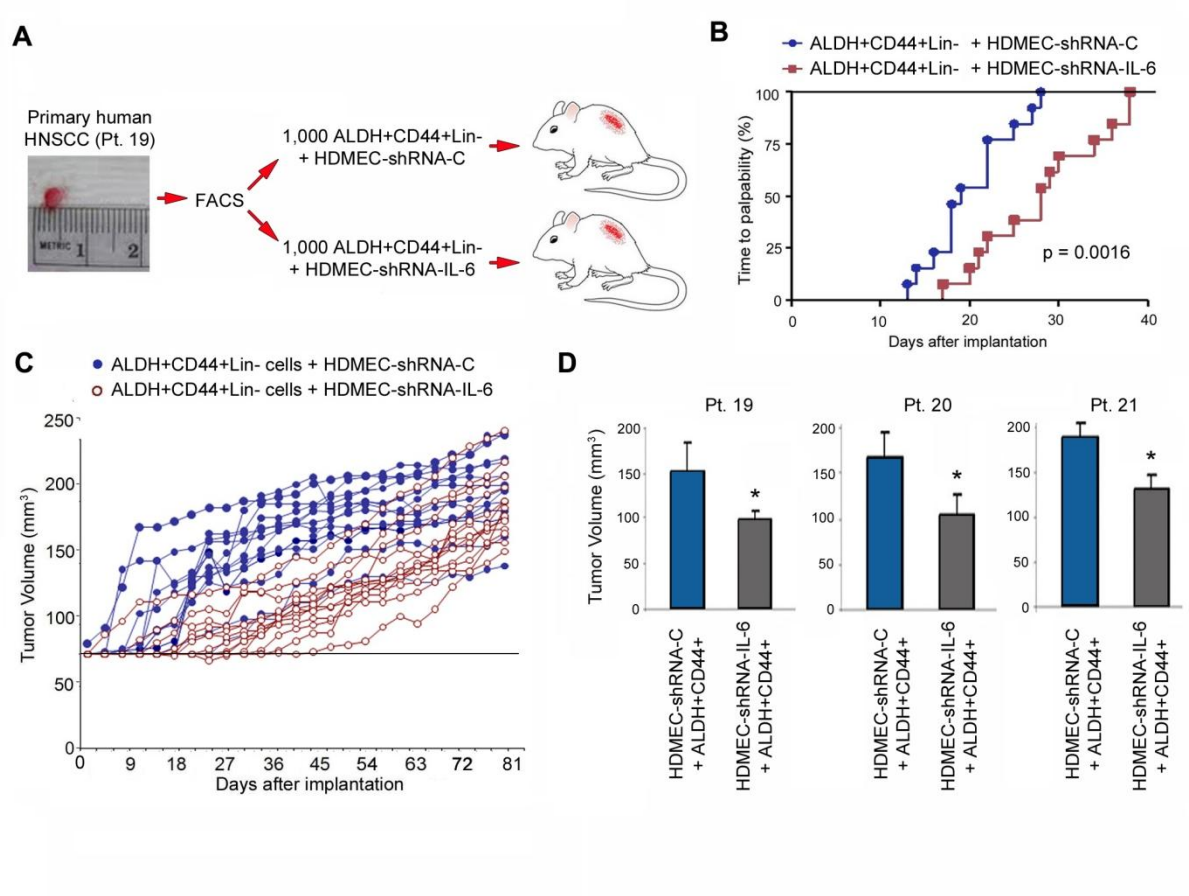
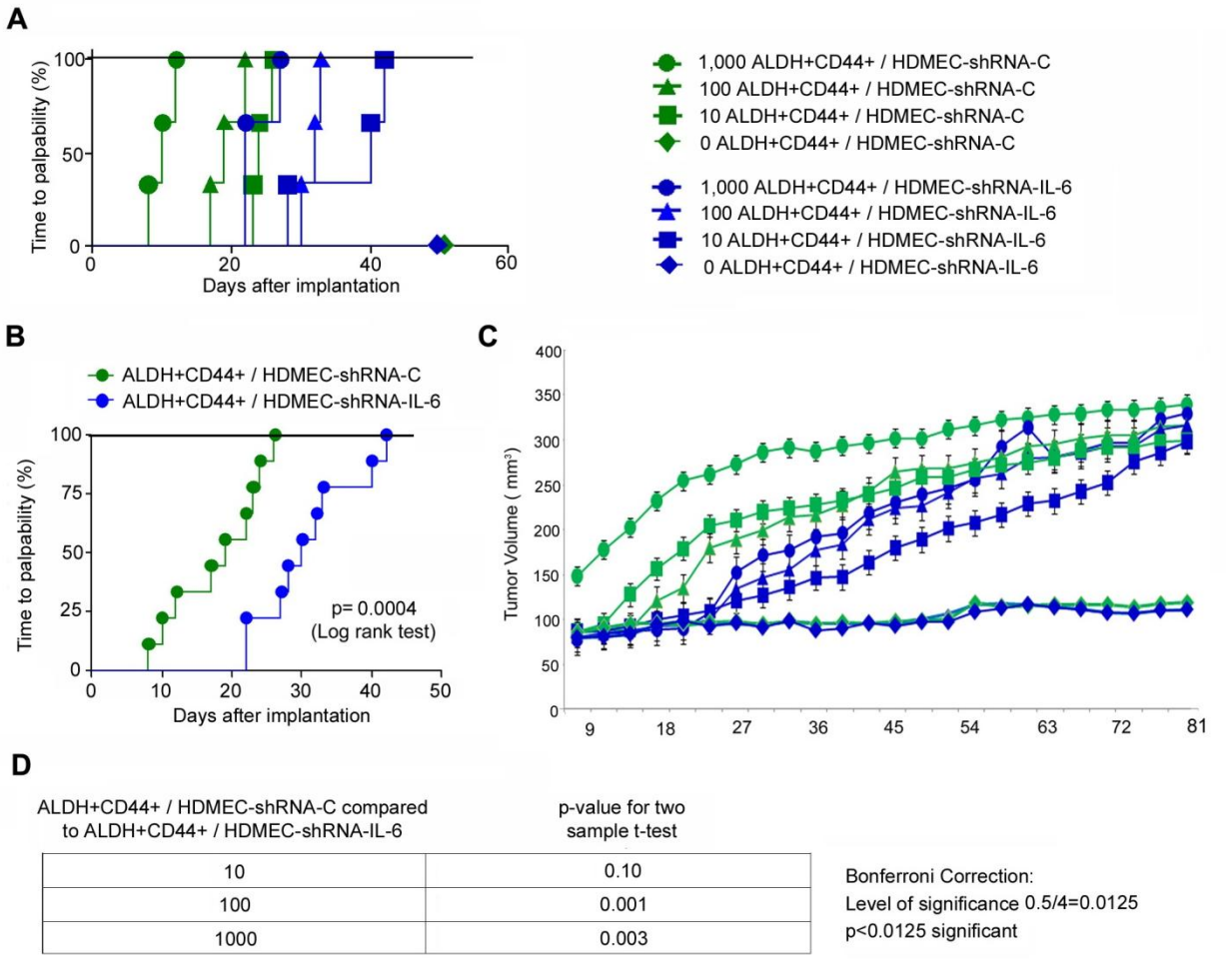


Figure 4.2. Endothelial cell secreted IL-6 promotes tumor initiation and growth of head and neck cancer stem-like cells. (**A)** Schematic representation of the approach used for testing the role of endothelial cell secreted IL-6 on the tumorigenic potential of ALDH+CD44+ cells in primary tumors. ALDH+CD44+Lin- cells were isolated from 3 different primary HNSCC and implanted in immuno-deficient mice along with HDMEC-shRNA-C or HDMEC-shRNA-IL-6. (**B**) Graph shows the relative occurrence of time to palpability of tumors (i.e., the time at which tumors are initiated) in the ALDH+CD44+Lin- tumors with HDMEC-shRNA-C or with HDMEC-shRNA-IL-6. (**C**) Graph shows the tumor volume of the individual mice (n=13) obtained by the implantation of ALDH+CD44+cells with HDMEC-shRNA-C or HDMEC-shRNA-IL-6. (**D**) Graph shows the relative tumor volumes of the two groups obtained from the three different patients at Day 45. *p<0.05.



D

ALDH+CD44+ / HDMEC-shRNA-C compared to ALDH+CD44+ / HDMEC-shRNA-IL-6	p-value for two sample t-test
10	0.10
100	0.001
1000	0.003

Bonferroni Correction:
Level of significance 0.5/4=0.0125
p<0.0125 significant

Figure 4.3. Endothelial cell secreted IL-6 promotes tumor initiation of ALDH+CD44+cells relative to their cell numbers. (**A**) ALDH+CD44+Lin- cells were isolated from a primary HNSCC (Patient 26) and implanted in serial dilutions of 1000, 100, 10 or 0 cells along with 500,000 cells of HDMEC-shRNA-C or HDMEC-shRNA-IL-6 in immunodeficient mice. Graph shows the relative occurrence of tumor initiation in the various individual groups. (**B**) Tumors generated from ALDH+CD44+cells with HDMEC-shRNA-C or with HDMEC-shRNA-IL-6 group irrespective of the ALDH+CD44+cell numbers was expressed as an overall tumor initiation graph after eliminating tumors that did not occur (0 group). (**C**) Graph shows the tumor volume of the individual groups obtained by the implantation of ALDH+CD44+cells with HDMEC-shRNA-C or HDMEC-shRNA-IL-6. (**D)** The pairwise statistics for the different frequencies of ALDH+CD44+cells (10,100, 1000) that created tumors was determined using the Bonferroni correction and established to be *p<0.0125.

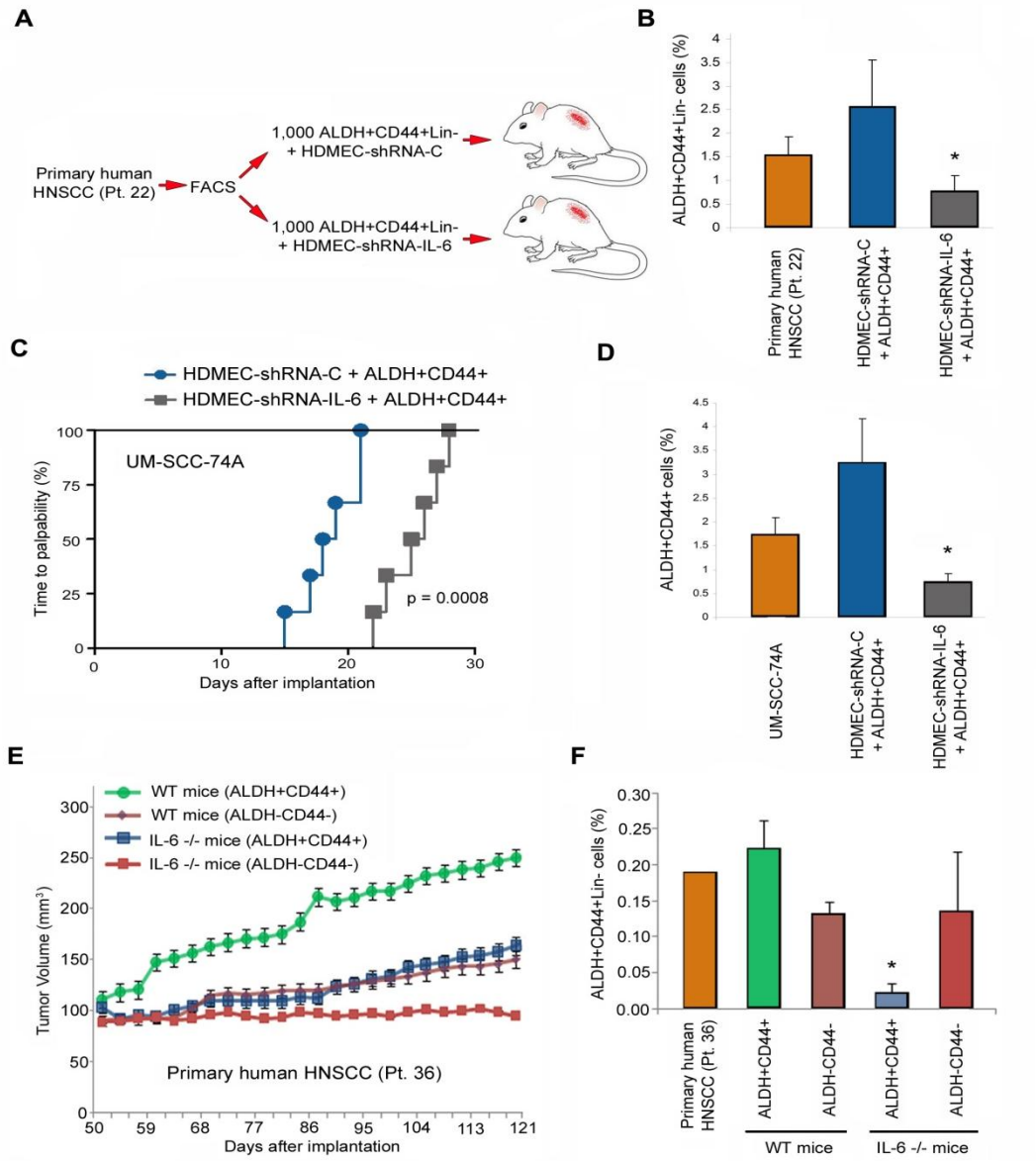


Figure 4.4. Endothelial cell secreted IL-6 promotes survival and self-renewal of head and neck cancer stem cells *in vivo*. (**A**), ALDH+CD44+Lin- cells isolated from a primary HNSCC (Patient 22) and implanted with HDMEC-shRNA-C or HDMEC-shRNA-IL-6. (**B**) Tumors retrieved after 30 days and percentage of ALDH+CD44+ fraction is evaluated in primary tumors and xenografts. (**C**) Graph represents the tumor initiation of ALDH+CD44+cells from UM-SCC-74A, implanted with HDMEC-shRNA-C or HDMEC-shRNA-IL-6 (n=6) (**D**) percent of ALDH+CD44+cells in the xenografts compared to the parental cell line (**E**) Tumor volumes from ALDH+CD44+Lin- cells and ALDH-CD44-Lin- cells in WT type and IL-6-/- immunodeficient mice from a primary HNSCC (Patient 36). (**F**) Cancer stem fraction (ALDH+CD44+Lin-cells) in the tumor xenografts compared to the primary tumor.

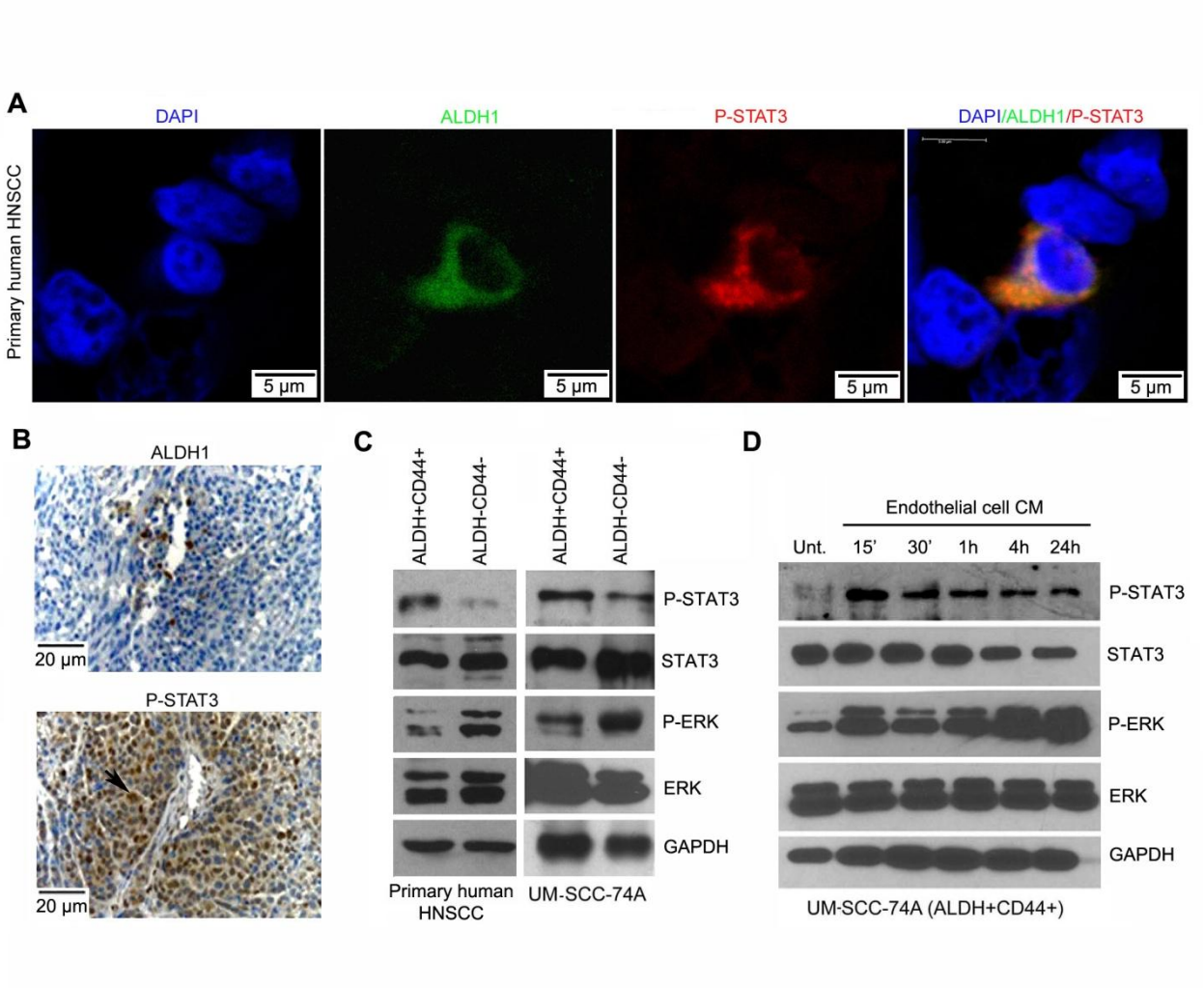


Figure 4.5. Endothelial cell-derived factors activate STAT3 pathway in head and neck cancer stem-like cells (**A**) Single cell co-localization of head and neck cancer stem-like cells (ALDH1+) cell with phospho-STAT3 as visualized by confocal imaging in a primary HNSCC. DAPI identifies the nuclei and the merged image shows the co-localization of ALDH1 and P-STAT3. (**B**) Immunohistological representation of ALDH1+cells and p-STAT3+ in a peri-vascular localization (arrows) in tumor xenografts generated from primary ALDH+CD44+cells. (**C**) Baseline phosphorylation of STAT3 in head and neck cancer stem-like cells (ALDH+CD44+) compared to non-cancer stem-like cells (ALDH-CD44-) in a primary HNSCC (Patient 28); and a head and neck cancer cell line, UM-SCC-74A. (**D**) Western blot for phosphorylated and total STAT3, ERK in ALDH+CD44+ cells on treatment with endothelial cell conditioned medium.

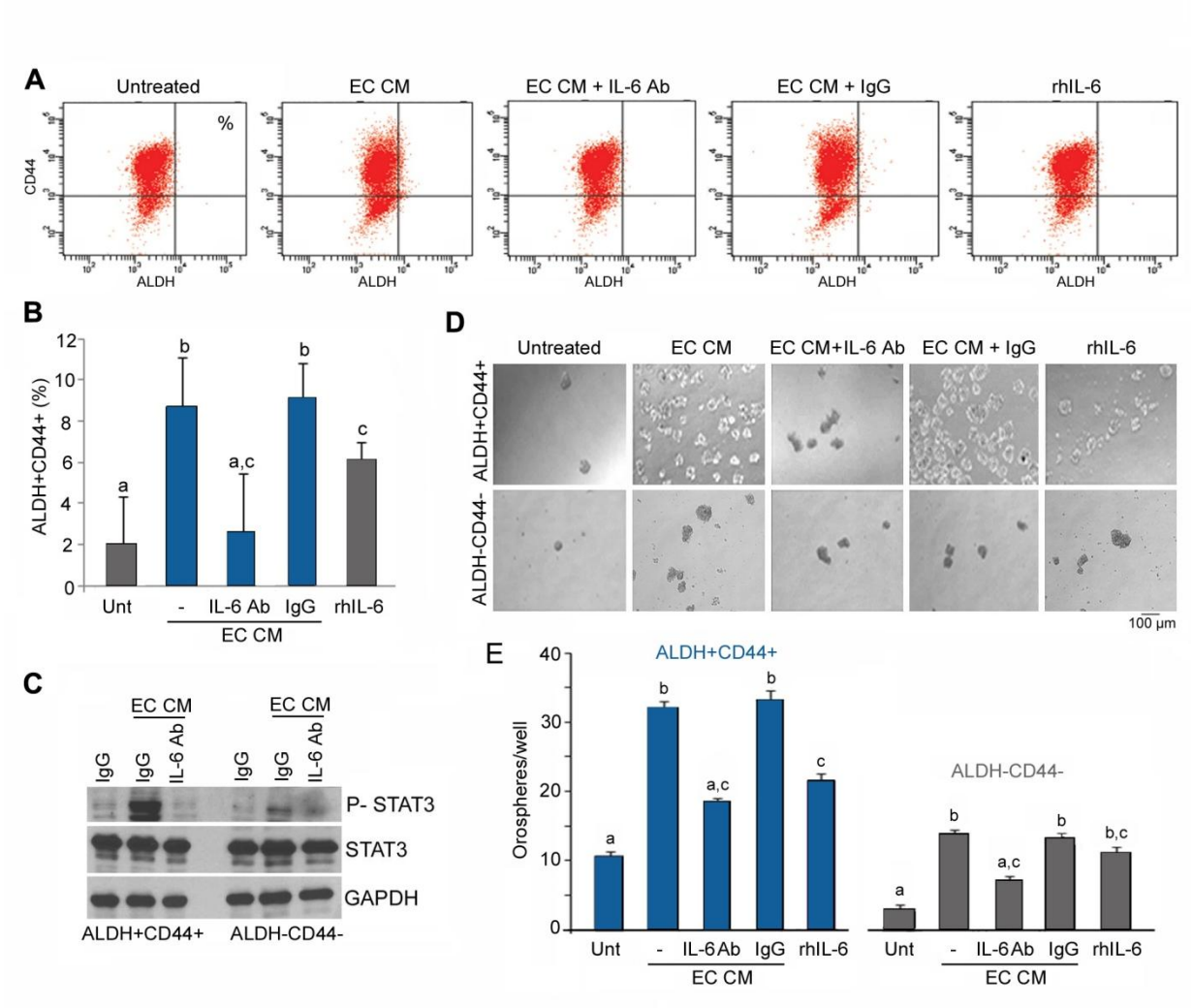


Figure 4.6. Endothelial cell secreted IL-6 signaling promotes self-renewal of head and neck cancer stem-like cells *in vitro.* (**A**) FACS analysis of UM-SCC-74A cells treated with endothelial cell CM with or without neutralizing antibody to IL-6 for 48 hours. The quantification of triplicates of treatment is depicted in (**B**). (**C**) ALDH+CD44+ and ALDH-CD44- cells, serum starved and exposed to endothelial cell CM with and without neutralizing antibody to IL-6. Phosphorylation of STAT3 was detected by Western Blot. (**D**) photomicrographs of representative orospheres arising from ALDH+CD44+ and ALDH-CD44-cells and cultured under low attachment plates. Cells were treated with endothelial cell CM with and without neutralizing antibody to IL-6 and quantified in (**E**).

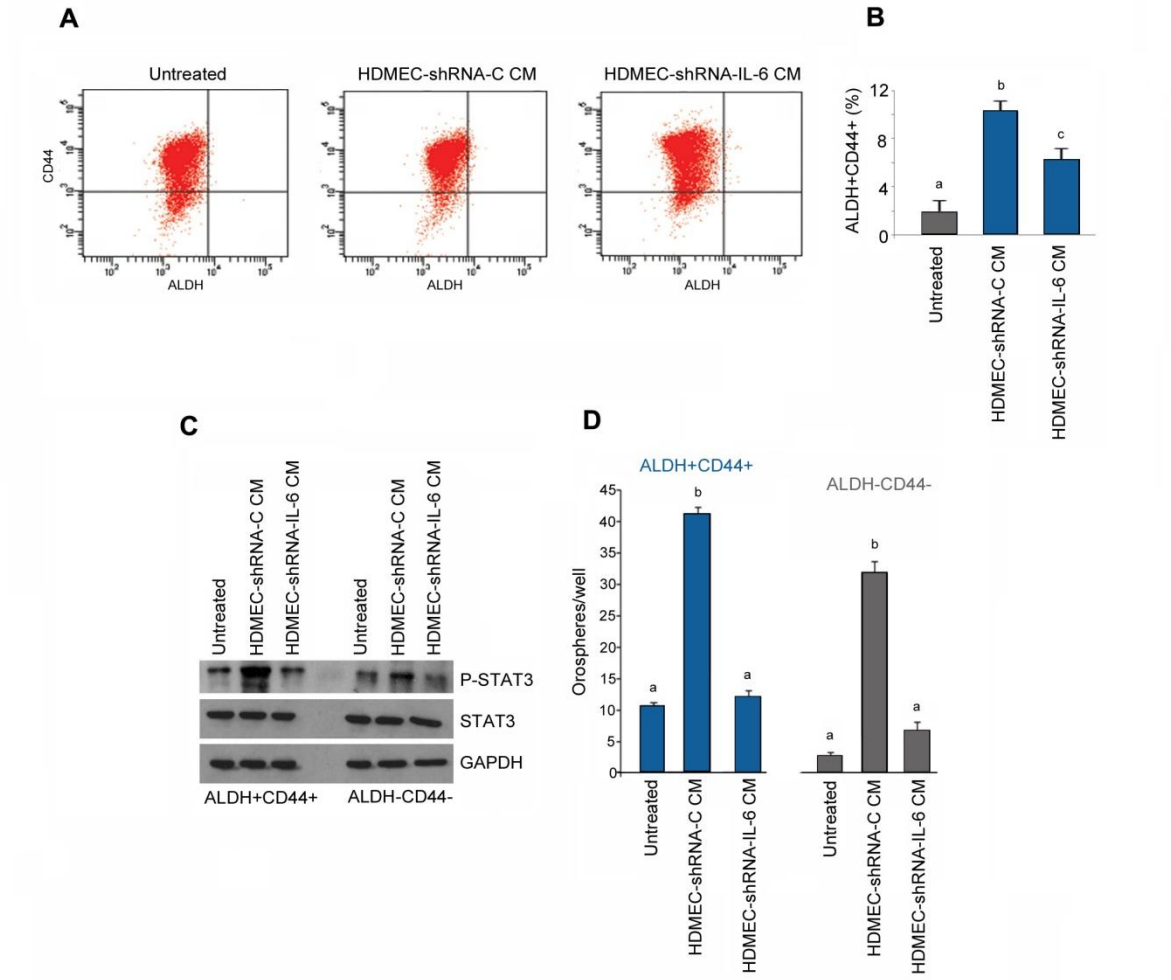


Figure 4.7. Effect of HDMEC-shRNA-IL-6 on self-renewal of head and neck cancer stem-like cells. (**A**) UM-SCC-74A treated with HDMEC-shRNA-C or HDMEC-shRNA-IL-6 CM for 48 hours and the cancer stem cell fraction is determined by FACS. The quantification is seen in (**B**). (**C**) ALDH+CD44+ and ALDH-CD44- cells were serum starved and exposed to HDMEC-shRNA-C or HDMEC-shRNA-IL-6 CM for 30 minutes. Phosphorylation of STAT3 was detected by Western Blot. (**D**) Orospheres arising from ALDH+CD44+ and ALDH-CD44- cells cultured under low attachment plates and treated with HDMEC-shRNA-C, HDMEC-shRNA-IL-6 or unconditioned DMEM and is quantified.

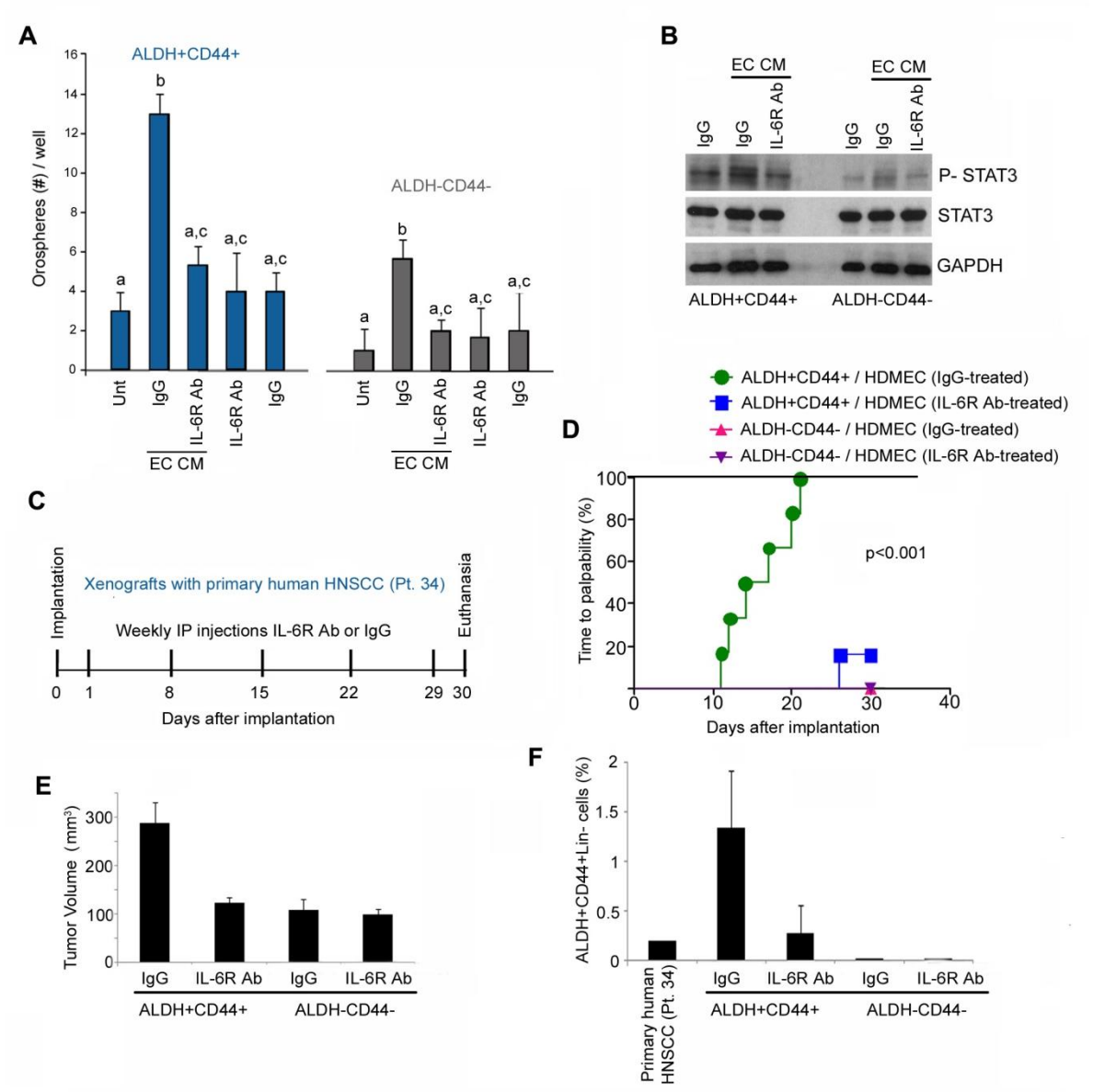


Figure 4.8. Tocilizumab or humanized anti-IL-6R antibody inhibits self-renewal and tumor initiation of head and neck cancer stem cells *in vitro* and *in vivo*. (**A**) Graph represents the relative number of orospheres arising from ALDH+CD44+ and ALDH-CD44- cells in the presence and absence of anti-IL-6R (10µg/ml) with HDMEC CM. (**B**) represents the phosphorylation of STAT3 on treatment with anti-IL-6R (10µg/ml). (**C**) Scheme of the preventive regimen for tumor initiation of ALDH+CD44+Lin-cells and ALDH-CD44-Lin-cells from a primary HNSCC (Patient 34). Mice are treated every week IP with IgG or anti-IL-6R for 4 weeks from Day 1. (**D**) Graph shows the time for tumor initiation in the IgG group and the anti-IL-6R treatment group in one month. (**E**) Represents the relative tumor volumes at retrieval in the individual groups and the cancer stem cell fraction (i.e., ALDH+CD44+Lin-cells) in (**F**).

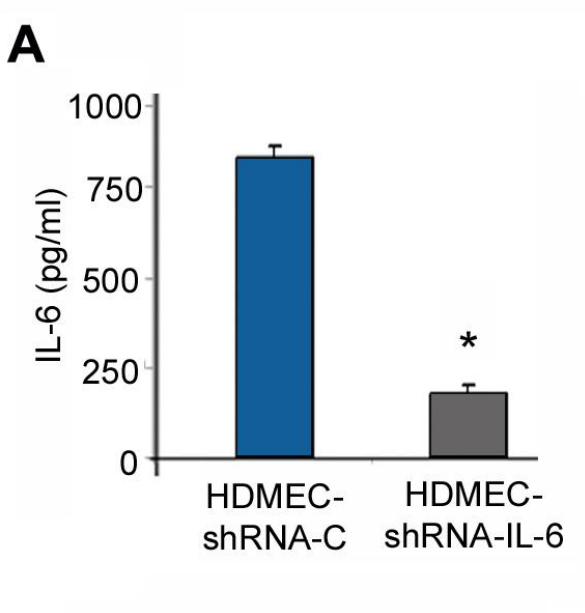


Figure 4.S.1 ELISA for IL-6 expression in HDMEC transfected with shRNA-C or shRNA-IL-6. (**A**) HDMEC were transfected with shRNA-C or shRNA-IL-6 for 48 hours and cells were selected for two weeks. Data is normalized by cell number.

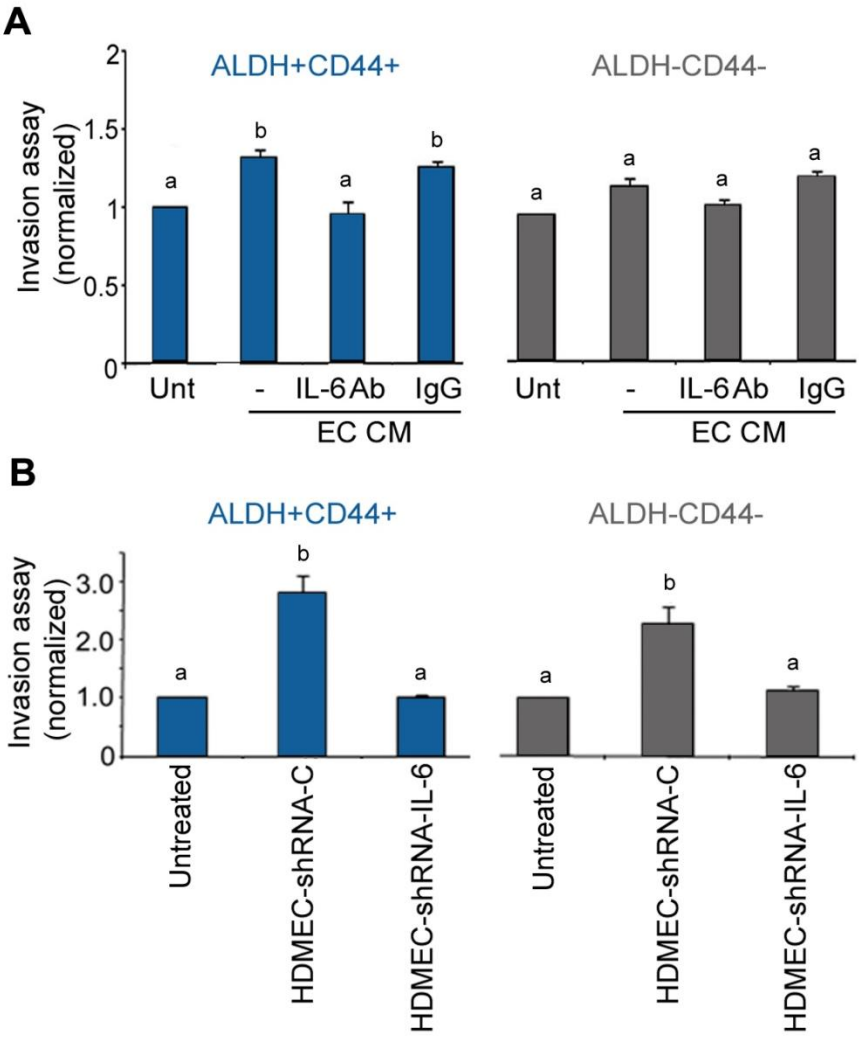


Figure 4.S.2 Head and neck cancer stem cell invasion. (**A**) Head and neck cancer stem cell (ALDH+CD44+) invasion was evaluated in the presence of endothelial cell CM with or without neutralizing antibody to IL-6. (**B**) ALDH+CD44+ cell invasion was evaluated in the presence of HDMEC-shRNA-C, HDMEC-shRNA-IL-6 or unconditioned DMEM.

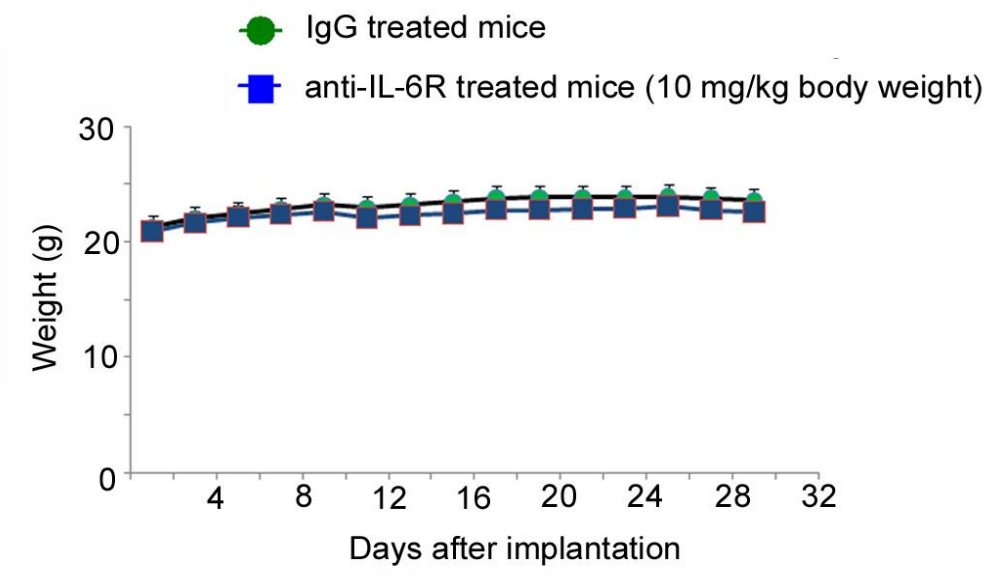


Figure 4.S.3 Comparable mice weights on treatment with anti-IL-6R. Graph shows the relative weights of the mice in the IgG group and the anti-IL-6R treatment group during the course of the study (30 days).

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CHAPTER V

CONCLUSION

Head and neck squamous cell carcinomas contain a sub-population of highly tumorigenic cells that exhibit self-renewal and multipotency. These cells can be isolated from primary head and neck squamous cell carcinomas using Aldehyde Dehydrogenase (ALDH) activity and CD44 expression, and have been named head and neck cancer stem cells (HNCSC). It has been proposed that the HNCSC are the “drivers” of head and neck tumor progression, and therefore have to be eliminated to achieve cancer cure. However, little is known about mechanisms underlying the survival and self-renewal of HNCSC. The hypothesis underlying this dissertation is that head and neck cancer stem cells (*i.e.* ALDH+CD44+) are localized in the perivascular niche and depend on endothelial cell-secreted factors for their survival and self-renewal.

Here, we observed that HNCSC are found in close proximity to blood vessels in primary head and neck squamous cell carcinomas. Endothelial cell-secreted factors promoted the proliferation, self-renewal and survival of HNCSC *in vitro,* as evidenced by the increase number of orospheres (i.e. non-adherent colonies of cells) formed in soft agar or ultra-low attachment plates. *In vivo*,

selective ablation of tumor endothelial cells caused a marked decrease in the fraction of HNCSC suggesting that cancer stem cells depend on intact perivascular niches for their survival.

In our search for a possible mechanism to explain the dependency of HNCSC on endothelial cells, we observed that cancer stem cells have a constitutively active IL-6R/STAT3 pathway, while tumor endothelial cells secrete high levels of IL-6. Inhibition of endothelial cell IL-6 caused a decrease in the self-renewal and tumorigenicity of the HNCSC. Notably, a humanized antibody to IL-6R delayed tumor initiation and decreased the survival of HNCSC *in vivo*. Collectively, these results unveil the endothelial cell-secreted factors as critical regulators of HNCSC survival and self-renewal, and suggest that patients with head and neck cancer might benefit from targeted therapies against cancer stem cells.

Our understanding of the pathobiology of head and neck squamous cell carcinomas has been steadily increasing in the last couple of decades. Despite these various advances in prevention and treatment of HNSCC, the 5-year survival rate has not significantly increased. This poor prognosis is primarily due to the increased recurrences seen in head and neck cancers. The cancer stem cells, the sub-population of cells which are believed to be tumorigenic, are hypothesized to be responsible for the recurrences seen in cancers. With this perspective in mind, we wanted to explore the characteristics of the cancer stem cells in HNSCC and try to identify the micro-environment in which they exist. Little is known about the molecular mechanisms that distinguish the head and

neck cancer stem cells from the rest of the tumor cells. The identification of novel molecular mechanisms that regulate these cells would be critical in developing targeted therapy against head and neck cancer stem cells.

In Chapter III, we demonstrated that ALDH+CD44+Lin- cells from primary HNSCC had a cancer stem-like phenotype. We demonstrated that only a 1000 cells of the ALDH+CD44+ cells could generate tumors consistently compared to the 10,000 cells of the ALDH-CD44- cells. Moreover, the tumor xenografts we generated from the ALDH+CD44+ cells resembled the primary tumors histologically. We demonstrated their self-renewal and developed an assay to study and expand cancer stem cells *in vitro* which we termed the “orosphere assay” in Chapter II. These ALDH+CD44+ cells could also be differentiated to their parental cell line on culturing under regular attachment conditions thus satisfying the two tenets of making the ALDH+CD44+ cells as cancer stem cells in head and neck squamous cell carcinomas.

Cancer stem cells are being targeted in various cancers today either in lieu of their altered genetic signature or due to their dependence on their micro-environment or their niche. The cancer stem niche in HNSCC had not been identified. In Chapter III, we set out to identify a possible peri-vascular niche for the ALDH+CD44+ cells. In our study of the normal mucosa, we identified the stem cells (ALDH1+) cells to be found in the basal layer of the stratified epithelium as seen in the stem cells of the skin. However, in primary cancers we observed a spatial relationship of the ALDH1+cells to the vasculature. We observed that every ALDH1+ cell in 8 different patients with HNSCC was found

within a distance of 100 μm of a blood vessel. We were intrigued by this localization and decided to study the possible role of endothelial cells on the cancer stem cells.

One of the first things we decided to explore in the possible crosstalk between the endothelial cells and head and neck cancer stem cells was to study non-contact cell-cell interaction. As a result, we collected Conditioned media from endothelial cells and studied the effects of endothelial cell secreted factors on the cancer stem cells. We found that the factors from endothelial cell Conditioned medium had an induction in the expression of Bmi-1 and increased number of orospheres; demonstrative of self-renewal in the head and neck cancer stem cells. At this time, it was necessary to perform *in vivo* assays showing the importance of the endothelial cells in the tumorigenicity and the survival of the cancer stem cells. We addressed this by two independent strategies, we generated tumor xenografts from ALDH+CD44+ cells either in the presence or absence of the endothelial cells and we showed that the tumor growth is greater in the presence of endothelial cells. Alternatively, we generated tumor xenografts with endothelial cells transduced with an inducible caspase-9 which we could selectively induce apoptosis, and then examined the survival of the cancer stem cell fraction and saw that the loss of tumor endothelial cells decreased the survival of the cancer stem cell fraction.

In Chapter IV, we tried to dissect the endothelial-stem cell crosstalk and identify the possible factors in the endothelial cell CM which promoted the self-renewal and survival of the head and neck cancer stem cells. In primary HNSCC,

we demonstrated the increased IL-6 mRNA expression in the tumor endothelial cells than tumor cells. We also observed that ALDH+CD44+ cells have an activated STAT3 pathway, a downstream target of IL-6. Using an antibody against IL-6 and shRNA-IL-6 approaches, we demonstrated that endothelial cell secreted IL-6 induced self-renewal of the ALDH+CD44+ cells. There was an increased tumorigenicity in the presence of IL-6 which was significantly lost or delayed in the absence of IL-6 as demonstrated by our transgenic mice (IL-6-/- mice and our shRNA-IL-6). We also observed a significant reduction of the cancer stem fraction (ALDH+CD44+) in the tumor xenografts suggesting the importance of IL-6 in maintenance of the cancer stem cells *in vivo*.

At this point, we were interested in developing an agent to interfere in the endothelial cell–cancer stem cell interaction to potentially translate as an adjuvant therapy to delay recurrences. With this long-term perspective, we used Tocilizumab, an FDA approved humanized anti-IL-6R antibody (Chugai Pharmaceuticals), in an *in vivo* study and showed that interfering with the IL-6 signaling causes a delay in the tumor initiation and a concomitant decrease in the cancer stem cell fraction. In Figure 5.1, we propose a model that has been described in this dissertation. The cancer stem cells (ALDH+CD44+) in head and neck squamous cell carcinomas are found in a vascular niche. The endothelial cells secrete various factors which promote the self-renewal and survival and tumorigenicity of the cancer stem cells (Chapter III). One such molecule is the inflammatory cytokine IL-6. IL-6 secreted from the tumor endothelial cells bind to the cancer stem cells via IL-6R and activates STAT3. Self-renewal is induced as

a result of this, cancer stem cells increase in number, and tumor growth ensues. Interference of the IL-6/IL-6R signaling by either antibody against IL-6 or use of the anti-IL-6R antibody, caused a decrease in the survival of the cancer stem cells; loss of the tumor initiating cells and delay in tumor growth and recurrences.

The specific conclusions of this dissertation are:

1. ALDH+CD44+ cells are cancer stem-like cells in head and neck cancers.
2. ALDH+CD44+cells exist in vascular niches and the endothelial cells secrete factors that promote their self-renewal and tumorigenicity.
3. The orosphere assay might prove to be a valuable tool to study and expand cancer stem cells *in vitro*.
4. Endothelial cell initiated IL-6/IL-6R/STAT3 pathway promotes self-renewal, survival and tumor initiation of the head and neck cancer stem cells *in vivo.*

In summary, our work demonstrates that endothelial cells play an integral role in the maintenance and survival of head and neck cancer stem cells. Targeting cancer stem cells either *via* their niche or by exploring the specific molecular mechanisms that regulate them would result in newer, better therapeutics benefiting patients with HNSCC. Though beyond the scope of this project, the concept of re-programming (differentiated cells re-acquiring the characteristics of stem cells) would also be an area that merits detailed study. In fact, the fact that ALDH-CD44-cells could induce limited tumorigenicity and scope of forming tumors would lend credence to this theory.

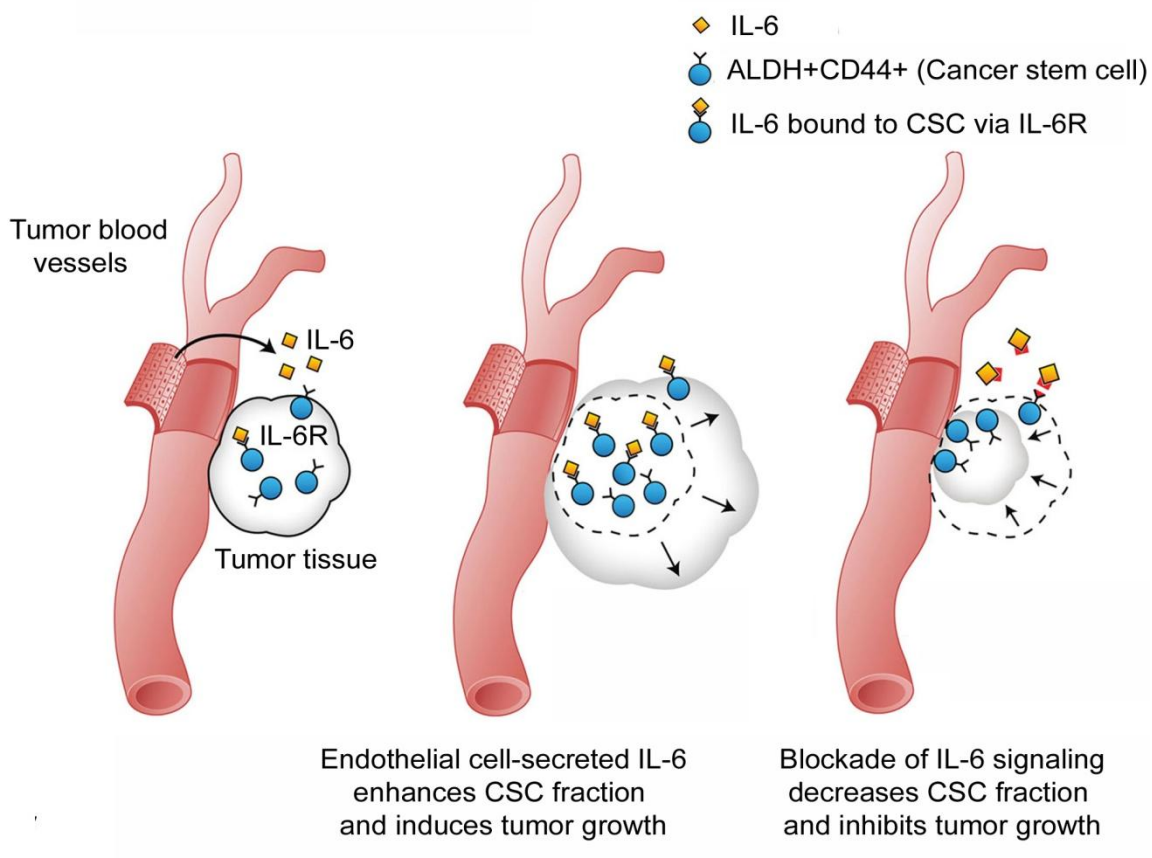

Figure 5.1. Illustrative representation of IL-6-IL-6R signaling in head and neck cancer stem cells. HNSCC is a heterogenous tissue with a few cancer stem cells (ALDH+CD44+). Tumor associated blood vessels provide a vascular niche and secrete factors including IL-6 which binds to IL-6R on the cancer stem cells. IL-6-IL-6R activates STAT3 pathway and results in self-renewal of ALDH+CD44+ cells and tumor growth. Inhibition of activation of IL-6 pathway either by antibodies to IL-6 or to IL-6R prevents the activation of the pathway, decreased survival and proliferation of the cancer stem cells and tumor initiation and volume decreased.

APPENDIX

LIST OF PROTOCOLS

1. Tumor Dissociation Protocol: for primary tumors and xenografts
2. Head and neck cancer stem cell isolation via Fluorescence Activated Cell Sorting (FACS) for ALDH and CD44
3. Suspension Cultures:

   A, Orospheres

   B, Colony Formation Assay
4. Immunohistochemistry for ALDH1
5. Cell localizations using Confocal Imaging for ALDH1, CD44, P-STAT3, IL-6R, gp130

Tumor Dissociation Protocol

Purpose: To obtain single cell suspensions of primary head and neck squamous cell carcinomas or tumor xenografts

Materials Required:

1. Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% Fetal Bovine Serum (FBS Gibco 10082; Invitrogen), 1% Antibiotic Antimycotic Solution (AAA, Sigma A5955; St. Louis, MO)
2. DMEM/F12 (no supplements, Hyclone, Waltham, MA)
3. Collagenase /Hyaluronidase (Stem Cell Technologies 07912; Vancouver, BC, Canada)
4. ACK Lysing Buffer (Gibco A10492)
5. 40 µm nylon mesh or sieve (BD Falcon 352340; Franklin Lakes, NJ)

General Instructions:

Please use a disposable gown, mask, gloves while working with primary samples.

Procedure:

1. Obtain tumor specimens (primary samples from OR or xenografts from mice) and place it immediately in complete DMEM or transport media.

   Note: This complete DMEM has 10% FBS and 1% AAA

2. Pour off most of the media leaving about 5 ml with the sample. Wash the sample with the DMEM complete media, 3-4 times (essential with the

primary samples so that we can surface clean the samples for bacterial and fungal pathogens).

Note: i) This is vital especially if we culture primary samples subsequently as it helps minimize contamination.

ii) Please do not vacuum out the media during washing, decant the old media and add fresh media to wash gently, this helps in preventing possible contamination and also minimizes cell lysis.

3. Place tumor and 5 ml of the same complete media in a sterile petri dish.

   Note: 1. Do not use tissue culture dishes.

   Mince the tumor with sterile scalpel/razor blade until no large chunks are seen. The tumor pieces should pass through a 25ml pipette.

Note: This is a critical step; chop the tumor in one direction to small pieces and then re-cross them in the opposite direction as minimally as possible. Use of too much force lyses the cells resulting in low number of viable cells.

4. Place the minced tumor in a 50ml conical tube and fill up to 25-30 ml with plain DMEM/F12.

   Spin at 800 rpm, 4˚C for 3 minutes to wash.

5. Carefully decant media and place tumor pellet in the petri dish.
6. Dilute 1 ml of 10X collagenase/Hyaluronidase in 9 ml DMEM/F12. Take 5 ml of the solution. Wash the 50 ml tube with enzyme solution and pipette gently on the sample. Repeat with the final 5 ml of enzyme solution.

Note: this is done so that there are no tumor chunks/cells left in the tube. You can go down to about 6-7 ml of this solution to conserve the enzyme especially with tumor xenografts.

7. Mix the tumor/enzyme solution well so that you have a homogenous mixture and place in the incubator at 37˚C for 15 minutes. Remove from the incubator and pipette the entire mixture through a 25 ml pipette to mechanically dissociate tumor chunks. Repeat this every 15-20 minutes for a maximum of about 1 hour. As you observe tumor dissociation move from a 25 ml pipette down to a 10 ml pipette to ensure proper dissociation.

Note: i) Do not leave the tumor sample in collagenase more than an hour and 15 minutes as this becomes too traumatic to the cells and ensures poor cell viability.

ii) While mechanically dissociating using pipettes use a little pressure to suck in the tumor pieces and splash it down against the wall of the Petri dish for maximal dissociation.

8. Place a 40μm sieve in a fresh 50 ml conical tube. Collect the tumor/single cell suspension and pipette into the sieve. If flow stops, place the pipette on the bottom of the sieve and “suck up” to create a vacuum to ensure flow through the sieve.

   [Note: To start or establish cell lines *in vitro*, place chunks of tumor pieces in a T-25 with appropriate medium with AAA. Change media daily to prevent contamination and observe].

9. The filtrate has the single cell suspension. Neutralize the collagenase with FBS at approx. the ratio of 80-20% (to the filtrate solution say 8 ml add 2 ml of FBS). Spin for 3 minutes at 800 rpm at 4˚C.

10. Observe the cell pellet after centrifugation. Decant neutralized enzyme mixture to obtain cell pellet.

    Note: The cell pellet may not be visible especially in biopsy samples. So, decant the supernatant carefully to avoid losing the cancer cell suspension.

11. Re-suspend the cell pellet in 5 ml of ACK lysing buffer for 1 minute for RBC cell lysis. Spin down at 800 rpm for 3 minutes.

    Note: You will see a change in color of the cell pellet from pinkish to a clearer white. Continue with any protocol you are working with.

Head and neck cancer stem cell isolation via Fluorescence Activated Cell Sorting (FACS) for ALDH and CD44

Materials Required:

1. Aldefluor substrate and its inhibitor, DEAB (Aldefluor kit, Stem Cell Technologies, 01700; Vancouver, BC, Canada).
2. CD44 antibody, conjugated with APC (BD Pharmingen, 559942; Franklin Lakes, NJ)
3. IgG antibody conjugated with APC (BD Pharmingen, 550931)
4. 7AAD (eBioscience, 00-6993-50; San Diego, CA)

Procedure:

1. Obtain single cell suspension from cell lines either by trypsinizing or digesting tumor specimens from patient/xenografts.
2. Count the cells and distribute cells in Flow cytometry tubes (11×75 tubes) at 1×10^6 cells per tube in 1 ml of Aldefluor Buffer.

   Note: i) You can go up to a maximum of 4-5 million cells for the same staining efficiency. I personally recommend using about 3- 3.5 million cells per tube.

   ii) Cell count is very important for reproducible, consistent response of aldefluor activity.

iii) Aldefluor is very sensitive to light exposure, volume of buffer and temperature.

3. Label the flow cytometry tubes per condition along with controls as follows:

A. a Blank tube (only cells in PBS for negative control)

B. a 7AAD tube (for cell viability)

C. an IgG-APC tube (IgG control for CD44)

D. a DEAB tube (a negative inhibitor for Aldefluor substrate) Keep this tube empty no cells added.

E. Test tubes are labeled from T1, T2 and so on according to the number of cell availability and requirement.

4. In the tubes, add cells (3- 3.5 million cells) in Aldefluor buffer to all the tubes except the DEAB. Turn off the lights in the hood.

5. Step-wise activation of Aldefluor:
6. Add 5 μl of DEAB solution to the DEAB tube and 5 μl of the activated aldefluor substrate to T1. Transfer 0.5 ml of this activated solution with cells to the DEAB tube.

   Note: i) This is done in this order for the DEAB to be functional. Quick addition of the activated solution to the DEAB tube is important for inhibition.

ii) All fluorochromes being light sensitive please work under dark conditions (no lights on in the hood).

7. Add 5 μl of activated Aldefluor substrate to the rest of the test tubes. Incubate the DEAB and the test tubes for about 45 minutes at 37˚C covered in Aluminium foil (minimum time 30 minutes, do not exceed 1 hour, best result is at 35-45 minutes)

Note: Aldefluor is light sensitive, work in the dark, keep wrapped in aluminum foil.

After the incubation, spin down the cells at 800 rpm for 5 minutes and re-suspend the cell pellets in 1 ml of Aldefluor Buffer.

Note: This step is important to terminate the activation of the aldefluor.

8. Add 5 μl of CD44-APC antibody (1:50) to the test tubes and the DEAB tube and IgG to the control tube. Store on ice.
9. Add the 7AAD to the test tubes and DEAB tube in the same ratio along with the 7AAD control tube.
10. Keep all the tubes in ice, covered in Aluminium foil
11. Collection tubes are labeled as (ALDH+CD44+) and ALDH-CD44- with 1 ml of complete media (DMEM Low glucose, 10% FBS, 1% Penicillin Streptomycin).
12. The cells are ready to be sorted now using FACS. The Aldefluor activity can be detected for a maximum of 24 hours. Store and transport samples for sorting in ice covered with Aluminium foil for best results.

General Instructions:

1. For sorting of primary tumors, please use the Lineage specific cocktail to eliminate lineages.

   CD2 (BD Pharmingen, 555328; Franklin Lakes, NJ) – to detect T-cells and N-Killer cells

   CD3 (BD Pharmingen, 555334; Franklin Lakes, NJ)- to detect T-lymphocytes

   CD10 (BD Pharmingen, 555376; Franklin Lakes, NJ)- to detect B-cells

   CD16 (BD Pharmingen, 555408; Franklin Lakes, NJ)- to detect neutrophils, monocytes, macrophages

   CD18 (BD Pharmingen, 557528; Franklin Lakes, NJ)- to detect integrins associated with macrophages and lymphocytes

   Note: all these antibodies are pre-conjugated with PE-cy5 (1:50). They detect cells of the hematopoietic origin like T-cells, Natural Killer cells, monocyte-macrophages, B-cells, neutrophils which is eliminated. Use about 3 µl/sample.

2. During sorting of mouse xenografts, to eliminate mouse cells, please add to test tubes and to a separate color control tube for PE,

   anti H2kD Biotinylated (1:100); (BD Pharmingen, 553564; Franklin Lakes, NJ) and

   PE streptavidin (BD Pharmingen, 554061; Franklin Lakes, NJ).

   Use about 4 µl of each and wait for 10-15 minutes on ice between them.

3. To eliminate human endothelial cells from the xenografts use 5 µl of PE- CD31 (BD Pharmingen, 560983; Franklin Lakes, NJ) in test tubes and add a control tube as well.

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3. Aaron Robida bida@umich.edu

Suspension Cultures: Orospheres

Materials Required:

1. Dulbecco's modified Eagle's medium Low Glucose (DMEM, Invitrogen) with 10% Fetal Bovine Serum (FBS Gibco 10082; Invitrogen), 1% Penicillin/Streptomycin or 1% Antibiotic Antimycotic Solution for primary cultures(AAA, Sigma A5955; St. Louis, MO)
2. Ultra-low attachment plates (Corning 3471; Corning NY)

Procedure:

1. FACS sorted cells (ALDH+CD44+ and ALDH-CD44-) are seeded as triplicates in 6 well Low attachment plates at about 5×10^3 cells/well in 2-3 ml of media.

   Note: Use 3 ml if there is no treatment subsequently.

   If you want to treat the cells (for example, endothelial cell CM), suspend the cells in 2 ml of media, wait 6 hours in incubation and add 1 ml of treatment on top gently without disturbing the cells very much.
2. Incubate at 37˚C for 3 days, observe every day with minimal shaking to prevent dissociation of the forming spheres.
3. Orospheres can be seen in 3 days under a light microscope or a phase contrast microscope. Orospheres can be quantified by counting the

number of spheres/well. Spheres which are smaller than (25-50 cells) are not counted.

To generate secondary and tertiary orospheres,

1. orospheres are dissociated mechanically using a pipette.
2. Single cell suspensions are again counted and re-seeded in a new 6-well Low Attachment Plate
3. Check under the microscope for thorough dissociation
4. Observe after 3 days for secondary and tertiary orospheres.

General Instructions:

1. The time for sphere generation can be cell type specific. Some cell types form spheres earlier or later, the average being 2-5 days.

Suspension Cultures: Colony Formation Assay

Materials Required:

1. Ultrapure Low Melting point agarose (24-28˚C)(Invitrogen 15517-022; Carlsbad, CA)
2. 10X Minimum Essential Medium (MEM, Gibco 11430; Invitrogen)
3. 1X DMEM (no supplements)

Procedure:

1. Preparation of 2X DMEM , 2.5% FBS, 1% P/S to make up a volume of 50 ml.
   a) 5.55 mL of 10X MEM
   b) 2.5 mL of FBS
   c) 1 mL of P/S
   d) 40.95 mL of 1X DMEM

   Mix all the above to obtain 2X DMEM complete media to use.
2. Preparation of soft agarose plates
   a) Prepare 1.2% and 0.6% agarose solutions, sterilize and store them at room temperatures.
   b) Heat the agarose “gels” to melt them in the microwave.
   c) Keep the melted agarose gels in 37˚C waterbath.
   d) Now, it is ready to use.

3. In a 6 well plate, to form the inert feeder layer, for each well, mix 1.5 mL of 2X DMEM and 1.5 mL of melted 1.2% agarose (equal volumes are important). Leave the plates in the hood for 30-40 minutes in the hood for the agarose to gel.
4. Add equal volumes of 2X DMEM (1.5 mL) and 0.6% agarose (1.5 mL) with the required cell suspension (approx. 500-5000 cells/well) and add it on to the solidified feeder layer.

   Note: the melted agarose should not be too hot when the cells are added to ensure cell viability.
5. Leave the plates again in the hood for 30 minutes to one hour for the second layer to solidify.
6. Add a layer of 1X DMEM on the surface (about 500 µl) to maintain moisture. Any treatments like Conditioned Medium can also be added to the surface.

   Note: Add treatment or fresh media of about 500 µl every 2-3 days.
7. Observe the agarose under a light microscope for colonies; colonies usually appear in about a week's time.
8. The number of colonies in an entire well can be quantified in triplicates accordingly.

Immunohistochemistry for ALDH1

Materials Required:

1. ALDH1 antibody (BD Biosciences, 61195; Franklin Lakes, NJ)
2. Deparaffinizing materials (Xylene, Ethanol)
3. Dako Retrieval Solution 10X (Dako S1699; Carpinteria, CA)
4. Background Sniper – (Biocare Medical BS99H; Concord, CA)
5. DAB/AEC Chromogen kits (DAB - DS854H; AEC - RAEC-810; Biocare Medical, Concord, CA)
6. Secondary antibody MACH 3 (MP53G, MH53G, Biocare Medical, Concord, CA)

Procedure:

1. Bake paraffin embedded sections at 37˚C for 30 minutes
2. Bake slides at 59˚C for 30 minutes.
3. Xylene 100% - 10 minutes, 2 times. (Please do not change the order of the xylenes if being re-used.), check for complete removal of paraffin.
4. Ethanol – 100% 5 minutes, 2 times
5. Ethanol - 95% 3 minutes, 1 time
6. Ethanol - 75% 5 dips
7. Rinse the slides thoroughly for 5 minutes, 2 times in DDW.
8. Wash in 1X PBS for 5 minutes.

9. Antigen Retrieval Solution: Immerse slides in Dako Retrieval Solution 1X solution in a water bath.

   Note: it helps to do slow heating from about 45˚C – to a maximum of 98˚C over a period of 40-45 minutes. Please do not let the tissue sections to boil.

10. Let the slides cool at room temperature for 20-30 minutes.
11. Rinse the slides in distilled water.
12. Circle the tissues with a PAP pen to contain the reagents.
13. To block for non-specific binding, incubate in 3% hydrogen peroxidase (prepared in methanol or PBS), for 6-8 minutes, once.
14. Rinse the slides in PBS, 5 minutes.
15. Background Sniper, incubate for 6-8 minutes, once.

    Note: not strictly necessary, use if the tissue sections have too much background).

16. Wash slides with PBS, 5 minutes, twice.
17. Incubate slides in primary antibody, ALDH1 (1:50) in PBS/diluent solutions overnight at 4˚C.

Note: If tissue sections have a good staining, you can go up to 1:100 or 1:200, but in most hard to stain tissue sections, 1:50 works best.

18. The next day, wash slides in PBS, 2 times for 10 minutes, with gentle shaking.

19. Secondary antibody: mouse probe (room temperature for 20 minutes). We use MACH 3 linker (MP530G) from Biocare medical.
20. Wash 2 times, 10 minutes, in PBS with shaking.
21. Mouse-HRP polymer, room temperature for 20 minutes. We use MACH 3 HRP polymer (MH530G) from Biocare Medical.
22. Wash slides with PBS, twice 10 minutes, shaking.
23. Color development (DAB or AEC)

    DAB substrate 1 ml + 1 drop DAB chromogen (Biocare Medical)

    AEC buffer 5 ml + 1 drop Solutions A, B, C (Romulin AEC kit, RAEC 810L)

    Note: AEC and DAB are carcinogenic, please be careful while handling them.

24. Control the staining in the reaction, check for optimal staining in the microscope. Stop the reaction immersing the slides in DDW.
25. Counterstain with Hematoxylin in quick dips and wash with flowing water for 15-20 minutes.
26. Mount the slides with aqueous mounting media and cover with cover glass.

Preparation of slides for Confocal Imaging

Materials Required:

1. Zenon Alexafluor 488 (Molecular Probes, Z25102; Invitrogen)
2. Zenon Alexafluor 594 (Molecular Probes, Z25007; Invitrogen)
3. Prolong Gold labeling with DAPI- antifade (Invitrogen P36931)
4. Deparaffinizing materials (Xylene, Ethanol)
5. Dako Retrieval Solution 10X (Dako S1699; Carpinteria, CA)
6. Background Sniper – (Biocare Medical BS99H; Concord, CA)
7. Antibodies to proteins of interest

   ALDH1 (BD Biosciences, 61195; Franklin Lakes, NJ)

   CD44 (abcam, 51037; San Francisco, CA)

   P-STAT3 (santa cruz, 7993-R; Santa Cruz, CA)

   IL-6R (abcam, 47173; San Francisco, CA)

   Gp130 (abcam, 59389; San Francisco, CA)

Procedure:

1. Bake paraffin embedded sections at 37˚C for 30 minutes
2. Bake slides at 59˚C for 30 minutes.
3. Xylene 100% - 10 minutes, 2 washes. (Please do not change the order of the xylenes if being re-used.), check for complete removal of paraffin.
4. Ethanol – 100% 5 minutes, 2 washes
5. Ethanol - 95% 3 minutes, 1 wash

6. Ethanol - 75% 5 dips
7. Rinse the slides thoroughly for 5 minutes, 2 times
8. Antigen Retrieval Solution: Immerse slides in Dako Retrieval Solution 1X solution in a water bath.

   Note: it helps to do slow heating from about 45˚C – to a maximum of 95˚-98˚C over a period of 40-45 minutes. Please do not let the tissue sections to boil.

9. Let the slides cool at room temperature for 20-30 minutes.
10. Rinse the slides in distilled water.
11. To block for non-specific binding, wash in 3% hydrogen peroxidase (prepared in methanol and not water), for 6-8 minutes, once.
12. Rinse the slides in PBS, 5 minutes.
13. Background Sniper, wash for 6-8 minutes, once.

    Note: not strictly necessary, use if the tissue sections have too much background).
14. Wash slides with PBS, 5 minutes, twice.
15. Prepare the Zenon complex for labeling antibodies.
    a) Prepare 1 µg of the antibody of interest in PBS, (to approximately 10 µl of PBS).
    b) Add 5 µl of the Zenon human IgG labeling reagent A to the antibody solution.

Note:i) Please take care that if the antibody is mouse, the Zenon labeling is mouse as well

ii) For different antibodies, use different colors and species of Zenon. For example, ALDH1 is mouse antibody and CD44 is rabbit antibody. Use green mouse for ALDH1 and red rabbit for CD44 from the Zenon labeling kit.

c) Incubate the mixture for 5 minutes in room temperature

d) Add 5 µl of the appropriate Component B of the Zenon Labeling kit.

e) Incubate the mixture for 5 minutes in room temperature.

Note: The Zenon reagents are fluorochromes; light sensitive, cover the tube with aluminium foil during incubation.

f) The complexes are ready to use. Use within 30 minutes.

16. Dilute this mixture at the appropriate dilutions (for ALDH1, 1:50; CD44, 1:200, P-STAT3, 1:200; IL-6R, 1:200, gp130, 1:200) in PBS or diluents and add it to the tissue sections.
17. Cover the slides holder with Aluminium foil (the reagents are light sensitive) and incubate in 4˚C overnight.
18. The next day, wash the slides with PBS for 15 minutes three times.
19. Mount the slides with Prolong Gold with DAPI and store in -20C until imaging.

General Instructions:

1. The concentration of the antibody needs to be standardized for individual antibodies.
2. Imaging needs to be done as soon as possible.
3. Depending on the application, use the appropriate scope for confocal. For example, single cell co-localizations are best done with the Leica Inverted Microscope and the Z-stacking images to study several microns of thickness with the Zeiss confocal.