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# Cisplatin Induces Bmi-1 and Enhances the Stem Cell Fraction in Head and Neck Cancer[1,2]

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## Abstract

Recent evidence has unveiled a subpopulation of highly tumorigenic, multipotent cells capable of self-renewal in head and neck squamous cell carcinomas (HNSCCs). These unique cells, named here cancer stem cells (CSCs), proliferate slowly and might be involved in resistance to conventional chemotherapy. We have shown that CSCs are found in perivascular niches and rely on endothelial cell–secreted factors [particularly interleukin-6 (IL-6)] for their survival and self-renewal in HNSCC. Here, we hypothesized that cisplatin enhances the stem cell fraction in HNSCC. To address this hypothesis, we generated xenograft HNSCC tumors with University of Michigan–squamous cell carcinoma 22B (UM-SCC-22B) cells and observed that cisplatin treatment increased ($P = .0013$) the fraction of CSCs [i.e., aldehyde dehydrogenase activity high and cluster of differentiation 44 high ($ALDH^{high}CD44^{high}$)]. Cisplatin promoted self-renewal and survival of CSCs *in vitro*, as seen by an increase in the number of orospheres in ultralow attachment plates and induction in B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1) and octamer-binding transcription factor 4 expression. Cisplatin-resistant cells expressed more Bmi-1 than cisplatin-sensitive cells. IL-6 potentiated cisplatin-induced orosphere formation generated when primary human HNSCC cells

Abbreviations: HNSCC, head and neck squamous cell carcinoma; CSC, cancer stem cell; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; Bmi-1, B lymphoma Mo-MLV insertion region 1 homolog; Oct-4, octamer-binding transcription factor 4; UM-SCC, University of Michigan–squamous cell carcinoma

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were sorted for ALDH$^{high}$CD44$^{high}$ immediately after surgery and plated onto ultralow attachment plates. IL-6–induced signal transducer and activator of transcription 3 (STAT3) phosphorylation (indicative of stemness) was unaffected by treatment with cisplatin in UM-SCC-22B cells, whereas IL-6–induced extracellular signal–regulated kinase (ERK) phosphorylation (indicative of differentiation processes) was partially inhibited by cisplatin. Notably, cisplatin-induced Bmi-1 was inhibited by interleukin-6 receptor blockade in parental and cisplatin-resistant cells. Taken together, these results demonstrate that cisplatin enhances the fraction of CSCs and suggest a mechanism for resistance to cisplatin therapy in head and neck cancer.

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## Introduction

Drug resistance is a critical problem in cancer treatment. Head and neck squamous cell carcinoma (HNSCC), one of the most common types of cancer afflicting more than half a million people worldwide each year, exhibits an unacceptably poor patient survival [1,2]. The standard of care for head and neck tumors used to be surgery and radiation. The addition of platinum-based drugs led to an improvement in local disease control and organ preservation [3]. However, the overall survival of patients with HNSCC has not changed significantly for the last three decades. This has been attributed to the development of chemoresistance and incidence of distant metastasis [4–6]. Understanding the mechanisms underlying resistance to chemotherapy might have a profound impact in the survival of patients with head and neck cancer.

This clinical scenario suggests that some tumor cells are unaffected by chemotherapy and have the capability to migrate and initiate a new tumor. Considering that tumors are complex "organs" constituted by many subtypes of tumor cells, it is plausible that resistance to therapy is related to a subpopulation of less differentiated, multipotent, and self-renewing cells, named cancer stem cells (CSCs), that are uniquely resistant to therapy [7,8]. Such CSC cells are capable of initiating tumors and disseminate from the primary tumor to promote metastasis [9–11]. Notably, current therapies targeted to highly proliferative cells can affect tumor bulk by killing differentiated tumor cells but may spare the CSCs that are believed to exhibit slow proliferation rates. Indeed, it has been recently shown that stem cells survive anticancer treatment in some tumor types, as for example lung cancer [12], glioblastoma [13], and breast cancer [11]. These findings are aligned with the concept that CSCs are key mediators of resistance to therapy leading to recurrence or metastasis [14]. Head and neck tumors contain a small and highly tumorigenic subpopulation of cells with stem and self-renewal properties [15,16]. It is possible that therapies targeting CSCs, used in combination with conventional "tumor debulking" agents, may become a more efficient strategy in the treatment of patients with head and neck cancer.

The goal of targeting CSC has remained elusive. CSC shares many characteristics with normal stem cells, which makes specific targeting very difficult. In addition, CSC exhibits high drug efflux through ATP-binding cassette transporters [17]. Indeed, this feature has been used to identify stem cells [18,19]. Because the direct target of CSC seems to be difficult, other approaches have been considered, as for example to target supportive cells of the CSC niche. It has been found that CSC depends on the tumor microenvironment for their growth and survival and proposed that therapeutic targeting of the supportive niche might result in ablation of CSC [20,21]. We have shown that head and neck CSCs reside in perivascular niches and that their cross talk with vascular endothelial cells is critical for their survival and self-renewal [16]. This suggests that the blockade of the cross talk between endothelial cells and CSC might sensitize these cells to conventional chemotherapy. We have previously reported that interleukin-6 (IL-6) expression is upregulated in endothelial cells cocultured with HNSCC cells and that blockade of endothelial cell–derived IL-6 inhibits signal transducer and activator of transcription 3 (STAT3) signaling and survival and migration of unsorted HNSCC cells [22]. IL-6 signaling through IL-6 receptor (IL-6R) and activation of the Janus kinase (JAK)/STAT3 pathway have been implicated in the tumorigenic behavior of CSC, at least in breast cancer models [11,23,24]. We have also observed that IL-6 expression is higher in vascular endothelial cells than in tumor cells in human HNSCC and that IL-6R expression is higher in the CSC than in the non-CSC in these tumors. Notably, there is a strong correlation between increased pretreatment serum IL-6 levels and poor survival of patients with HNSCC [25]. Here, we tested the hypothesis that cisplatin enhances the fraction of CSCs in head and neck cancer.

## Materials and Methods

### *Cell Culture*

Cells derived from primary human HNSCC tumors were prepared within 12 hours after surgery. Tumors were cut into small pieces, minced with a sterile scalpel, resuspended in a mix of 9:1 Dulbecco's modified Eagle's medium–F12 (DMEM-F12; Hyclone, Waltham, MA) and collagenase/hyaluronidase (STEMCELL Technologies, Vancouver, British Columbia), and incubated for 1 hour at 37°C. The mixture was filtered through a 40-μm nylon mesh (BD Falcon, Franklin Lakes, NJ) and resuspended in low-glucose DMEM, as we described [16]. The primary tumors were obtained from patients on signature of informed consent under an approved Institutional Review Board (IRB) protocol. HNSCC cell lines [University of Michigan–squamous cell carcinoma 1 (UM-SCC-1), UM-SCC-22A, and UM-SCC-22B; gift from Dr Carey, University of Michigan, Ann Arbor, MI) were cultured in DMEM (Invitrogen, Grand Island, NY), 10% FBS (Invitrogen), and 100 U/ml penicillin-streptomycin (Invitrogen). The identity of all tumor cell lines was confirmed by genotyping. Human dermal microvascular endothelial cells (HDMECs;

Lonza, Walkersville, MD) were cultured in endothelial cell growth medium 2 (EGM-2MV; Lonza).

### *Severe Combined Immunodeficient Mouse Model of Human Tumor Angiogenesis*

Xenograft HNSCC tumors with humanized vasculature were generated in severe combined immunodeficient (SCID) mice (CB17 SCID; Taconic, Germantown, NY) as we described [26]. Briefly, $1 \times 10^5$ tumor cells (UM-SCC-22B) were seeded with $9 \times 10^5$ HDMEC in poly-(L-lactic) acid (Medisorb, Nicosia, Cyprus) biodegradable scaffolds. Mice were anesthetized with ketamine (Hospira, Inc; Lake Forest, IL)/xylazine (Lloyd Laboratories; Shenandoah, IA), and bilateral scaffolds were implanted subcutaneously in the dorsum of each mouse ($n$ = 12). Mice were monitored daily for tumor growth, and treatment (weekly intraperitoneal injection, 5 mg/kg cisplatin (Bedford Laboratories; Bedford, OH) or vehicle) started when the average volume of the tumors reached 200 $mm^3$ ($n$ = 12 tumors per experimental condition). At termination of the experiment, mice were killed, and tumors were retrieved, measured, weighed, and processed for histology and flow cytometry. The care and treatment of experimental animals were in accordance with University of Michigan institutional guidelines.

### *Immunohistochemistry*

Paraffin-embedded tissues were treated with peroxidase (Dako, Carpinteria, CA) and submitted to antigen retrieval for 30 minutes at 95°C. Tissues were exposed to 1:100 dilution of the anti–B lymphoma Mo-MLV insertion region 1 homolog (anti–Bmi-1; Abcam, Cambridge, United Kingdom) or 1:50 of the anti-cluster of differentiation 44 (CD44; Cell Signaling Technology, Danvers, MA) overnight at 4°C.

### *Flow Cytometry*

Single-cell suspensions were obtained either from trypsinization of cell lines or digestion of tumor specimens from patients. ALDEFLUOR kit (STEMCELL Technologies) was used to identify cells with high aldehyde dehydrogenase (ALDH) activity, as previously reported [16]. Cells were suspended with activated ALDEFLUOR substrate aminoacetaldehyde (BAAA) or the ALDH inhibitor diethylaminobenzaldehyde (DEAB) for 45 minutes at 37°C. Then, cells were exposed to anti-CD44 antibody (clone G44-26BD; BD Pharmingen, Franklin Lakes, NJ) and lineage markers (i.e., anti-CD2, anti-CD3, anti-CD10, anti-CD16, and anti-CD18; BD Pharmingen) preconjugated with phycoerythrin-cyanine dye 5 (PE-cy5). Mouse cells were identified using anti-H2K antibody (BD Biosciences, Franklin Lakes, NJ) and eliminated. 7-Aminoactinomycin (BD Pharmingen) was used to select live cells.

### *Cytotoxicity Assay*

Sulforhodamine B (SRB) cytotoxicity assay was performed, as we described [27]. Briefly, cells were seeded at $2 \times 10^3$ cells per well in 96-well plates, allowed to attach overnight, and treated with cisplatin for 48 to 96 hours. Cells were fixed with 10% TCA and stained with 0.4% SRB (Sigma-Aldrich, St Louis, MO) in 1% acetic acid, and plates were read in a microplate reader at 560 nm (GENios; Tecan, Männedorf, Switzerland). Data were obtained from triplicate wells per condition and represent three independent experiments.

### *Orospheres*

Orospheres, i.e., nonadherent spheroids of ≥25 cells, were generated by seeding one cell per well in one ultralow attachment plate per experimental condition (Corning, Corning, NY), as we showed [28]. Cells were plated immediately after sorting and treated with 0, 0.02, 0.2, or 2 μM cisplatin with or without recombinant human IL-6 (rhIL-6; R&D Systems, Minneapolis, MN) for 10 days when the spheres were counted under microscope.

### *Western Blot Analysis*

UM-SCC-1, UM-SCC-22A, and UM-SCC-22B cell lines were plated, serum starved overnight, and treated with cisplatin at the indicated concentrations, 0 to 20 ng/ml rhIL-6 and 0 to 10 μg/ml anti–IL-6 neutralizing antibody (R&D Systems) or 0 to 10 μg/ml anti–IL-6R antibody (tocilizumab; Genentech, San Francisco, CA), and Western blot analyses were performed. Primary antibodies were as follows: mouse anti-human phospho-STAT3, rabbit anti-human STAT3, rabbit anti-human phospho-extracellular signal–regulated kinase (ERK)1/2, mouse anti-human ERK1/2, rabbit anti-human phospho-AKT, rabbit anti-human AKT, rabbit anti-human Bmi-1, mouse anti-human CD44 (Cell Signaling Technology), and mouse anti–glyceraldehyde-3-phosphate dehydrogenase (Chemicon/Millipore, Billerica, MA). Immunoreactive proteins were visualized by Super-Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

### *Cisplatin-Resistant HNSCC Cell Lines*

Cisplatin-resistant derivative cell lines were generated from UM-SCC-22B cells, as described [29]. Briefly, the parent cell line was submitted to repeated subcultures in the presence of increasing concentrations of cisplatin from 0.5 to 12 μM during a 3-month period. Each concentration was applied twice, and exposure was continuous for more than 3 days. Four cisplatin-resistant cell lines were generated as follows: UM-SCC-22BCis1 (UM-SCC-22B resistant to 1 μM cisplatin), UM-SCC-22BCis4 (UM-SCC-22B resistant to 4 μM cisplatin), UM-SCC-22BCis6 (UM-SCC-22B resistant to 6 μM cisplatin), and UM-SCC-22BCis12 (UM-SCC-22B resistant to 12 μM cisplatin).

### *Statistical Analysis*

One-way analysis of variance and $t$ test were performed using the SigmaStat 3.1 software (SPSS, Chicago, IL). Statistical significance was determined at $P < .05$.

## Results

### *Cisplatin Enhances the Fraction of CSCs in HNSCC*

To evaluate the effect of cisplatin on the head and neck CSC fraction, we generated HNSCC xenografts with humanized vasculature, as shown [16,26]. When tumors reached 200 $mm^3$, we began weekly injections with 5 mg/kg cisplatin for 2 weeks and killed the mice on the day after the last dose (Figure 1*A*). This treatment regimen did not cause a significant decrease in tumor volume (data not shown). Qualitative immunohistochemical analysis showed strong staining for Bmi-1 and CD44 in tumors retrieved from cisplatin-treated mice (Figure 1*B*). Importantly, we observed a significant increase in the fraction of CSCs identified as double staining [i.e., aldehyde dehydrogenase activity high and cluster of differentiation 44 high ($ALDH^{high}CD44^{high}$)] in

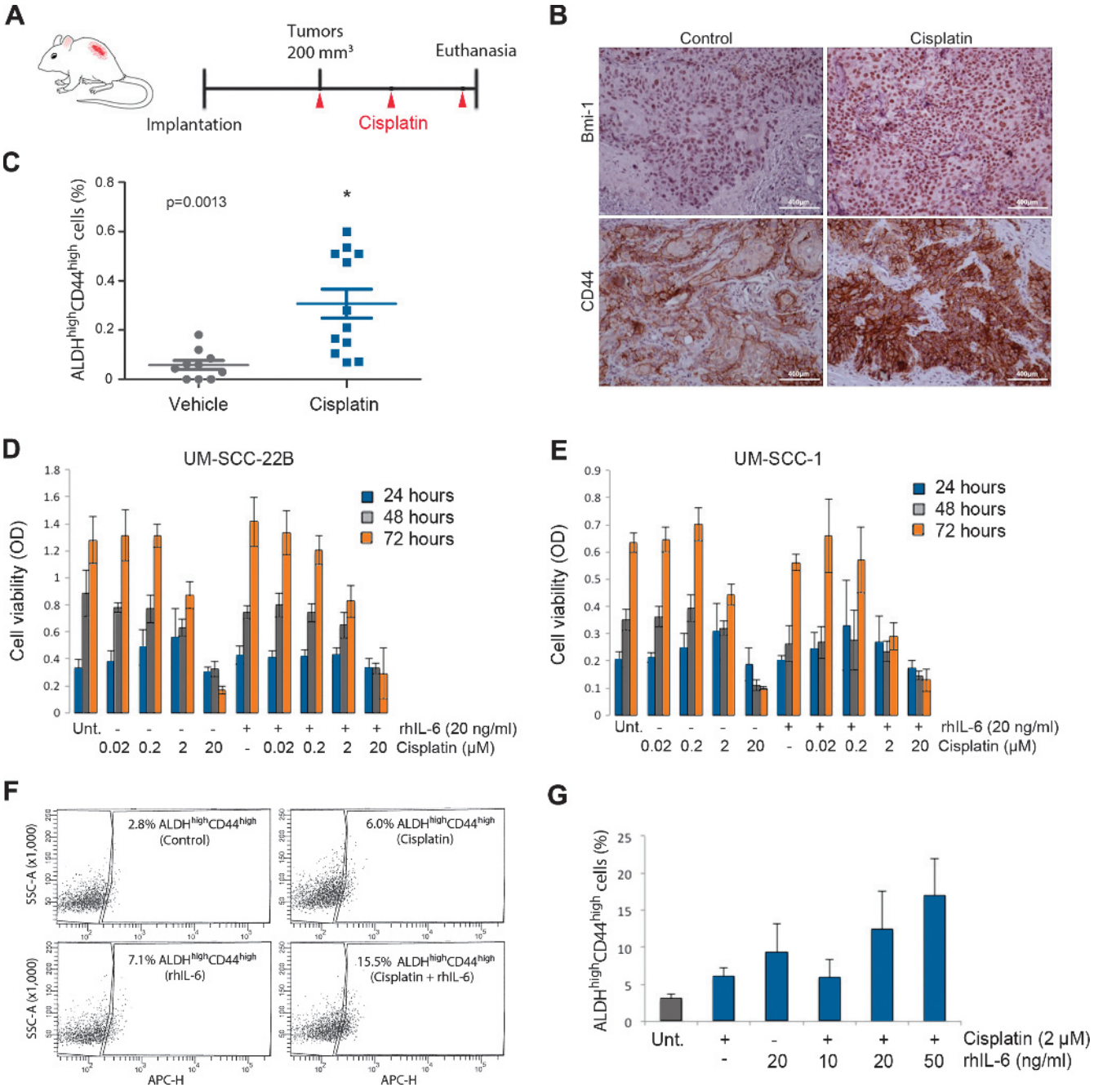


**Figure 1.** Cisplatin enhances the fraction of CSCs in HNSCC. (A) Schematic drawing depicts the experimental design. Xenograft tumors were generated by the transplantation of human HNSCC cells (UM-SCC-22B) and primary human endothelial cells (HDMEC) in SCID mice. When tumors reached an average size of 200 $mm^3$, mice began to receive weekly intraperitoneal injections (5 mg/kg cisplatin or vehicle). (B) Photomicrographs of histologic sections immunostained for CD44 and Bmi-1 from xenograft tumors treated with cisplatin or vehicle controls. (C) Graph depicts the fraction of CSCs (as defined by $ALDH^{high}CD44^{high}$) in xenograft tumors treated with cisplatin or vehicle control ($n = 11$). Asterisk depicts $P = .0013$. (D and E) Graphs depict the viability of UM-SCC-22B (D) and UM-SCC-1 (E) cells, as determined by the SRB assay. Cells were preincubated for 1 hour with 20 ng/ml rhIL-6 and then coincubated with cisplatin (0-20 $\mu$M) and rhIL-6 for 24 to 72 hours. (F and G) Flow cytometric analysis of ALDH/CD44 status of UM-SCC-22B cells treated with rhIL-6 and/or cisplatin. Cells were preincubated for 1 hour with rhIL-6 and then coincubated with cisplatin (0 or 2 $\mu$M) and rhIL-6 (0-50 ng/ml) for 24 hours.

mice treated with cisplatin, when compared with vehicle control mice (Figure 1*C*). To begin to understand possible mechanisms underlying these results, we performed SRB assays that showed the expected dose-dependent cytotoxicity of cisplatin at 48 and 72 hours, using two well-established HNSCC cell lines (UM-SCC-1 and UM-SCC-22B) from the Carey laboratory (Figure 1, *D* and *E*). We determined that the half maximal inhibitory concentration ($IC_{50}$) for unsorted UM-SCC-1 was 2.305 μM cisplatin and for UM-SCC-22B was 2.400 μM cisplatin at 72 hours (Figure W1). Knowing that high IL-6 expression in serum correlates with poor survival of patients with HNSCC [25] and that IL-6 is important for the survival and stemness of CSCs in breast cancer [23], we interrogated the effect of

IL-6 on cisplatin-induced death of unsorted HNSCC cells. We observed that IL-6 did not protect unsorted HNSCC cells against cisplatin-mediated cell death (Figure 1, *D* and *E*). In contrast, single-agent cisplatin enhanced the fraction of CSCs *in vitro*, as determined by FACS analysis for ALDH activity and CD44 expression (Figure 1, *F* and *G*). Furthermore, when both cisplatin and IL-6 were combined, we observed a dose-dependent increase (for IL-6) in the fraction of $ALDH^{high}CD44^{high}$ cells (Figure 1*G*). Collectively, these data demonstrate that the combination of cisplatin treatment with the high expression of IL-6, which is typically observed in HNSCC [30], leads to a dramatic increase in the fraction of CSCs.

### *Cisplatin Enhances the Stemness of Primary Human Head and Neck CSCs*

To evaluate the effect of cisplatin on the stemness of human HNSCC cells, we obtained specimens immediately after surgery and sorted these cells for ALDH activity and CD44 expression. The average fraction of $ALDH^{high}CD44^{high}$ in these primary human tumors was 3.2 +/– 2.6% (Figure W2). Single $ALDH^{high}CD44^{high}$ cells were plated onto ultralow attachment 96-well plates and observed for 10 days as shown [28]. Qualitative assessment showed a trend for larger orospheres in the wells treated with cisplatin and IL-6, when compared with controls (Figure 2*A*). Notably, we observed a dose-dependent increase in the number of orospheres when primary human CSCs were treated with cisplatin and IL-6 together (Figure 2*B*). Notably, the highest dose of cisplatin (20 μM) was very cytotoxic to the cells cultured in ultralow attachment plates, killing most (if not all) cells (data not shown).

To begin to understand possible mechanisms involved in this increase in the number of orospheres, we exposed a pair of HNSCC cell lines (UM-SCC-22A and UM-SCC-22B) to increasing concentrations of cisplatin in the presence/absence of IL-6. We observed that cisplatin by itself mediated a dose-dependent increase in the expression of Bmi-1 (Figure 2*C*), a member of the polycomb group family of transcriptional regulators that plays an essential role in stem cell fate decisions and regulates the self-renewal capacity of normal and CSCs [31,32]. Notably, combined use of cisplatin and IL-6 further enhanced expression of Bmi-1 in these cells (for concentrations up to 0.2 μM cisplatin), when compared to single-agent cisplatin (Figure 2*C*). We observed a trend for increasing expression of CD44 with increasing concentrations of cisplatin and further potentiation by combination of cisplatin and IL-6 (Figure 2*D*). To validate these results using an alternative approach, we used a third cell line (UM-SCC-1) and observed that either IL-6 or cisplatin induces strong expression of Bmi-1 and that treatment with IL-6 or cisplatin induced expression of the stem cell marker CD44 (Figure W3).

### *Effect of Cisplatin on Key Signaling Pathways Involved in the Regulation of Stemness*

STAT3 is the prototypic downstream signaling molecule for IL-6 and is constitutively activated in several malignancies including head

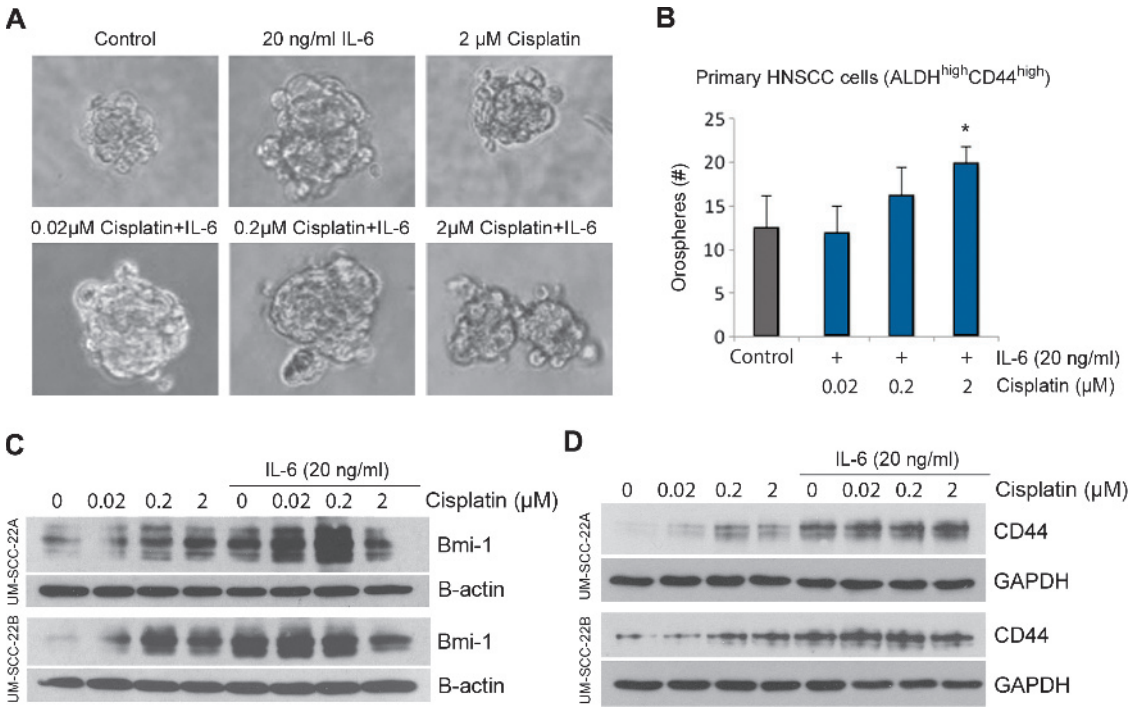


**Figure 2.** Cisplatin and IL-6 enhance Bmi-1 and the stemness of HNSCC cells. (A and B) Primary human HNSCC cells were sorted for ALDH and CD44 immediately after surgery. $ALDH^{high}CD44^{high}$ cells were cultured in ultralow attachment plates for 10 days. (A) Photomicrographs depict orospheres (nonadherent spheres of >25 HNSCC cells) after treatment with cisplatin (0-2 $\mu$M) and/or rhIL-6 (20 ng/ml) or vehicle control. (B) Graph depicts the number of orospheres generated with primary human HNSCC cells treated with cisplatin (0-2 $\mu$M) and/or rhIL-6 (0 or 20 ng/ml). Asterisk depicts $P < .05$. (C) Western blot analysis depicts the expression of Bmi-1 in UM-SCC-22A and UM-SCC-22B cells treated with cisplatin (0-2 $\mu$M) and/or rhIL-6 (0 or 20 ng/ml) for 24 hours. (D) Western blot analysis depicts the expression of CD44 in UM-SCC-22A and UM-SCC-22B cells treated with cisplatin (0-2 $\mu$M) and/or rhIL-6 (0 or 20 ng/ml) for 24 hours.

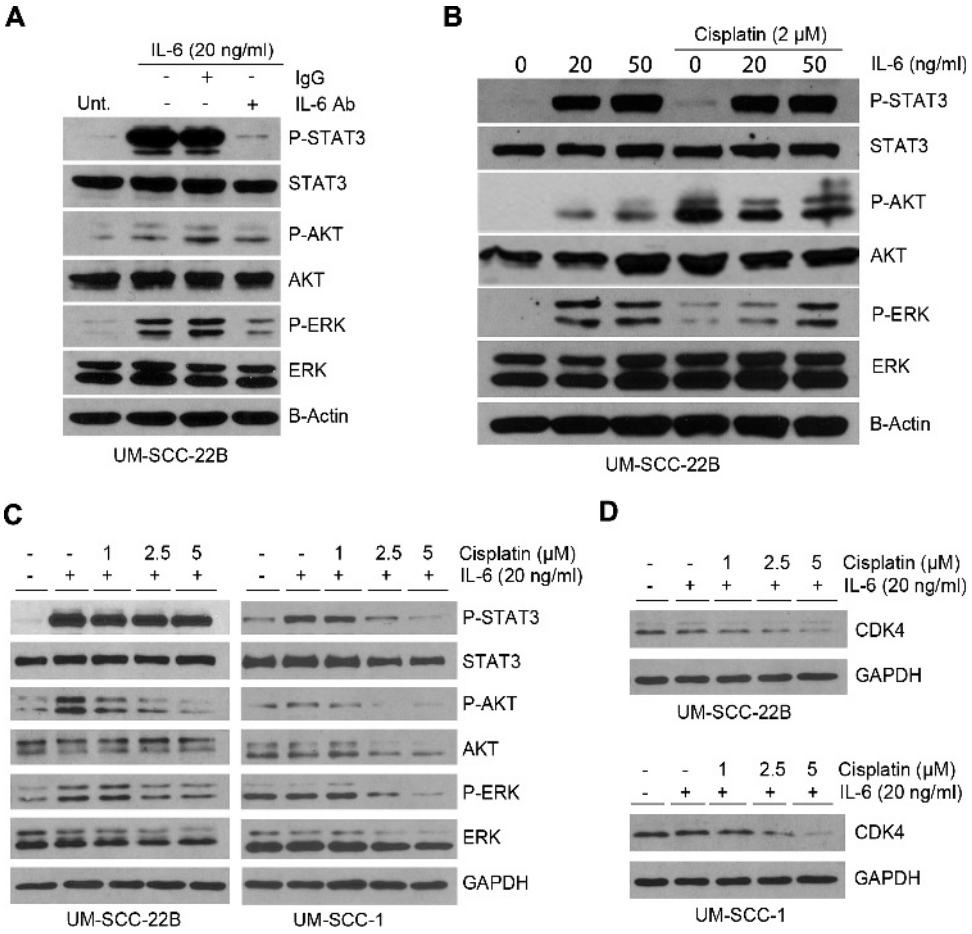


**Figure 3.** Effect of cisplatin on key signaling pathways involved in the regulation of stemness. HNSCC cells were serum starved overnight and then treated with cisplatin (0-5 $\mu$M), rhIL-6 (0-50 ng/ml), and/or anti–IL-6 neutralizing antibody (0-10 $\mu$g/ml) for 30 minutes. (A–C) Western blot analysis for phosphorylated and total STAT3, ERK, and AKT in UM-SCC-22B and UM-SCC-1 cells. (D) Western blot analysis for CDK4 in UM-SCC-22B and UM-SCC-1 cells.

and neck cancer [33,34]. It is known that the activation of the STAT3 signaling pathway is critical for the maintenance of the stemness of mammary and brain CSCs [23,24,35,36]. Here, we first confirmed that, indeed, IL-6 specifically activated STAT3 signaling, as well as the critical prosurvival AKT and ERK pathways (Figure 3*A*). We observed that IL-6–induced STAT3 phosphorylation was not affected by cisplatin (Figure 3*B*) in the cell line that was used for the *in vivo* experiment shown in Figure 1 (UM-SCC-22B), even when a high concentration of cisplatin (i.e., 5 μM) was used (Figure 3*C*). Interestingly, in UM-SCC-1, the phosphorylation of STAT3 was inhibited at the highest concentrations of cisplatin (Figure 3*C*). In contrast, we observed a dose-dependent partial inhibition of AKT and ERK phosphorylation with increasing concentrations of cisplatin in both cell lines (Figure 3*C*). Interestingly, ERK phosphorylation has been observed in cells undergoing differentiation [37]. Furthermore, we observed that cisplatin mediated a dose-dependent inhibition of the expression of cyclin-dependent kinase 4 (CDK4; Figure 3D), a Ser/Thr protein kinase that is important for $G_1$ cell cycle progression [38]. Together, these data suggest that IL-6 enhances the stemness of HNSCC cells and that cisplatin cannot overcome IL-6–induced signaling pathways that are involved in the acquisition of a stemlike phenotype in UM-SCC-22B cells.

### *Cisplatin-Resistant Cells Are Endowed with Cancer Stemlike Features*

To further investigate the effect of cisplatin on stemness in HNSCC, we generated cisplatin-resistant cell lines by exposing HNSCC to increasing concentrations of this drug (Figure 4*A*). The UM-SCC-22BCis1 is resistant to 1 μM cisplatin, UM-SCC-22BCis4 is resistant to 4 μM, and so forth. We observed that UM-SCC-22BCis12 form more orospheres when cultured in ultralow attachment plates, when compared to the parental UM-SCC-22B (Figure 4*B*). Cisplatin alone increases orosphere number in the parental cell line (Figure 4*B*). We also observed that, in the UM-SCC-22BCis12 cells, either IL-6 or cisplatin does not induce further the number of orospheres (Figure 4*B*). Notably, unstimulated cisplatin-resistant HNSCC cells express higher levels of Bmi-1 and octamer-binding transcription factor 4 (Oct-4), two markers of stemness (Figure 4, *C*–*E*).

To further examine the effect of cisplatin resistance on response to IL-6, we exposed cisplatin-resistant cells to this cytokine and observed that it did not induce Bmi-1 or Oct-4 expression beyond what was observed in the parental cells (Figure 4, *D* and *E*). Surprisingly, we observed that 2 μM cisplatin had a somewhat inhibitory effect on Bmi-1 and Oct-4 expression in the UM-SCC-22BCis12 cells (Figure 4, *D* and *E*). IL-6 potently activated STAT3 phosphorylation in both parental cells and cisplatin-resistant cells (Figure 5*A*). Interestingly,

the most resistant cells (i.e., UM-SCC-22BCis12) were the ones that expressed the lower levels of the differentiation signaling molecule p-ERK (Figure 5*A*). These findings cannot be attributed to differential levels of IL-6R expression levels in both parental cells and cisplatin-resistant cells, treated and untreated (Figure 5*B*). Blockade of IL-6R signaling with the Food and Drug Administration (FDA)-approved humanized anti–IL-6R antibody tocilizumab partially inhibited Bmi-1 expression in parental cells and in cells resistant to cisplatin (Figure 5*C*). Interestingly, the same effect was not observed when we used an anti–IL-6 antibody (Figure 5*C*). Collectively, these data demonstrate that cisplatin-treated and cisplatin-resistant cells exhibit enhanced stemness and suggest that therapeutic IL-6R blockade might overcome this effect by downregulating Bmi-1 expression.

## Discussion

Cisplatin is the most frequently used chemotherapeutic drug for the treatment of HNSCC [39]. When combined with radiotherapy, cisplatin improves organ preservation and enhances the quality of life of patients with head and neck cancer [40]. However, the overall survival of patients with head and neck cancer has not improved significantly for the last 30 years [2], despite the extensive use of platinum-based therapies for the last two decades. This appears to be related to the observation that some tumor cells exhibit resistance to cisplatin and are able to regrow and regenerate tumors locally and/or at distant sites. Here, we propose a putative explanation for cisplatin resistance using the CSC hypothesis as mechanistic underpinning. Indeed, we observed that cisplatin enhances the fraction of head and neck CSCs ($ALDH^{high}CD44^{high}$ cells), which are known to be highly tumorigenic [16]. We also observed that IL-6 further enhances the stemness of HNSCC cells resistant to cisplatin, as demonstrated by higher levels of Bmi-1 and CD44 expression when IL-6 and cisplatin are combined. These findings provide preclinical support for a combination therapy that includes cisplatin and a targeted drug (e.g., IL-6R inhibitor) that inhibits important CSC functions in patients with head and neck cancer.

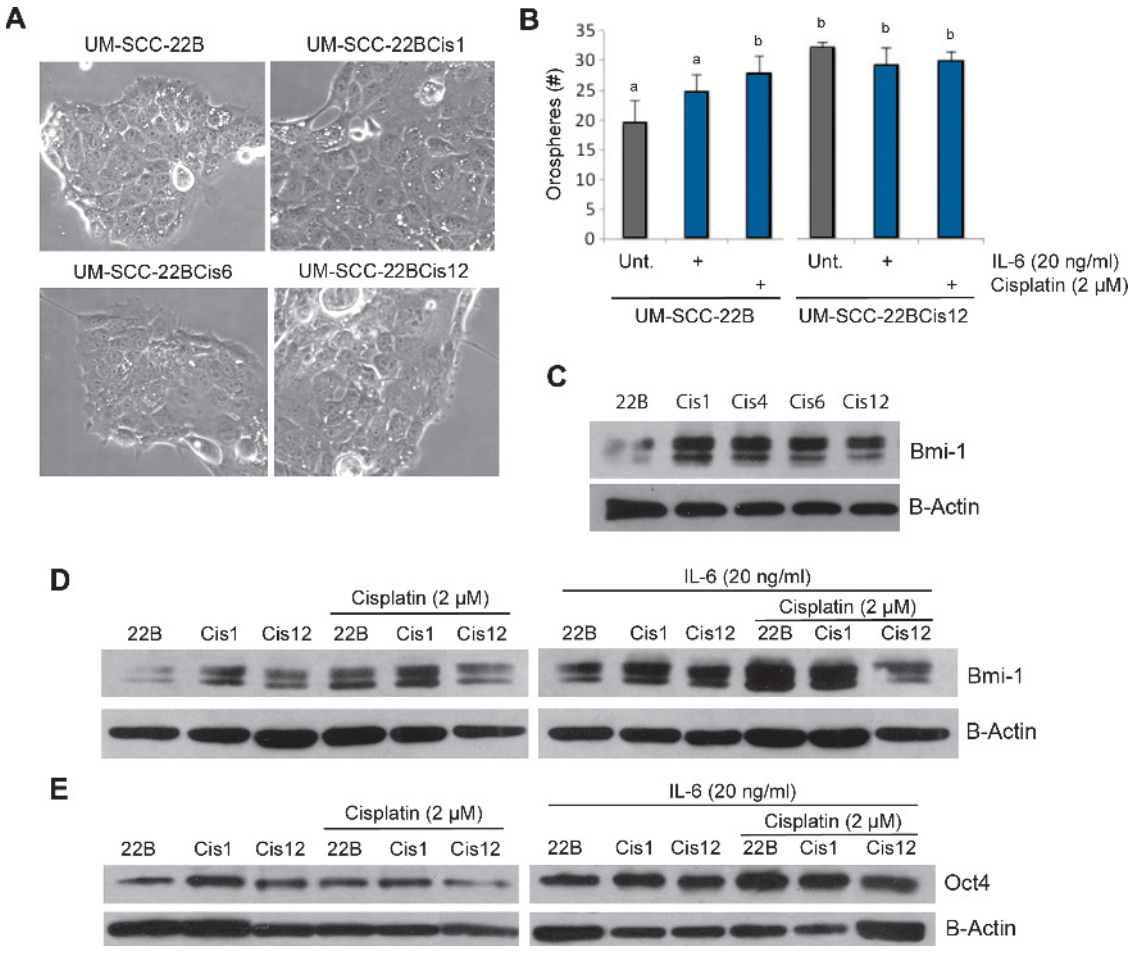


**Figure 4.** Cisplatin-resistant cells display cancer stemlike features. Cisplatin-resistant cells were generated by subculturing these cells in presence of increasing concentrations of cisplatin from 0.5 to 12 $\mu$M during a 3-month period. (A) Photomicrographs of parental cells (UM-SCC-22B) and cells resistant to 1 $\mu$M cisplatin (UM-SCC-22BCis1), 6 $\mu$M cisplatin (UM-SCC-22BCis6), or 12 $\mu$M cisplatin (UM-SCC-22BCis12). (B) Graph depicts number of orospheres generated with parental UM-SCC-22B cells or cisplatin-resistant cells (UM-SCC-22BCis12) treated with cisplatin (0 or 2 $\mu$M) and/or rhIL-6 (0 or 20 ng/ml) for 10 days in ultralow attachment plates. Different lowercase letters depict $P < .05$. (C) Western blot analysis of the expression of Bmi-1 in cisplatin-resistant cells (UM-SCC-22BCis1, UM-SCC-22BCis4, UM-SCC-22BCis6, and UM-SCC-22BCis12), compared to parental cells (UM-SCC-22B). (D and E) Western blot analysis of the expression of Bmi-1 (D) or Oct-4 (E) in cisplatin-resistant cells (UM-SCC-22BCis1 and UM-SCC-22BCis12), compared to parental cells (UM-SCC-22B). Cells were treated with cisplatin (0-2 $\mu$M) and/or rhIL-6 (0-20 ng/ml) for 24 hours.

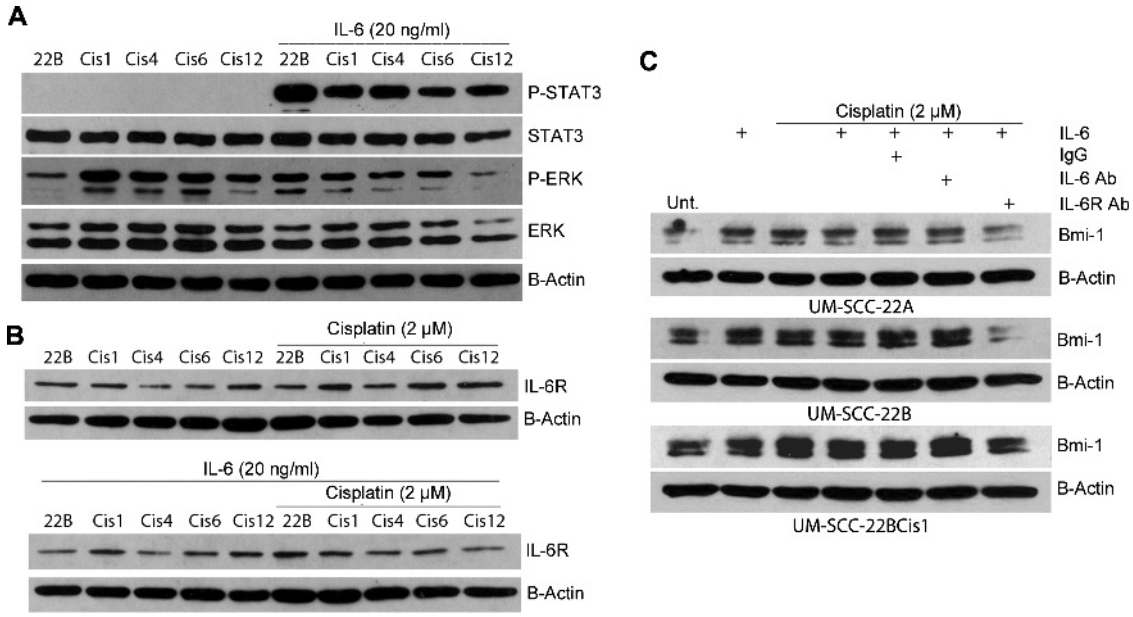


**Figure 5.** IL-6 activates STAT3 signaling and induces Bmi-1 expression in cisplatin-resistant cells. (A) Western blot analyses for phosphorylated and total STAT3 and ERK in cisplatin-resistant cells (UM-SCC-22BCis1, UM-SCC-22BCis4, UM-SCC-22BCis6, and UM-SCC-22BCis12), compared to parental cells (UM-SCC-22B). (B) Western blot analyses for expression of IL-6R in cisplatin-resistant cells (UM-SCC-22BCis series), compared to parental cells (UM-SCC-22B). Cells were treated with cisplatin (0-2 $\mu$M) and/or rhIL-6 (0 or 20 ng/ml) for 24 hours. (C) Western blot analysis of Bmi-1 in cisplatin-resistant cells (UM-SCC-22BCis1), compared to parental cells. Cells were treated with cisplatin (0-2 $\mu$M) and/or rhIL-6 (0 or 20 ng/ml) in presence of anti–IL-6 or anti–IL-6R antibody for 24 hours.

CSCs are resilient cells that play a major role in resistance to chemotherapy and radiotherapy in several tumor types [41–43]. For example, a relatively quiescent subpopulation of glioma cells with CSC properties was implicated in tumor regrowth after treatment of glioblastomas with temozolomide [13]. Furthermore, long-term trastuzumab treatment enriches the CSC population in breast cancer [11]. We observed here that cisplatin enhanced the fraction of head and neck CSCs *in vivo*, despite the lack of a significant change in the overall tumor volume with the dosing used here. In search for a mechanism for this response, we performed a series of *in vitro* studies that revealed the following: 1) A 24-hour exposure to 2 μM cisplatin does not mediate a decrease in the survival of unsorted HNSCC cells, when compared to untreated controls. However, we observed that a 24-hour exposure to 2 μM cisplatin doubled the fraction of ALDH+CD44+ cells. Notably, we evaluated 10,000 events for all the experimental conditions in the flow cytometry experiments. Therefore, cisplatin was able to increase the fraction of CSCs despite the fact that the total number of cells remained unchanged. 2) We observed a significant increase in the number of orospheres on treatment of parental HNSCC cells with 2 μM cisplatin. These data support the notion that cisplatin enhances the ALDH+CD44+ fraction because only these cells survive/proliferate under low attachment conditions. And 3) even very low concentrations of cisplatin (subapoptotic) were capable of inducing expression of the self-renewal of Bmi-1 and stem cell marker CD44 in unsorted HNSCC cells. Collectively, these data suggest that cisplatin was able to transform cells to be more ALDH+ and CD44+. Importantly, these data indicate that $ALDH^{high}CD44^{high}$ cells are resistant to cisplatin therapy. Considering the highly tumorigenic nature of these cells [16], this finding might explain, at least in part, the frequent occurrence of tumor relapse and distant metastases observed in patients with head and neck cancer treated with cisplatin.

It is well established that IL-6 signaling through the STAT3 pathway is employed by tumor cells to circumvent apoptosis and maintain proliferation and neoplastic growth [34,44]. Thus, it has been postulated that IL-6 participates in resistance to chemotherapeutic drugs [45,46]. It has been shown that the expansion of the CSC population observed in trastuzumab-treated mammary tumors is mediated by IL-6 signaling [11]. In head and neck cancer, cisplatin induces expression of IL-6, which mediates a transient increase in the tumorigenic potential of cancer cells [47]. Furthermore, sensitivity to chemotherapy appears to be modulated by lethal-7 (Let-7) through the regulation of IL-6/STAT3 pathway in head and neck cancer [48]. Notably, high serum IL-6 level is a strong predictor of the poor survival of patients with head and neck cancer [25]. We have recently observed that CSCs reside in perivascular niches in head and neck cancer [16] and that tumor-associated endothelial cells express high levels of IL-6, whereas $ALDH^{high}CD44^{high}$ cells express higher levels of IL-6R than $ALDH^{low}CD44^{low}$ cells (unpublished observations). Here, we observed that IL-6 contributes to cisplatin-induced stemness (Bmi-1 and CD44 expression and number of orospheres) of head and neck cancer cells and that therapeutic blockade of IL-6R signaling with a humanized monoclonal antibody partially inhibits cisplatin-induced Bmi-1 expression. Interestingly, the blockade of the receptor IL-6R was more effective than blockade of the ligand IL-6 in inhibiting Bmi-1 expression in response to cisplatin. These data suggest that an autocrine loop perhaps mediated in part by another IL-6R ligand (i.e., ciliary neurotrophic factor) contributes to the effect of cisplatin on HNSCC self-renewal. Notably, even cells pretreated with cisplatin for a long period of time (e.g., UM-SCC-22BCis12) still showed higher expression of

Bmi-1 on exposure to IL-6, suggesting that the effect of cisplatin persists over time.

Collectively, these data demonstrate that cisplatin enhances the fraction of putative head and neck CSCs (defined as $ALDH^{high}CD44^{high}$ cells), which are known to be highly tumorigenic in preclinical models of HNSCC [16]. We have also observed that IL-6 contributes to cisplatin-induced stemness, suggesting that a combination therapy involving a platinum-based drug and an IL-6R inhibitor might be beneficial for patients with HNSCC.

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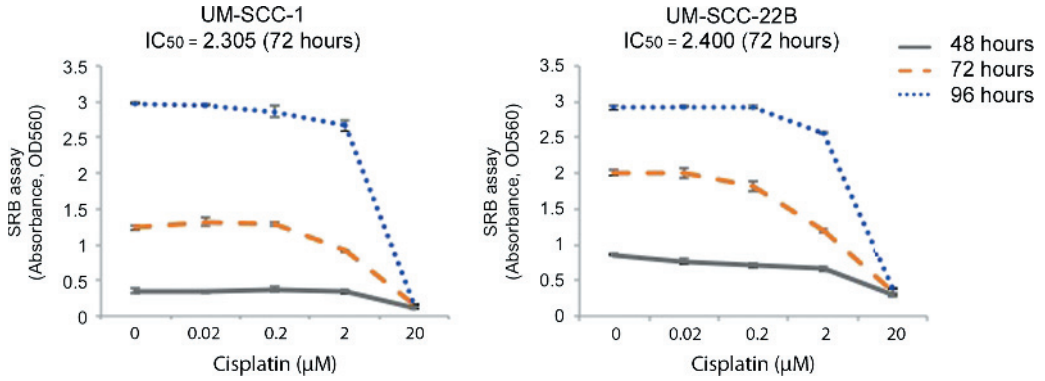


**Figure W1.** Cisplatin enhances the fraction of CSCs in HNSCC. Graphs depict the viability of UM-SCC-22B and UM-SCC-1 cells, as determined by the SRB assay. Cells were treated with cisplatin (0-20 $\mu$M) for 48 to 96 hours. The IC50 was determined at 72 hours.

| | | | | | Number of cells sorted | | Percentage (%) | |
|---|---|---|---|---|---|---|---|---|
| Specimen* | Gender | Age | Site | TNM | $ALDH^{high}CD44^{high}$ | $ALDH^{low}CD44^{low}$ | $ALDH^{high}CD44^{high}$ | $ALDH^{low}CD44^{low}$ |
| UM-HNSCC-1 | male | 60 | right parotid gland | T4N2bM0 | 1,570 | 243,940 | 0.086 | 94.634 |
| UM-HNSCC-2 | male | 25 | tongue | T4N2bM0 | 7,210 | 12,940 | 8.523 | 60.697 |
| UM-HNSCC-3 | female | 82 | floor of mouth | T2N0M0 | 6,510 | 90,430 | 2.470 | 63.880 |
| UM-HNSCC-4 | male | 60 | lip, mandible, tongue, floor of mouth | T4N2cM0 | 46,000 | 303,000 | 2.830 | 69.569 |
| UM-HNSCC-5 | female | 71 | mandible, floor of mouth, lip | T4N0M0 | 7,550 | 77,980 | 1.287 | 77.753 |
| UM-HNSCC-6 | male | 60 | left tongue | T4N0M0 | 4,600 | 54,040 | 3.270 | 70.815 |
| UM-HNSCC-7 | male | 60 | left tongue, floor of mouth | T4N1M0 | 27,920 | 187,112 | 4.153 | 50.987 |
| | | | | **Average** | **14,480** | **138,492** | **3.231** | **69.792** |
| | | | | **s.d.** | **16,344** | **107,559** | **2.685** | **13.861** |

* University of Michigan-Head and Neck Squamous Cell Carcinoma (UM-HNSCC)

**Figure W2.** The table depicts the demographic and stage information of the patients from whom we obtained the primary human HNSCC specimens that were used here. These tumors were dissociated by mechanic and enzymatic digestion, and nonviable and lineage cells were excluded. The total number and the percentage of $ALDH^{high}CD44^{high}$ and $ALDH^{low}CD44^{low}$ cells sorted are depicted.

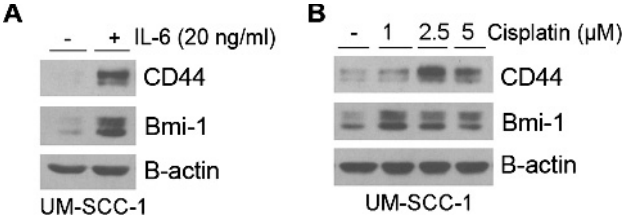


**Figure W3.** Western blot analysis depicts the expression of CD44 and Bmi-1 in UM-SCC-1 cells treated with (A) rhIL-6 (0 or 20 ng/ml) or (B) cisplatin (0-5 $\mu$M) for 24 hours.