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Los Angeles

Histone demethylase JMJD6 enhances cancer stem cell phenotype in oral squamous cell carcinoma cells

A thesis submitted in partial satisfaction

of the requirements of the degree Master of Science

in Oral Biology

by

Chang-Ryul Lee

2014

ABSTRACT OF THESIS

Histone demethylase JMJD6 enhances cancer stem cell phenotype

in oral squamous cell carcinoma cells

By

Chang-Ryul Lee

Master of Science in Oral Biology

University of California, Los Angeles 2014

Professor Ki-Hyuk Shin, Co-Chair

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Jumonji Domain Containing 6 (JMJD6) is a histone arginine demethylase that preferentially removes methyl groups from dimethylated arginine 2 of histone 3 (H3R2me2) and arginine 3 of histone 4 (H4R3me2), thereby enabling dynamic regulation of transcription. JMJD6 also regulates gene expression by modulating RNA splicing, suggesting that JMJD6 is a multifaceted epigenetic regulator. Clinically, JMJD6 overexpression is strongly linked to poor prognosis in various human cancers, including breast and lung. JMJD6 is also reported to promote cancer cell migration/invasion and angiogenic sprouting, two phenotypes of which are well known for cancer stem cell (CSCs; also known as cancer initiating cells) characteristics. JMJD6 is also known to be essential for the differentiation of multiple tissues and cells during

embryogenesis. Recent studies revealed that histone methylation played a critical role in controlling stemness of normal stem cells. Although CSCs share molecular and phenotypic characteristics with normal stem cells, a considerable knowledge gap remains in our understanding of epigenetic regulation of oral CSCs particularly by histone demethylases.

To investigate potential involvement of histone demethylases in oral CSCs, we screened expression of 22 known histone demethylases in CSC-enriched oral squamous cell carcinoma (OSCC) populations. Among them, JMJD6 was unequivocally overexpressed in all tested CSC-enriched OSCC populations. Subsequent functional analysis showed that knockdown of endogenous JMJD6 in OSCC strongly suppressed CSC phenotypes (*e.g.*, decreased self-renewal capacity and migration). Conversely, ectopic expression of JMJD6 enhanced the CSC phenotypes. Interestingly, expression of CSC factors (*e.g.*, pluripotency transcription factors and cytokines) was markedly affected by modulating JMJD6 expression. We further found that JMJD6 regulates the expression of CSC-specific cytokines (*i.e.,* IL-4, IL-5, MIP-1α, MIP-1β, IFNα, and IFNβ) by binding to their promoter regions. Our study suggests that JMJD6 promotes OSCC CSC phenotype by epigenetically regulating its target genes.

The thesis of Chang-Ryul Lee is approved.

Mo Kwan Kang

Ki-Hyuk Shin, Committee Co-chair

Reuben Kim, Committee Co-chair

University of California, Los Angeles
2014

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Introduction

Oral cancer represents an important problem not only because of its high incidence and significant mortality rate associated with the disease but also the functional and cosmetic defects that accompany the treatment. Oral cancer is the sixth most prevalent cancer worldwide, and it affects nearly 29,000 individuals each year in the United States alone. Oral squamous cell carcinoma (OSCC) is the most common type oral cancer representing more than 90% of all oral cancers [1]. Despite the improvement of surgical techniques and adjuvant treatments, the prognosis of OSCC remains poor, and more than 50% of OSCC patients decease within 5 years after the outbreak of the disease [2]. Also serious oral and maxillofacial defects associated with OSCC devastate the patients' physical and psychological welfare.

Although deeper understanding of molecular basis of oral cancer development is yet to be achieved, spontaneous accumulation of genetic predisposition and epigenetic alterations are known to be the main cause of oral cancer. The aim of our study was to investigate the epigenetic regulation of OSCC.

Accumulation of epigenetic alterations has an important implication in human cancer, and the importance of epigenetic changes that occur during multiple step oral cancer development has been recognized [3]. Epigenetic changes are associated with both DNA methylation and histone modifications, such as acetylation, phosphorylation, ubiquitination and methylation that provide a mechanism that controls the access of the transcriptional machinery to DNA [4]. Emerging evidence suggests that histone modifications can activate or repress the expression of downstream target proteins by altering chromatin accessibility [5,6]. Therefore, understanding the mechanism of these epigenetic modifications can identify novel cancer-related genes and provide new insights into the biology of oral cancers.

Methylation of histone tails is an important epigenetic modification that regulates various biological processes such as heterochromatin formation, X-chromosome inactivation, DNA repair and transcriptional regulation [7]. This methylation can occur on either lysine or arginine residues in the tails of histones 2, 3, and 4, and this modification is catalyzed by protein methyltransferases [8]. Histone methylation was thought to be irreversible until recently. However, two types of mechanism for histone demethylation have now been identified. A group of enzymes called histone demethylases regulates posttranscriptional activation or repression by removing these histone methylation marks [9,10]. The nuclear amine oxidase KDM1 was the first histone demethylase identified, and it mediated demthylation of H3K4 and H3K9 through a flavin dependent reaction [11-13]. Second group of histone demethylases includes members of the Jumonji C domain containing proteins that remove methyl groups on histone tails through an oxidative hydroxylation reaction [15-17]. Therefore, histone methylation can be regulated through the recruitment of methyltransferases and demethylases activating and repressing individual genes.

Recently, it has been demonstrated that histone demethylases play an important role in the development of various diseases, especially cancer. Histone demethylase KDM1 is overexpressed in various cancers including bladder cancer, colorectal cancer, oestrogen-receptor-negative breast cancer, and prostate cancer [14-16]. Also, the demethylase activity of KDM2B promotes the proliferation of acute myeloid leukemia and pancreatic ductal adenocarcinoma in which KDM2B is also highly expressed [17]. In addition, shRNA-mediated knockdown of KDM2B attenuated the growth of pancreatic cancer cells and acute myeloid leukemia cells [18]. It has been reported that KDM4C stimulates the growth of multiple cell lines in squamous cell carcinoma, prostate carcinoma, and breast carcinoma while depletion of KDM4B decreases the

proliferation of breast and colorectal cancer cell lines [19-23]. KDM5B is highly expressed in breast, prostate and bladder cancers, and KDM5B is reported to promote the growth of breast cancer and melanoma cells [24-29]. Interestingly, the latter study reported that KDM5B is expressed in slow-cycling tumor melanoma cells suggesting that the histone demethylase has an implication in a particular subpopulation of cancer cells called cancer stem cells (CSCs).

Recent studies have revealed the physiological role of CSCs in relapse and malignancy of cancers [30]. CSCs are considered as the seed of cancer for their crucial role in tumor formation and metastasis. CSCs possess characteristics associated with embryonic and normal adult stem cells and may generate tumors through stem cell processes of self-renewal and differentiation into multiple cell types [31]. CSCs are drug-resistant to cancer therapeutic reagent and highly metastatic. They form tumors upon injection in immunodeficient mice and grow as tumor spheres in undifferentiating medium [32].

There are several CSC factors that play an important role in the survival and sustenance of CSCs. The positive correlations of Oct4 and Nanog expression in oral cancer stem-like cells and high-grade OSCC cells have been revealed [33]. KLF4 is required for the maintenance of breast CSCs and cell migration [34]. Lin28A plays an important role in the regulation of CSC properties in OSCC by increasing cell proliferation, colony formation and invasion [35]. Also, transcription factors Zeb1 and Zeb2 have shown to maintain CSC properties such as self-renewal capacity in head and neck cancer [36]. All these CSC factors play a critical role in the CSC sustenance, and we used these factors to characterize CSCs in our present study.

Since CSCs maintain cellular heterogeneity of cancer tissues thereby driving the perpetuity of cancer, CSCs have become an important target of anti-cancer therapies. In our study, we examined the role of histone demethylases in the epigenetic regulation of CSCs.

Histone demethylase KDM1 has been reported to sustain the oncogenic potential of leukemia CSCs by preventing differentiation and apoptosis [37]. Inhibition of KDM1 using a specific bioactive inhibitor or siRNA attenuated the growth of CSCs in tetratocarcinoma, embryonic carcinoma, and seminoma that expressed stem cell markers such as Oct4. Interestingly, these inhibitory effects were CSC specific because the inhibition of KDM1 displayed minimum growth-inhibitory effects on non-CSCs [38]. All these results present histone demethylases as the novel regulators of CSCs.

Our study focuses primarily on a histone demethylase called Jumonji domain-containing 6 (JMJD6). Sequence analysis has shown that JMJD6 is a protein within a highly conserved, cupin fold-containing enzyme family that contains a Jumonji C domain [39]. The majority of proteins in this family are histone demethylases. However, the cellular activity of JMJD6 still remains unexplored compared to the other members of the family. Interestingly, JMJD6 was first identified as a phosphatidylserine receptor on cell membrane that recruits phagocytic cells to sites of apoptosis [40]. Subsequently, the involvement of JMJD6 in embryonic development has been reported. JMJD6 knockouts resulted in early postnatal lethality and growth retardation in mice [41]. Also, targeted knockout of JMJD6 in mice and morpholino silencing of JMJD6 in zebrafish caused embryonic lethality with hematopoiesis defect and abnormal development of eye, brain and heart [42-45]. Later, JMJD6 protein was described as a JmjC-containing iron- and 2-oxoglutarate-dependent dioxygenase that removed methyl moieties on histone H3 at arginine 2 (H3R2) and histone H4 at arginine 3 (H4R3) [46]. JMJD6 protein was also shown to interact and hydroxylate RNA splicing factor U2AF65 that binds to VEGF receptor (Flt1) mRNA, and siRNA-mediated knockdown of JMJD6 impaired angiogenic potential of endothelial cells by altering gene expression and splicing of VEGF receptor (Flt1) [47].

High expression level of JMJD6 protein has shown to be linked to poor prognosis and aggressive behavior of breast cancer and lung adenocarcinoma. Lee *et al.* have examined patients with breast cancer and revealed that JMJD6 expression was positively associated with decreased time to distant metastasis in eight of the 14 breast cancer cohorts studied. Also, Lentiviral-based overexpression of JMJD6 significantly increased proliferation of breast cancer cells. On the other hand, siRNA silencing of endogenous JMJD6 significantly decreased breast cancer cell proliferation. JMJD6 was also reported to influence cell scattering and motility. A degree of scattering is an indication of cellular motility and epithelial-mesenchymal transformation (EMT), and JMJD6 overexpression increased the degree of scattering in breast cancer cells [48]. Also, JMJD6 mRNA and protein expression were found to be significantly higher in lung adenocarcinoma tissues than they were in non-tumorous lung tissues, and lung adenocarcinoma patients with high JMJD6 expression had a significantly lower median overall survival rate compared to those with low JMJD6 expression suggesting the role of JMJD6 in aggressive clinical behavior of lung cancer [49].

These various observations indicate that the function of JMJD6 may be multidimensional. However, very little is known about the role of JMJD6 in the initiation and development of cancer and its molecular mechanism. Therefore, we first set out to investigate the link between JMJD6 and cancer focusing on CSCs, and we found substantial evidence that JMJD6 might play an important role in regulation of CSCs.

Next, we examined the possible mechanism of the regulation and find JMJD6 target genes. We decided to find JMJD6 target cytokines because increasing evidence suggested the role of cytokines in cancer development. Cytokines are broad and general category of messenger molecules with growth, differentiation, and activation functions that regulate and determine the

nature of immune responses. They are made by immune cells such as macrophages, B lymphocytes, T lymphocytes and mast cells, endothelial cells, fibroblasts, and various stromal cells. Cytokines include chemokines, interfereons, interleukins, lymphokines, tumour necrosis factor but not hormones or growth factors. Cytokines such as tumour necrosis factor (TNF) and interleukin 6 (IL6) have been characterized to exhibit tumorigenic potential, and they are suspected to activate oncogenic transcription factors NF-κB, AP-1 (TNF) and STAT3 (IL6) in epithelial cells [50,51]. Expression of IL6 in patients' serum correlates with the risk of colorectal adenomas development, and elevated expression of IL6 in tissue samples of patients with cancer or tumour-bearing animals is linked to poor prognosis [52-54]. Furthermore, genetic ablation of IL6 in mice resulted in a larger reduction of both the multiplicity and the size of colon carcinomas tumour load in mice [55]. TNF is also known as an important tumour-promoting cytokine that activates cancer cell survival and proliferation pathways, causes chronic inflammation and angiogenesis, and promotes tumour cell migration and invasion [56]. Elevated levels of IL4 and IL4R expression have been detected in various tumour cells of thyroid, colon, breast and ovarian cancers [57-59]. In a nutrient-deficient environment, IL4 induced proliferation and tumour growth of prostate cancer [60]. On the other hand, neutralizing IL4 significantly attenuated the growth of fibrosarcoma [61]. Also, the expression of IL5 and IL-5Rα was enhanced in patients with muscle invasive bladder cancers, and IL5 increased migration of bladder cancer cell lines via activation of transcription factors NF-κB and AP-1 [62].

Interestingly, some cytokines have been reported to play an important role in the regulation of CSCs. Studies have shown that the exposure of tumor cells to proinflammatory cytokines, TGFβ and TNFα, generated cells with CSC-phenotypes such as self-renewal capacity, tumorigenicity, and resistance to chemotherapeutic drugs [63]. Also, interaction of CSCs and

cells in the tumor microenvironment is shown to involve cytokines such as IL1, IL6 and IL8, which activate Stat3/NF-κB pathways in both tumor and stromal cells. Activation of these pathways enhances further cytokine production activating positive feedback loops that promote CSC self-renewal. Animal studies have shown that production of IL1 by tumor-associated macrophages increased angiogenesis, tumor growth, and metastatic potential, and blocking IL1 receptor decreased these processes in mouse models [64,65]. Furthermore, elevated IL1 expression was found in breast CSCs compared to their non-CSC counterparts [66]. IL6 is reported to play an important role in the regulation of breast CSC self-renewal and bone marrow mesenchymal stem cells [67,68]. Also, high expression of IL8 receptor CXCR1 was detected in breast CSCs, and recombinant IL8 enhanced breast CSC self-renewal and tumor growth. At the same time, blocking CXCR1 using a small molecular inhibitor repertaxin significantly decreased breast CSC population and reduced tumorigenicity and metastasis [69]. It has been suggested that IL4 may play a crucial role in the survival of colon CSCs, and blocking IL4 signal using a neutralizing antibody sensitized these colon CSCs to apoptotic stimuli and increased the *in vivo* efficacy of chemotherapeutic drugs [70]. All these reports suggest the important role of cytokines in regulation of CSCs, and we decided to investigate cytokines as the potential target of the CSC-specific histone demethylase JMJD6 that we identified in our study. In fact, there have been a few studies that demonstrate the link between histone demethylases and cytokines. In human primary macrophages, inhibition of KDM6B significantly decreased the production of proinflammatory cytokines such as TNFα [71]. Also, there was a report on the repressive role of histone demethylases in cytokine production. KDM1A binds to the promoter region of cytokines IL1α, IL1β, IL6 and IL8 and reduces the expression of these cytokines [72].

Thus, there have been a number of studies that reveal the potential role of histone

demethylases in cancer therapy and regulation of CSCs. Also, the strong association between several histone demethylases and cytokines has been reported. Although JMJD6 has been reported to be involved in various cancer malignancies, studies on the role of JMJD6 in CSCs are very limited. In our present study, we investigated the effect of JMJD6 on CSC property in OSCC. Our study demonstrated that JMJD6 enhances CSC phenotypes such as self-renewal capacity and metastatic potential by activating various cytokines in oral squamous cell carcinoma cells.

Materials and Methods

Cells and cell culture.

SCC4 and SCC9 cancer cell lines were purchased from the American Type Culture Collection (ATCC). SCC4 and SCC9 were cultured in Dulbecco's Modified Eagle's Medium (DMEM/F12) (Invitrogen) supplemented with 10% super calf serum (Germini Bio-products) and 0.4 μg/ml hydro-cortisone (Sigma–Aldrich), and 5 μg/ml Gentamycin aminoglycoside antibiotic (Invitrogen). SCC9 was treated with 5 ng/ml of TNFα (Sigma–Aldrich) for 2 months to establish SCC9/TNFα cancer cell line. HOK16B, POE9n, and OKF6/tert immortalized pre-cancer cell lines were cultured in EpiLife Medium (M-EPICF-500; Invitrogen) supplemented with Human Keratinocyte Growth Supplement (S-001-5; Invitrogen).

Tumor sphere formation assay.

Tumor sphere formation assays have been widely used to identify stem cells to evaluate their self-renewal capacity and differentiation at the single cell level *in vitro*. The first sphere formation assay was performed in 1992 when Reynolds and Weiss first cultured cells that exhibited stem cell properties as free-floating spheres from the adult brain. They dissected striatal tissue and enzymatically dissected the tissue to single cells. Then, they plated these cells in non-adherent conditions in serum-free medium supplemented with epidermal growth factor (EGF) [73]. Since this early experiment, tumor sphere formation assay has been used as a simple retrospective assay to identify cells exhibiting stem cell-phenotypes, self-renewal and differentiation. Tumor sphere formation assay was used for two major purposes in our study. First, we performed tumor sphere formation assay to obtain SCC4 and SCC9/ TNFα sphere cells. For this purpose, 10,000 cells per well were plated in two Ultralow Cluster Plates (Fisher) and

grown in tumor sphere medium for 7 days. The tumor sphere cells were collected and checked for gene expression.

Tumor sphere formation assay was also used for a functional study to examine the effect of histone demethylases modulation on self-renewal capacity of SCC4 CSCs. The cells were cultured in 6-well Ultralow Cluster Plates (Fisher) at a density of 3,000 cells per well. The number of tumor spheres formed were observed and counted under a microscope (Olympus) after 7 days. Assay was done in triplicates for each tested cell type, and average number of spheres formed per well were calculated.

The cells were grown in tumor sphere medium, which contains DMEM/F12 media with 1:100 N2 supplement (Invitrogen), 10ng/mL EGF, 10ng/ml bFGF (Invitrogen), and 5ug/ml Gentamycin aminoglycoside antibiotic (Invitrogen).

Quantitative real-time PCR (qPCR).

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen), and the quality of RNA was assessed using NanoDrop Spectrophotometer (Thermo Fisher Scientifirc)**.** cDNA was generated from 5 μg of total RNA extracted using SuperScript first-strand synthesis system (Invitrogen). We amplified 1ul cDNA using SYBR Green I Master Mix (Roche Applied Sciences) with the LightCycler 480 II real-time PCR system following the manufacturer's protocol (Roche). We obtained the primer sequences from the Universal Probe Library database (Roche), and the sequences are available upon request. The samples were loaded in triplicates in LightCycler 96 well plates (Roche). We used PCR amplification of housekeeping gene GAPDH as internal control. Second derivative Cq values of genes of interest and GAPDH were compared to assess the fold-differences of amplification following the manufacturer's instruction (Roche).

Western blotting.

We isolated whole cell extracts from the cultured cells using the lysis buffer (1% Triton X-100, 20 mM Tris –HCl pH 7.5, 150 mM NaCl, 1mM EDTA, 1mM EDTA, 2.5 mM sodium pyrophosphate, 1 μM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mg/ml PMSF). These extracts were run through SDS-PAGE gel and transferred to protein membrane (Millipor, Billerica, MA). BenchMark Pre-Stained Protein Ladder (Invitrogen) and MagicMark XP Western Protein Standard (Invitrogen) were mixed in 1:1 ratio and used as a protein ladder. The membranes were blocked in 5% nonfat milk (Bio Rad) and incubated with the primary and the secondary antibodies. After incubation with antibodies, the membranes were exposed to the chemiluminescence reagent (Amersham) for detection of proteins. Following primary antibodies were used for the study: JMJD6 (H-7; Santa Cruz Biotech.), Oct4 (Santa Cruz Biotech.), and GAPDH (FL-335; Santa Cruz Biotech.). Horse radish peroxidase (HRP)-conjugated antibodies (Santa Cruz Biotech.) were used for secondary antibodies.

Knockdown of endogenous histone demethylases by siRNA.

18 to 24 hours prior to transfection, we plated SCC4 cells and SCC9/TNFα cells (ATCC) in 3.5ml culture medium containing DMEM/F12 medium (Invitrogen), 10% super calf serum (Germini Bio-products) and 0.4 μg/ml hydro-cortisone (Sigma–Aldrich) in 60mm plates without antibiotics such that they would be 80% confluent at the time of transfection. The cells were incubated in 37^{o}C in a CO_2 incubator. In these cells, JMJD6 expression was inhibited with siRNA targeting JMJD6 or the control, scrambeled siRNA (Santa Cruz). siRNAs were introduced using Lipofectamine RNAiMAX (Invitrogen). For each well to be transfected,

siRNA-Lipofectamine RNAiMAX complexes were prepared as follows: 15µl of siRNA (10µM) in 500µl Epilife (Invitrogen) was mixed with 10µl Lipofectamine RNAiMAX in 500ul Eplilife making the total volume of 1ml. This mixture was in incubated for 15 minutes at room temperature. We added this solution to each plate of cells and incubated 15 hours in 37^{o}C. After the incubation, the medium was changed with fresh medium containing 5µg/ml Gentamycin aminoglycoside antibiotic (Invitrogen). The cells were incubated at 37^{o}C overnight and harvested for a functional study (tumor sphere formation assay) and quantitative real-time PCR confirmation of successful knockdown. This transfection method let us transiently knockdown 6 histone demethylases (KDM3A, KDM4D, KDM5A, KDM7A, KDM8, and JMJD6) in SCC4 and 1 histone demethylase (JMJD6) in SCC9/TNFα.

Knockdown of endogenous JMJD6 by shRNA in SCC4.

5µg of shRNAs expression plasmids purchased from HuSH Origene were diluted in 50ul of dH_2O to make a final concentration of 100ng/µl. 18 to 24 hours prior to transfection, we seeded SCC4 cells (ATCC) in 3.5ml culture medium containing DMEM/F12 medium (Invitrogen), 10% super calf serum (Germini Bio-products) and 0.4 µg/ml hydro-cortisone (Sigma–Aldrich) in 60mm plates without antibiotics such that they would be 80% confluent at the time of transfection. The cells were incubated in 37^{o}C in a CO_2 incubator.

We inhibited JMJD6 expression in SCC4 cells using duplex shRNA expression plasmid targeting JMJD6 or the control, scrambeled shRNA (OriGene), which was introduced using DNAfectin2100 (Lamda Biotech). The transfection complexes were prepared as follows. 4 µg of shRNA was added to 460µl of serum-free, antibiotic-free medium so that the total is 500µl. 30µl of DNAfectin2100 (Lamda Biotech) was added to 470µl of serum-free, antibiotic-free medium

so that the total is 500µl of transfection complexes. These two solutions were added and incubated for 20 minutes at room temperature. 2.4ml of serum-free, antibiotic-free medium was added to the combined solution to make a total of 3.4ml transfection complexes. The growth medium was removed from the 60mm plates, and the 3.4ml transfection complexes were added to the 60mm plates.

The cells were incubated at 37°C overnight, and the medium was changed with fresh medium containing 5µg/ml Gentamycin aminoglycoside antibiotic (Invitrogen). The cells were selected with 0.5µg/ml puromycin for three weeks.

Overexpression of exogenous JMJD6 in SCC4.

18 to 24 hours prior to transfection, we plated SCC4 cells (ATCC) in 3.5ml culture medium containing DMEM/F12 medium (Invitrogen), 10% super calf serum (Germini Bio-products) and 0.4 µg/ml hydro-cortisone (Sigma–Aldrich) in 60mm plates without antibiotics such that they would be 80% confluent at the time of transfection.

On the day of transfection, a vector that expresses exogenous JMJD6 or the control empty vector (OriGene) was introduced into SCC4 using Lipofectamine 2000 (Invitrogen). We made a solution containing 4µg of shRNA expression plasmid and 11µl of Lipofectamine 2000 and added it into the plates of SCC4 cells. The cells were incubated at 37°C overnight, and the medium was changed with fresh medium containing 5µg/ml Gentamycin aminoglycoside antibiotic (Invitrogen). The cells were selected with 0.5 µg/ml puromycin for three weeks.

Transwell assays.

Transwell assays have been used widely to examine the migratory property of cancer cells. During this assay, cancer cells are placed on the permeable membrane of the upper chamber and the solution containing the serum is placed in the lower chamber to induce the migration of the cancer cells through the permeable filter. The cells that have migrated through the membrane are stained and counted. The advantage of this migration assay is its detection sensitivity. Low levels of angiogenic inducers (*i.e.*, fetal bovine serum) can induce migration through the permeable membrane.

Transwell chambers were used according to the manufacturer's instructions (Corning). The cells were suspended in serum-free DMEM/F12 medium at 2×10^5 cells/ml. A 100μl cell suspension was pipetted into the upper chamber of the Transwell inserts (6.5mm diameter, 8.0 μm pore size). 600μl DMEM/F12 medium with 1% fetal bovine serum was added to the lower chamber as angiogenic chemoattractant. The cells were incubated for 48 hours at 37 °C. We gently washed the transwell inserts with PBS. The cells that had invaded the lower surface were fixed with formaldehyde and stained with 0.25 % crystal violet for 90 minutes. Then the cells on the upper side of the membrane were removed with a cotton swab. The number of cells that had invaded through the transwell membrane was observed counted ×200 magnification under microscope (Olympus).

Wound-healing assay.

The wound-healing assay is one of the most simple and inexpensive methods to study cell migration *in vitro*. This assay mimics cell migration that occurs during wound-healing process *in vivo*. In this method, a "wound" is created in a cell monolayer, and

images are captured at the beginning and at regular time intervals. Then the images are compared to quantify the migration rate of the cells.

In our study, we transfected SCC4 cells to overexpress JMJD6 and cultured them in 60mm plates. The cells were grown to confluence and undergone serum-free condition for 24hours before the experiment. Linear wounds were made with a sterile 200-µl pipette tip. Cells were washed to remove the debris using PBS and incubated at 37 °C for 24 h. The serial images of cell migration at 0, 6 and 24 h after scratching were obtained using a microscope (Olympus).

Immunohistochemical staining of human head and neck SCC.

The use of normal human oral epithelia (NHOE) and oral squamous cell carcinomas (OSCC) samples for this study was approved by the University of California, Los Angeles Institutional Review Board. We deparaffinized paraffin-embedded slides in 60^{o}C and rehydrated with xylene and ethanol. Tissues were unmasked with citrated buffer. The cells were incubated with 3% hydrogen peroxidase in methanol to block endogenous peroxidase. We blocked non-specific signals with blocking buffer and stained the slides with anti-JMJD6 (1:100; H-7; Santa Cruz Biotech.) diluted in 5% BSA at 4°C overnight in a humid chamber. Then, the slides were washed and incubated with mouse secondary antibody (1:200) diluted 5% BSA for 40 minutes in a humid chamber. The slides were incubated with HRP-Avidin (1:1000) diluted in PBST then developed with 3,3' Diaminobenzidine (DAB) solutions (Abcam) to visualize the JMJD6 protein.

Chromatin immunoprecipitation assays.

ChIP assays were carried out with a MAGnify™ Chromatin Immunoprecipitation kit following the manufacturer's protocol (Invitrogen). SCC4 monolayer cells were prepared by

growing SCC4 cells (ATCC) in normal culture medium containing DMEM/F12 (Invitrogen), 10% super calf serum (Germini Bio-products) and 0.4 μg/ml hydro-cortisone (Sigma–Aldrich), and 5 μg/ml Gentamycin aminoglycoside antibiotic (Invitrogen) in a 100mm plate such that they would be 80% confluent at the time of harvest. SCC4 sphere cells were prepared by plating 1×10^4 cells per well in two 6 well Ultralow Cluster Plates (Fisher). SCC4 sphere cells were grown in sphere medium containing DMEM/F12 media with 1:100 N2 supplement (Invitrogen), 10 ng/mL EGF, 10ng/ml bFGF (Invitrogen), and 5μg/ml Gentamycin aminoglycoside antibiotic (Invitrogen) and harvested after 7 days. These cells were cross-linked with formaldehyde to ensure that the chromatin structure is preserved during the isolation and chromatin immunoprecipitation procedure. Lysis buffer with protease inhibitors were added to lyse the cells. Next, chromatin was sonicated and sheared into 200-500 bp fragments. A small fraction of the sonicated chromatin was put aside for input DNA and immunoprecipitation with antibodies. This sheared chromatin was diluted with dilution buffer with protease inhibitor and bound to the JMJD6 antibody-Dynabeads (Invitrogen) complexes.

ChIP complexes were immunoprecipitated with anti-JMJD6 (H-7; Santa Cruz Biotech.), or anti-Mouse IgG (Invitrogen) as a negative control. These chromatin-JMJD6 antibody-Dynabeads complexes were washed to remove any unbound product. Next, formaldehyde crosslinking was reversed using reverse crosslinking buffer (Invitrogen) and proteinase K (Invitrogen). Then, we purified the un-crosslinked DNA using the DNA purification magnetic beads (Invitrogen). The recruitment of JMJD6 proteins was measured by real-time qPCR, using specific primers listed below. The results were normalized with the levels of the input of samples and expressed as the percentage of input DNA.

All resulting precipitated DNA samples were quantified with a specific set of primers for individual genes by real-time PCR:

IL4, 5’-GCCTGTTATTCTGCCTCTATGC-3’ (forward) and

5’-TGGAAACTGTCCTGTCATGG-3’ (reverse);

IL5, 5’-TGGGACCCCACATTTCTAAG-3’ (forward) and

5’-TGTTCATGAGCAGCAGGAAA-3’ (reverse);

IL7, 5’-CACAAAACTGGCAGAGAGCA-3’ (forward) and

5’-TGAATGCATGCCACTGAAAT-3’ (reverse);

MIP-1α, 5’-ACGATGCTGGGTCAGGTATC-3’ (forward) and

5’-AGTGACTAGGGCGCTGTGTT-3’ (reverse);

MIP-1β, 5’-AGCAGAAGAAGGCACAGGAA-3’ (forward) and

5’-GAGAGTTGCAAACCCCAGAG-3’ (reverse);

IFNα, 5’-GGAACAAGATGGGGAAGACA-3’ (forward) and

5’-TGCTGAGATGGGTGACTCTG-3’ (reverse);

IFNβ, 5’-GAAGTTTCCAAGGCCCTCTC-3’ (forward) and

5’-GGGAATTGAGCATCCTCTGA-3’ (reverse);

IFNγ, 5’-TGGGTCTGTCTCATCGTCAA-3’ (forward) and

5’-TCCTCTGGCTGCTGGTATTT-3’ (reverse).

Results

Identification of CSC-specific histone demethylases.

In the beginning of our study, we wanted to see if there was association between histone demethylases and OSCC CSCs. We examined which histone demethylases were differentially expressed in CSC population compared to non-CSC population. In order to isolate CSC-enriched population, we utilized tumor sphere formation assay. We grew SCC4 sphere cells in tumor sphere medium for seven days and collected the spheres formed to isolate SCC4 CSCs. We also grew SCC4 cells in monolayer in normal culture medium. Tumor spheres represent CSC-enriched population while monolayer represents non-CSC population. Relative mRNA levels of 22 HDMs in SCC4 sphere cells and monolayer cells were compared to see if they were differentially expressed. We identified 6 histone demethyalases (KDM3A, KDM4D, KDM5A, KDM7A, KDM8, and JMJD6) that were overexpressed in SCC4 sphere cells compared to SCC4 monolayer cells, and we named these histone demethylases, CSC-specific histone demethylases (Fig. 1A). We suspected if the tumor sphere medium could be the cause of this differential histone demethylase expression. Therefore, we also grew SCC4 monolayer in tumor sphere medium and measured the expression level of histone demethylases as a control. We confirmed that difference in medium did not affect histone demethylases expression since SCC4 monolayer cells grown in normal culture medium and tumor sphere medium showed similar histone demethylases expression level.

We wanted to see if these 6 CSC-specifie histone demethyases were overexpressed in CSC-enriched population of other cancer cell lines. We performed a tumor sphere formation assay with SCC9/TNFα cells and confirmed the overexpression of these CSC-specific histone demethylases in SCC9/TNFα tumor spheres cells (Fig. 1B). Consistent upregulation of these

CSC-specific histone demethylases in SCC4 and SCC9/TNFα cell lines suggests that these histone demethylases may play an important role in the sustenance of CSCs. Especially, the expression of JMJD6 was significantly higher in both SCC4 and SCC9/TNFα CSC-enriched sphere populations. We also performed a western blot analysis of SCC4 tumor spheres and SCC4 monolayer and confirmed that JMJD6 protein level was overexpressed in CSC-enriched SCC4 tumor spheres (Fig. 1C).

The effect of CSC-specific histone demethylases knockdown on CSC self-renewal capacity.

Since we suspected that these CSC-specific histone demethylases might play an important role in CSC sustenance, we wanted to see if inhibition of these histone demethylases changed CSC phenotypes. We knocked down the histone demethyases in SCC4 cells using siRNA and performed a tumor sphere formation assay to see if these CSC-specific histone demethylases had an implication in CSC self-renewal capacity (Fig. 2A). Knockdown of some of these CSC-specific histone demethylases decreased the number of spheres formed, and knockdown of JMJD6 showed the most significance suppression of sphere formation. Both the number and the size of tumor spheres have decreased dramatically upon JMJD6 knockdown (Fig. 2 C). We also harvested cells and measured the expression levels of the histone demethylases using quantitative real-time PCR to confirm the knockdown (Fig. 2B).

Since the effect of JMJD6 knockdown showed the most significant phenotypic change, our subsequent experiments focused on this particular histone demethylase. We repeated sphere formation assays twice in SCC4 and once in SCC9/TNFα. JMJD6 knockdown consistently inhibited tumor sphere formation in both SCC4 and SCC9/TNFα confirming that JMJD6 knockdown did decrease CSC self-renewal capacity (Fig 2D).

Effect of JMJD modulation on SCC4 CSC self-renewal capacity.

We wanted to investigate the effect of JMJ6 modulation on the SCC4 CSC phenotypes. We knocked down JMJD6 in SCC4 using shRNA and made a stable cell line. Then, the cells were treated with puromycin to select for colonies with successful JMJD6 knockdown. We performed a western blot analysis and confirmed successful JMJD6 inhibition. We also measured the protein level of Oct4 an important CSC factor using a western blot analysis (Fig. 3A). Oct4 protein level was significantly reduced with JMJD6 knockdown suggesting that JMJD6 might be involved in CSC sustenance. Using this stable cell line, we repeated a tumor sphere formation assay. Inhibition of JMJD6 reduced relative number of tumor spheres formed (Fig. 3B).

Next, we amplified JMJD6 expression in SCC4 and observed the effect of this overexpression on SCC4 self-renewal capacity. We transfected SCC4 cells with a vector that expressed exogenous JMJD6. The cells were treated with puromycin to select for colonies successfully transfected with the vector. The western blot analysis confirmed successful JMJD6 overexpression (Fig. 3C). Using this system, we performed a tumor sphere formation assay. Overexpression of JMJD6 increased not only the number but also the size of spheres suggesting the involvement of JMJD6 in SCC4 self-renewal capacity, an important CSC phenotype. (Fig. 3D and E).

JMJD6 increased SCC4 migration.

One of the most well known characteristics of CSCs is that CSCs are highly metastatic. We wanted to see if JMJD6 modulation could affect metastatic potential of CSCs. We performed

a transwell assay using SCC4 cell line that overexpressed JMJD6, and counted the number of cells migrated after 48 hours to see the effect of JMJD6 overexpression on SCC4 migration. The length of incubation was limited to 48 hours to eliminate the possibility that proliferation of SCC4 affected the result. Our result showed that SCC4 cells overexpressing JMJD6 migrated significantly more than the control cells (Fig. 4A and B).

This increased migration capacity was further confirmed in a wound-healing assay. Prior to this migration assay, we cultured the cells in serum starvation condition for 24 hours to minimize the proliferation of SCC4 cells. SCC4 cells overexpressing JMJD6 migrated significantly faster than SCC4 control cells and filled up the gap completely after 24 hours (Fig. 4C). Based on the transwell assay and the would-healing assay results, we concluded that JMJD6 increased metastatic potential of CSCs. Thus, we have concluded that JMJD6 increased CSC self-renewal capacity and migration.

JMJD6 is associated with cancer carcinogenesis.

We wanted to see if JMJD6 was involved in cancer carcinogenesis. We compared JMJD6 protein expression level in two groups of cell lines: cancer cell lines and immortalized pre-cancer cell lines. Our western blot analysis indicated that JMJD6 protein level was consistently up-regulated in cancer cell lines UM17B and BapT while down-regulated in immortalized pre-cancer cell lines HOK16B, POE9n, and OKF6/tert (Fig. 5). This finding suggests the role of JMDJ6 in cancer carcinogenesis.

JMJD6 correlates positively with OSCC development.

Next, we wanted to see if JMJD6 was involved in OSCC development. We performed immunohistochemical staining for JMJD6 in normal human oral epithelia (NHOE) and oral squamous cell carcinomas (OSCC) to examine whether JMJD6 is associated with oral cancer development *in vivo*. We expected to see higher JMJD6 expression in malignant OSCC tissues compared to normal human oral epithelia. Although we could see moderate nuclear staining patterns in the basal cells in NHOE, strong and diffused patterns of JMJD6 expression was evident in OSCC tissues (Fig. 6). This data indicates that expression of JMJD6 correlates positively with cancer development.

JMJD6 regulates CSC factors.

After finding the strong association between JMJD6 and CSCs, we wanted to see if JMJD6 could modulate expression of various CSC factors. We inhibited or overexpressed JMJD6 in SCC4 and observed if this modulation can alter the mRNA expression of 5 well-known CSC factors (Oct4, KLF4, Lin28A, Zeb1, Zeb2). They were consistently up-regulated with JMJD6 overexpression and down-regulated with JMJD6 knockdown (Fig. 7A and B). This result shows that JMJD6 is an important regulator of CSCs.

Identification of JMJD6 target cytokines.

After finding the evidence that shows the association between JMJD6 and CSCs, we set out to find JMJD6 target genes and investigate its mechanism. We decided to investigate cytokines because of strong association between cytokines and CSCs reported. We investigated whether JMJD6 enhanced CSC phenotypes such as self-renewal capacity and metastatic potential by targeting certain cytokines.

To find JMJD6 target cytokines, we approached in two ways. First, we identified genes regulated by JMJD6. Second, we identified genes bound to JMJD6. We expected JMJD6 target cytokines to be regulated by as well as bound to JMJD6 (Fig. 8A).

Identifying cytokines regulated by JMJD6, we compared the expression of 22 different cytokines in SCC4 sphere cells SCC4 monolayer cells using quantitative real-time PCR. We identified 11 cytokines (IL-4, IL-5, IL-6, IL-7, IL-8, IL36Rn, IFN-α, IFN-β, IFN-γ, MIP-1α, and MIP-1β) that were significantly up-regulated in CSC-enriched SCC4 sphere compared to SCC4 monolayer (Fig. 8B).

Next, we compared the expression levels of these 11 CSC-specific cytokines in two different sets. In the first set, we measured the expression of CSC-specific cytokines in SCC4 cells overexpressing JMJD6. In the second set, we inhibited JMJD6 in SCC4 and measured the expression of these cytokines. We found that 8 out of these 11 CSC-specific cytokines were consistently regulated by JMJD6 in SCC4. They were up-regulated with JMJD6 overexpression and down-regulated with JMJD6 knockdown (Fig. 8C and D). We identified these cytokines (IL-4, IL-5, IL-7, MIP-1α, MIP-1β, IFN-α, IFN-β, and IFN-γ) as the potential JMJD6 target cytokines.

After identifying the cytokines regulated by JMJD6, we examined the physical interaction between JMJD6 and these cytokines promoters. We performed Chromatin immunoprecipitation assay with 8 cytokines regulated by JMJD6 (Fig. 9). We compared two groups: SCC4 monolayer cells and SCC4 sphere cells in this assay. We expected that JMJD6 would bind to its target cytokine promoter more in SCC4 tumor sphere cells in which JMJD6 was enriched. ChIP assay result showed that JMJD6 preferentially bound to the promoter of 6 cytokines (IL-4, IL-5, IFNα, IFNβ, MIP-1α and MIP-1β) in SCC4 tumor sphere cells compared

to monolayer cells. IgG was included as a negative control to ensure that binding of JMJ6 to the promoters of cytokines was specific. Based on these results, we identified IL-4, IL-5, IFNα, IFNβ, MIP-1α and MIP-1β as JMJD6 target cytokines.

Discussion

The aim of our study was to investigate the epigenetic regulation of CSCs. CSCs produce cellular heterogeneity of cancer tissues and drive the perpetuity of the disease, and they are becoming important targets of anti-cancer therapies. Their crucial roles in malignancy of cancer cells (*i.e.,* migration/invasion, resistance to chemotherapies, anchorage independent growth ability, and self-renewal capacity) have been reported [74]. In this study, we report that histone demethylases JMJD6 enhances CSC phenotype such as an increase in self-renewal capacity, migration, and CSC factor expression by activating various cytokines in OSCC cells.

A number of studies have shown the association between JMJD6 and human cancer. It has been reported that the expression of JMJD6 is markedly up-regulated in various types of human cancer including colon, breast, liver, lung, renal, pancreatic, colon, esophageal, rectal, and gastric cancers [75]. Also, high expression of JMJD6 protein is strongly linked to poor prognosis and aggressive clinical behavior of breast cancer and lung adenocarcinoma patients [48,49]. Our study presents the novel finding that JMJD6 may play an important role in oral cancer development by increasing CSC phenotype of OSCC.

In our preliminary experiments, we examined 22 histone demethylases mRNA expression level in SCC4 tumor sphere cells, which represent CSC-enriched population. Sphere formation assay has been used to obtain CSCs in cancer studies. Chiou et al. has enriched a subpopulation of oral cancer stem-like cells (OC-SLC) through tumor sphere formation by growing OSCC cells from established OSCC cell lines by cultivating OSCC cells within defined serum-free tumor sphere medium. They confirmed that these cancer stem-like cells obtained through sphere formation assay showed the characteristics of both stem cells and malignant tumors. OC-SLC showed the elevated expression level of stem/progenitor cell markers such as Nanog or Oct4 and

displayed induced differentiation abilities. Also, OC-SLC showed increased migration and invasion capabilities *in vitro* and *in vivo* [33]. Therefore, we utilized sphere formation assay to obtain CSC-enriched population in SCC4 and SCC9/TNFα cell lines in our present study.

We identified 6 histone demethylases (KDM3A, KDM4D, KDM5A, KDM7A, KDM8, and JMJD6) that were significantly up-regulated in SCC4 tumor sphere cells compared to SCC4 monolayer cells. Since tumor sphere cells represent CSC-enriched population, we named these 6 histone demethylases that were highly expressed in SCC4 sphere cells CSC-specific histone demethylases. The mRNA expression levels of these CSC-specific histone demethylases were also measured in SCC9/TNFα tumor sphere cells and monolayer cells. Since the CSC-specific histone demethylases were consistently up-regulated in tumor sphere cells compared to monolayer cells, we concluded that these CSC-specific histone demethylases might play an important role in CSC survival and maintenance and started functional studies to see if modulating these histone demethylases could induce CSC phenotypic changes.

Tumor sphere formation assay has also been used in stem cell biology to evaluate self-renewal capacity and differentiation of stem cells at the single cell level [73]. Self-renewal is an important CSC phenotype that enables cells to undergo numerous cycles of cell division while maintaining the undifferentiated state. This is the process by which CSCs perpetuate stem cell pool throughout life. Therefore, we knocked down the CSC-specific histone demethylases individually in SCC4 using siRNAs and performed a tumor sphere formation assay to see if inhibition of these histone demethylases had an effect on SCC4 CSC self-renewal capacity. Inhibition of all CSC-specific histone demethylases except KDM7A reduced SCC4 tumor sphere formation. Since inhibition of JMJD6 reduced sphere formation most dramatically, we concluded that JMJD6 had an effect on CSC self-renewal capacity and directed our aim to further

investigate the effect of JMJD6 modulation in CSC phenotypes. We inhibited JMJD6 in SCC9/TNFα and performed a tumor sphere formation assay. Knockdown of JMJD6 greatly reduced tumor sphere formation of SCC9/TNFα CSCs indicating that the effect of JMJD6 inhibition on CSC self-renewal capacity was not limited to SCC4 cell line.

To further investigate the role of JMJD6 in CSC phenotypes, we inhibited JMJD6 in SCC4 using shRNA and performed a tumor sphere formation assay. This stable knockdown of JMJD6 also reduced SCC4 self-renewal capacity. At the same time, we up-regulated JMJD6 in SCC4 cells by transfecting the cells with a vector that expressed exogenous JMJD6. JMJD6 overexpression increased SCC4 self-renewal capacity. All these results indicate that JMJD6 may enhance CSC self-renewal capacity.

CSCs are also known to exhibit high migratory and invasive characteristics. Therefore, we investigated whether JMJD6 affected OSCC CSC migration. There has been increasing evidence that reports the importance of JMJD6 in cell migration. Knockdown of JMJD6 in invasive breast cancer cell lines decreased cell migration, whereas overexpression promoted cellular motility [48]. It has been shown that JMJD6 interacts with splicing factor U2AF65 and modulate alternate splicing of VEGF receptor (Flt1) [76]. Alternate splicing of VEGF receptor (Flt1) by U2AF65 promoted endothelial cell migration, and si-RNA mediated silencing of JMJD6 in endothelial cells led to decreased migration [47].

Our finding is consistent with these recent reports demonstrating that JMJD6 enhances OSCC cancer cell migration. We overexpressed JMJD6 in SCC4 and performed a transwell assay and a wound-healing assay. Overexpression of JMJD6 significantly increased SCC4 migration. From these results, we concluded that JMJD6 increased SCC4 migration, an important CSC phenotype.

We also investigated whether JMJD6 is associated with cancer carcinogenesis. Carcinogenesis is a process by which normal cells are transformed into cancer cells. Normal cells go through the changes at the cellular, genetic, and epigenetic level that ultimately reprogram the cells to proliferate indefinitely and form a malignant mass. We measured JMJD6 protein levels in two groups: cancer cell lines (UM17B and BapT) and immortalized pre-cancer cell lines (HOK16B, OKF6/tert, and POE9n). JMJD6 protein level was consistently up-regulated in cancer cell lines suggesting that JMJD6 might play an important role in the transformation of pre-cancer cells to cancer cells.

Next, we investigated whether JMJD6 is involved in OSCC development *in vivo* and performed immunohistochemical staining for JMJD6 in normal human oral epithelia (NHOE) and oral squamous cell carcinomas (OSCC). The result showed that JMJD6 is highly expressed in OSCC compared to NHOE suggesting that expression of JMJD6 correlates positively with oral cancer development.

We also investigated whether JMJD6 could modulate CSC factor expression. We overexpressed or inhibited JMJD6 in SCC4 and measured the mRNA expression level of several important CSC factors (Oct4, KLF4, Lin28A, Zeb1 and Zeb2). Our finding shows that we may regulate the expression of these CSC factors by modulating JMJD6. Studies have shown the important role of these factors in synthesis and sustenance of CSCs. Oct4 has been detected in CSCs of various cancers including melanoma, prostate cancer, lung cancer, and oral cancer [33,77-79]. Also, Kumar et al. have showed that transmembrane delivery of Oct4 protein promoted melanoma cells to differentiate to CSC-like cells and acquire the ability to form tumor spheres. Also, subcutaneous tail vein injection of these cells had significantly increased tumorigenic capacity in mice [80]. Kruppel-like factor 4 (KLF4) is an important factor for

maintaining self-renewal of adult and embryonic stem cells. It has been used to induce pluripotent stem cells (iPS) from somatic cells. Also, it has been demonstrated that KLF4 acts as an oncogene in breast CSCs. Yu et al. have shown that the elevated level of KLF4 has been found in breast CSC-enriched population in mouse primary mammary tumor and cancer cell lines. They found that the inhibition of KLF4 in breast cancer cells decreased the proportion of CSCs. In addition, overexpression of KLF4 increased the proportion of CSC population in breast cancer cells. Furthermore, knockdown of KLF4 suppressed cell migration and invasion in breast cancer cells [34]. Furthermore, KLF4 is overexpressed in colon CSC-enriched sphere cells, and inhibition of KLF4 using shRNA significantly decreased the abilities of these cells to resist chemotherapeutic drugs, migrate, invade, and generate tumors [81]. Lin28A is another important CSC factor, and its role in CSCs of OSCC has been investigated. Hayashi *et al.* has found that Lin28A is markedly up-regulated in CSC-enriched OSCC population, and the overexpression of Lin28A in TOSCC23 oral cancer cells increased their proliferation, colony formation and migration showing the enhanced CSC characteristics [35]. Zeb1/Zeb2 are important epithelial-mesenchymal transcription factors, and they play important roles in the maintenance of CSC-like properties in head and neck cancer. It has been demonstrated that Zeb1/Zeb2 expression is significantly increased in head and neck CSCs compared to non-CSCs. Also, siRNA-mediated knockdown of Zeb1 and Zeb2 in head and neck cancer cells decreased their CSC-like properties such as self-renewal capacity, the expression of stemness markers, and drug resistance. On the other hand, co-overexpression of Zeb1 and Zeb2 in head and neck cancer cells enhanced their sphere-forming ability. Furthermore, delivery of siZeb1/2 to xenograft tumors in mice suppressed tumor growth and the rate of metastasis to distant site [36].

The result of our study is consistent with these various findings on CSC factors. These CSC-associated genes were up-regulated with JMJD6 overexpression and down-regulated with JMJD6 knockdown in SCC4 suggesting the importance of JMJD6 on CSC sustenance in OSCC. Indeed, JMJD6 may enhance cancer stemness by modulating various CSC factors.

Next, we set out to reveal the mechanism of this process. We speculated that JMJD6 might target cytokines to enhance CSC-like phenotypes. CSCs interact with and regulated by cells in the microenvironment, and this interaction involves various cytokines. The role of cytokines in CSCs has been widely studied, and the interaction between certain histone demethylases and cytokines has also been reported [71-72]. We measured the expression levels of 22 cytokines in SCC4 tumor sphere cells and SCC4 monolayer cells. We identified 11 CSC-specific cytokines that were highly expressed in CSC-enriched SCC4 sphere cells. To find the JMJD6 target cytokines, we first tested which of these CSC-specific cytokines were regulated by JMJD6. We identified 8 cytokines (IL-4, IL-5, IL-6, IL-7, IL36Rn, MIP-1α, MIP-1β, IFN-α, IFN-β, and IFN-γ) that are up-regulated with JMJD6 overexpression and down-regulated with JMJD6 knockdown.

Next, we examined the physical interaction between JMJD6 and the cytokines regulated by JMJD6 using chromatin immunoprecipitation assay. We compared two groups: SCC4 monolayer cells and SCC4 sphere cells. We expected that JMJD6 would bind to its target cytokine promoter more in SCC4 tumor sphere cells in which JMJD6 was enriched. We found that JMJD6 preferentially bound to the promoter of 6 cytokines (IL-4, IL-5, IFNα, IFNβ, MIP-1α and MIP-1β) in SCC4 tumor sphere cells compared to monolayer cells. Thus, we have identified 6 JMJD6 target cytokines that may enhance CSC properties in OSCC cells.

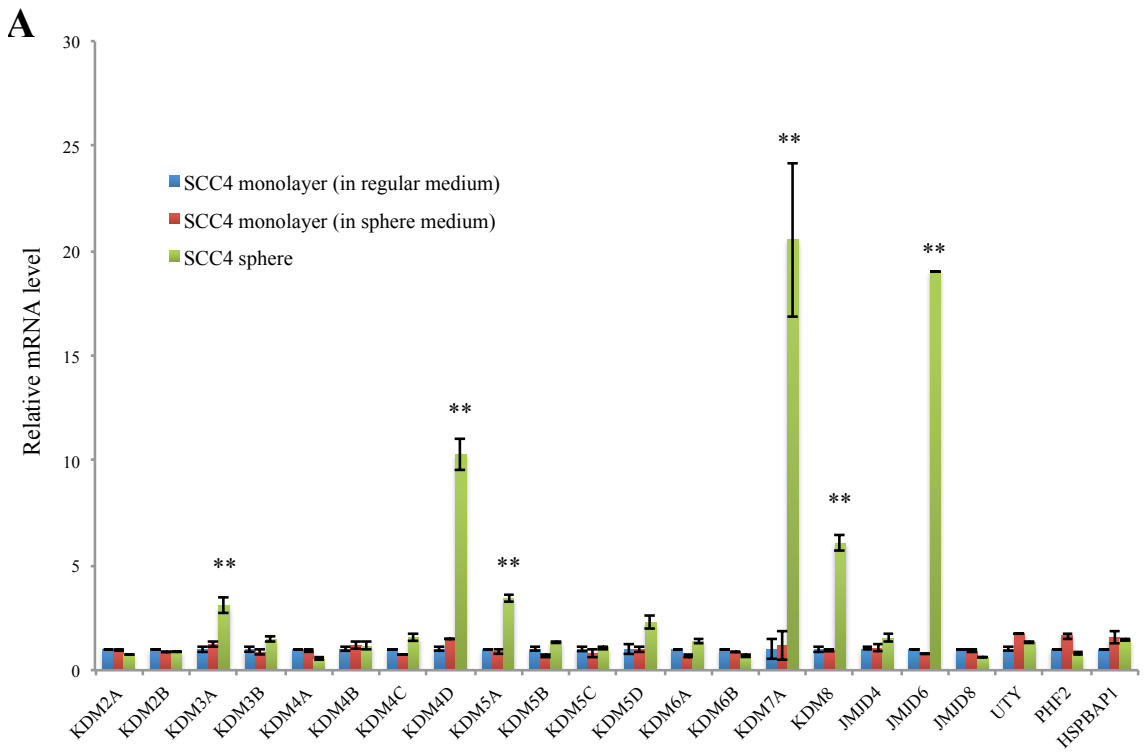
Some of these JMJD6 target cytokines have been associated with cancer malignancy and CSC survival. IL-4 production on colon CSC has been demonstrated to be essential for the survival of colon CSCs, and inhibition of IL-4 sensitized colon CSCs to apoptotic stimuli such as chemotherapeutic drugs [70]. Sivana *et al.* has demonstrated that MIP-1α plasma concentrations were strongly associated with established markers of lymphocytic leukemia suggesting MIP-1α as a potential prognostic marker of chronic lymphocytic leukemia [82]. The expression of IL-5 and IL-5Rα is elevated in patients with muscle invasive bladder cancer, and it has been demonstrated that IL-5 enhances migration and invasion of bladder cancer cells by activating transcription factors NF-κB and AP-1 [62]. These various findings suggest the potential of studying JMJD6 and its target cytokines for anticancer therapy.

In conclusion, this study provides new findings about the role of JMJD6 in OSCC progression. Histone demethylases JMJD6 enhances CSC phenotype by activating various cytokines in OSCC. JMJD6 up-regulates its target cytokines through epigenetic regulation, and we speculate that these JMJD6 target cytokines may play an important role in increasing cancer stemness. In the future, we can perform restoration/rescue experiments in which we treat various combinations of cytokines in SCC4/JMJD6shRNA cells to see if the supplementation of cytokines restores the self-renewal capacity of the cells. We can also perform *in vivo* animal study and see the effect of JMJD6 modulation on tumor growth. The potential of targeting JMJD6 in cancer therapy seems substantial, and finding chemical inhibitors of JMJD6 would be an important OSCC therapeutic modality.

Figure Legend and Figures

Figure 1. Differential expression of histone demethylases in non-CSC vs. CSC population.

The relative mRNA expression levels of histone demethylases were measured in SCC4 monolayer cells and SCC4 sphere cells. (A) Out of 22 histone demethylases, 6 histone demethylases (KDM3A, KDM4D, KDM5A, KDM7A, KDM8, and JMJD6) showed elevated levels in SCC4 sphere cells compared to monolayer cells. SCC4 monolayer cells were grown either in regular medium or in sphere medium to confirm that medium did not have effect on histone demethylases expression in this experiment. SCC4 monolayer cells grown in regular medium and SCC4 sphere cells were compared to determine statistical significance. (B) These 6 CSC-specific histone demethylases also showed increased expression level in SCC9/TNFα sphere compared to SCC9/TNFα monolayer. (C) Western blot analysis confirms the elevated JMJD6 expression in SCC4 sphere compared to SCC4 monolayer. GAPDH was used as a loading control. (** $P<0.01$, unpaired two-tailed Student's t test.)


A
Relative mRNA level
SCC4 monolayer (in regular medium)
SCC4 monolayer (in sphere medium)
SCC4 sphere
KDM2A
KDM2B
KDM3A
KDM3B
KDM4A
KDM4B
KDM4C
KDM4D
KDM5A
KDM5B
KDM5C
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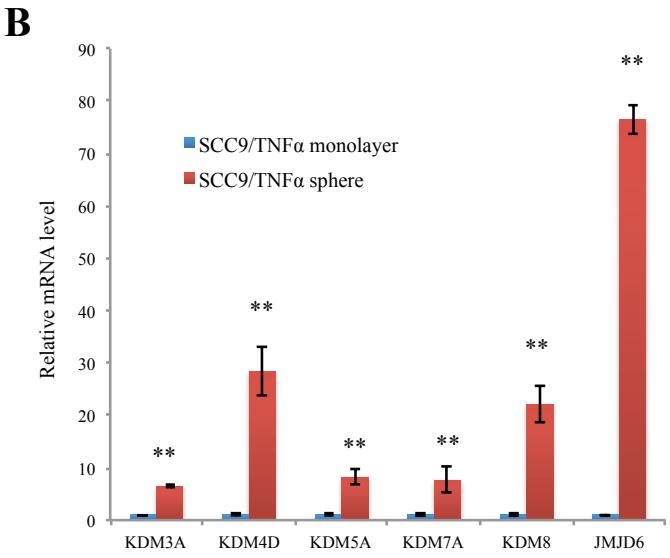
KDM7A
KDM8
JMJD4
JMJD6
JMJD8
UTY
PHF2
HSPBAP1


B
Relative mRNA level
SCC9/TNFα monolayer
SCC9/TNFα sphere
KDM3A
KDM4D
KDM5A
KDM7A
KDM8
JMJD6

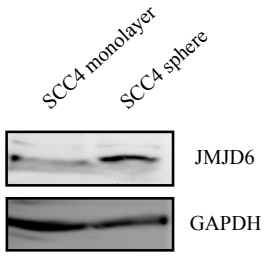
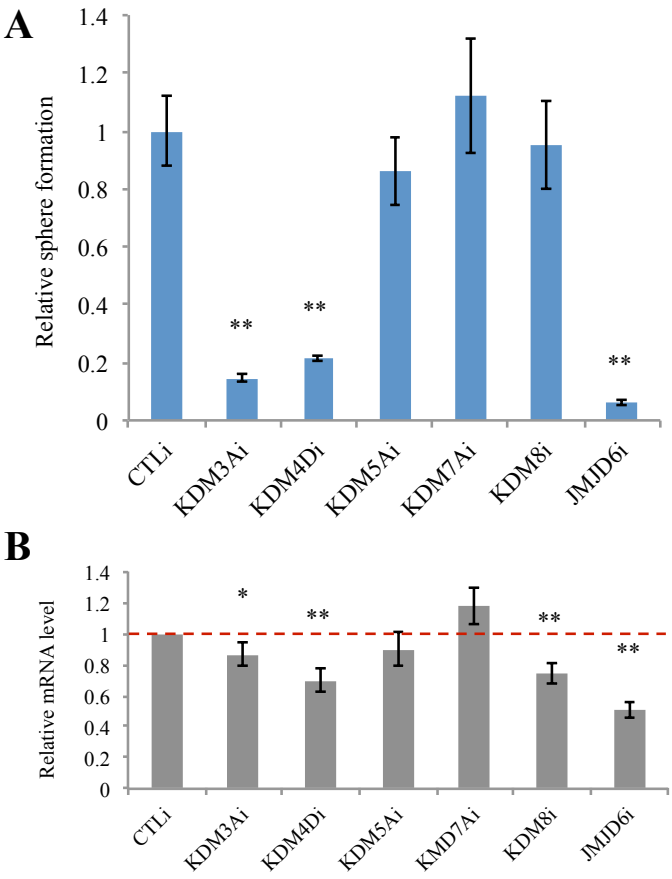
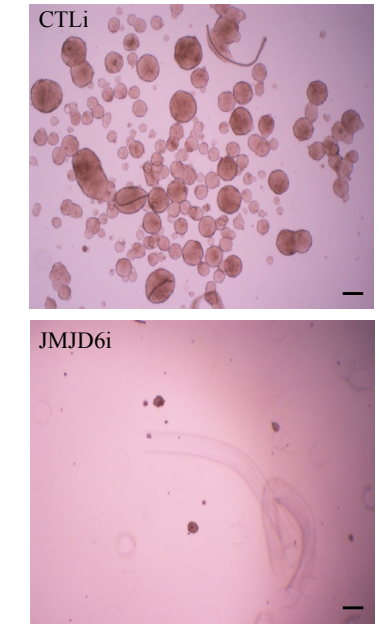

C
SCC4 monolayer
SCC4 sphere
JMJD6
GAPDH

Figure 2. Effect of CSC-specific histone demethylases knockdown on self-renewal capacity of SCC4.

CSC-specific histone demethylases were transiently knocked down using siRNA in SCC4. (A) We performed sphere formation assay using these cells, and relative sphere formation of SCC4 was most dramatically decreased with JMJD6 knockdown. Tumor sphere formation assay was performed in triplicate. Relative number of spheres formed by SCC4/CTLi and SCC4 cells with histone demethylases inhibition were compared to determine statistical significance. (B) Relative mRNA expression levels of these histone demethylases were measured using qPCR 24 hours after transfection to confirm the knockdown of the histone demethylases. Statistical significance was determined by comparing relative mRNA levels of each histone demethylases in SCC4/CTLi and SCC4 cells with histone demethylases inhibition. (C) Representative image of tumor spheres formed by SCC4 control and SCC4 with JMJD6 knockdown. The photographs were taken at a magnification of 40x. (Scale bar = 200nm) (D) Tumor sphere formation assay were repeated twice with SCC4 and once with SCC9/TNFα. JMJD6 knockdown consistently inhibited tumor sphere formation in both SCC4 and SCC9/TNFα. Relative number of spheres formed by SCC4/CTLi and SCC4/JMJD6i were compared to determine statistical significance. (* $P<0.05$, unpaired two-tailed Student's t test. ** $P<0.01$, unpaired two-tailed Student's t test.)


A
Relative sphere formation
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
CTLi
KDM3Ai
KDM4Di
KDM5Ai
KDM7Ai
KDM8i
JMJD6i
B
Relative mRNA level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**
**
CTLi
KDM3Ai
KDM4Di
KDM5Ai
KMD7Ai
KDM8i
JMJD6i


C
CTLi
JMJD6i

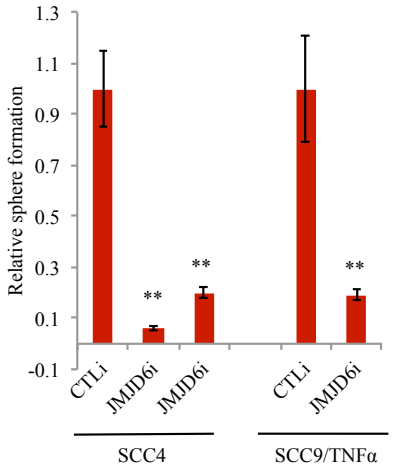

D
Relative sphere formation
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
**
**
**
CTLi
JMJD6i
JMJD6i
CTLi
JMJD6i
SCC4
SCC9/TNFα

Figure 3. Effect of JMJD modulation on self-renewal capacity of SCC4.

(A) JMJD6 was inhibited using shRNA in SCC4. The cells were treated with 0.5 μg/ml puromycin for three weeks to select for colonies with successful knockdown of JMJD6. Western blot analysis confirms the JMJD6 knockdown. Oct4 protein level is also reduced with JMJD6 knockdown. (B) This stable knockdown of JMJD6 decreased SCC4 sphere forming ability. Tumor sphere formation assay was performed in triplicate. Relative number of spheres formed by SCC4/EV and SCC4/JMJD6shRNA were compared to determine statistical significance. (C) SCC4 was transfected with a vector that expressed exogenous JMJD6. The cells were treated with 0.5 μg/ml puromycin for three weeks to select for colonies successfully transfected with the vector. Western blot analysis confirms the overexpression of exogenous JMJD6 which is flag-tagged. (D) Overexpression of JMJD6 increases SCC4 sphere forming ability. Relative number of spheres formed by SCC4/EV and SCC4/JMJD6 were compared to determine statistical significance. (E) Representative image of tumor spheres formed by SCC4/EV and SCC4/JMJD6. Spheres were photographed at a magnification of 40x. (Scale bar = 200nm) (* $P<0.05$, unpaired two-tailed Student's t test.)

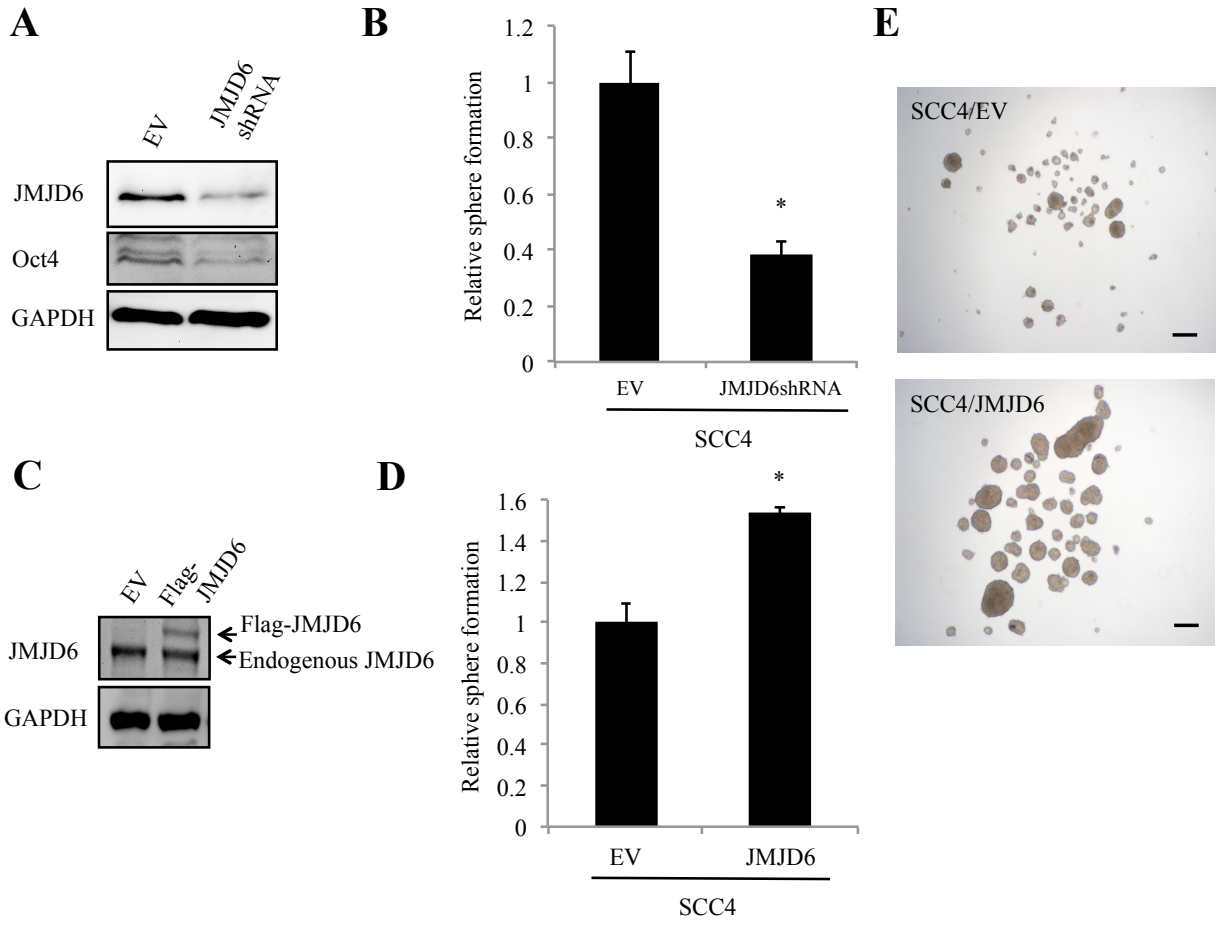

A
EV
JMJD6 shRNA
JMJD6
Oct4
GAPDH
B
Relative sphere formation
1.2
1
0.8
0.6
0.4
0.2
0
*
EV
JMJD6shRNA
SCC4
C
EV
Flag-JMJD6
JMJD6
Flag-JMJD6
Endogenous JMJD6
GAPDH
D
Relative sphere formation
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
EV
JMJD6
SCC4
E
SCC4/EV
SCC4/JMJD6

Figure 4. JMJD6 increased SCC4 migration.

SCC4 was transfected with a vector that overexpress exogenous JMJD6. (A) Transwell assay was performed, and number of cells migrated were counted. Overexpression of JMJD6 increased SCC4 migratory capacity. (B) Representative image of migrated cells. (Scale bar = 200nm) (C) Wound-healing assay was performed, and the serial images of cell migration were obtained at 0, 6, and 24 hours after scratching. (Scale bar = 200nm) Relative number of SCC4/EV cells and SCC4/Flag-JMJD6 cells migrated were compared to determine statistical significance. (** $P<0.01$, unpaired two-tailed Student's t test.)

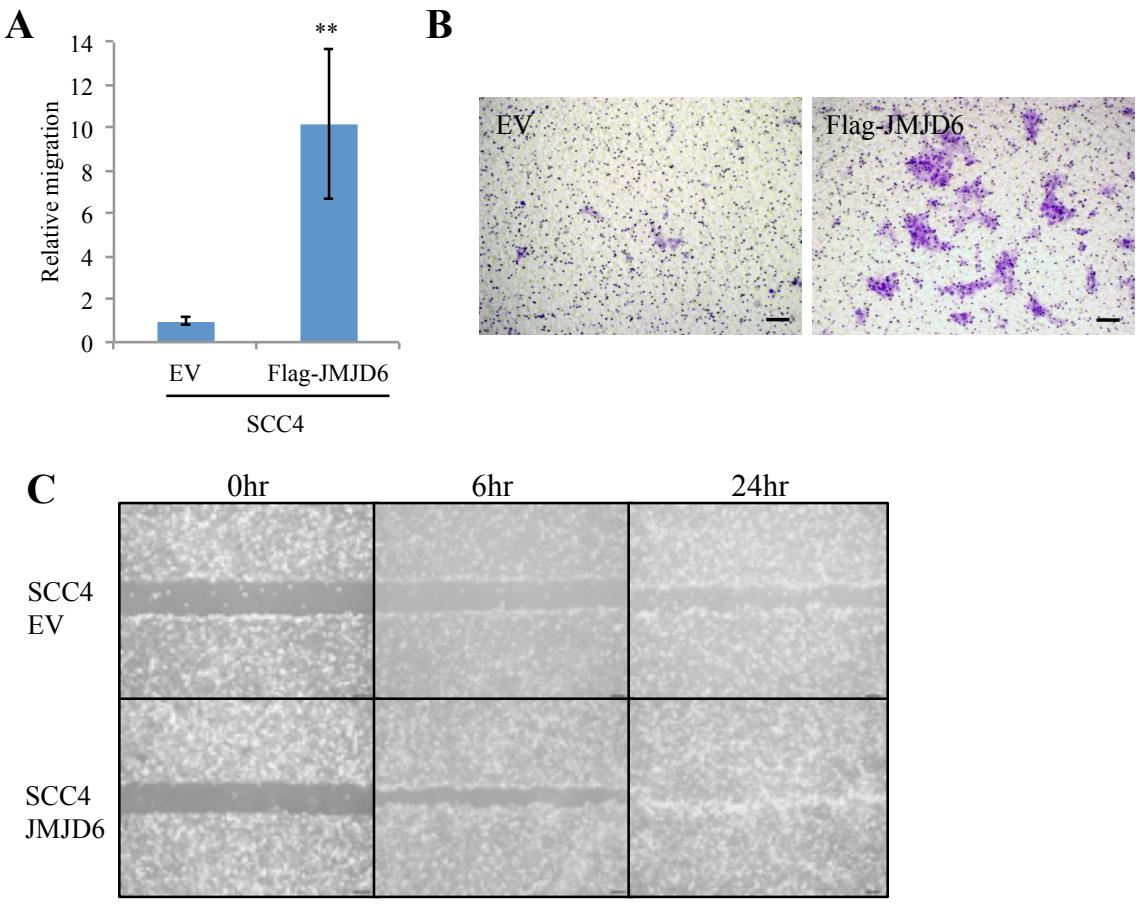

A
B
C
Relative migration
14
12
10
8
6
4
2
0
**
EV
Flag-JMJD6
SCC4
EV
Flag-JMJD6
0hr
6hr
24hr
SCC4
EV
SCC4
JMJD6

Figure 5. JMJD6 is associated with cancer carcinogenesis.

To see if JMJD6 is associated with cancer carcinogenesis, we performed a western blot analysis and measured JMJD6 protein level in two cancer cell lines (UM17B and BapT) and three immortalized pre-cancer cell lines (HOK16B, POE9n and OKF6/tert). We observed that JMJD6 expression was consistently down-regulated in immortalized pre-cancer cell lines and up-regulated in cancer cell lines. GAPDH was used as a loading control.

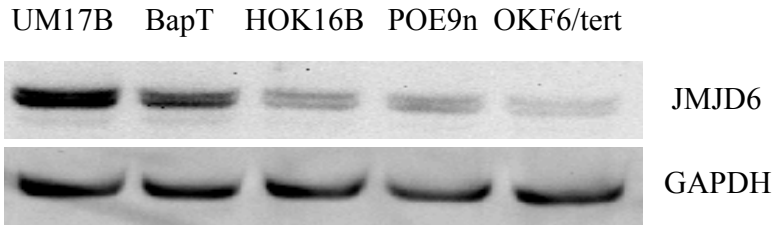

UM17B
BapT
HOK16B
POE9n
OKF6/tert
JMJD6
GAPDH

Figure 6. JMJD6 correlates positively with OSCC development.

To examine whether JMJD6 is associated with oral cancer development *in vivo*, we performed immunohistochemical staining for JMJD6 in normal human oral epithelia (NHOE) and OSCCs. Although moderate nuclear staining patterns were evident in the basal cells in NHOE, strong and diffused patterns of JMJD6 expression were noted in OSCCs indicating that expression of JMJD6 correlates positively with OSCC development. (Scale bar = 200nm)

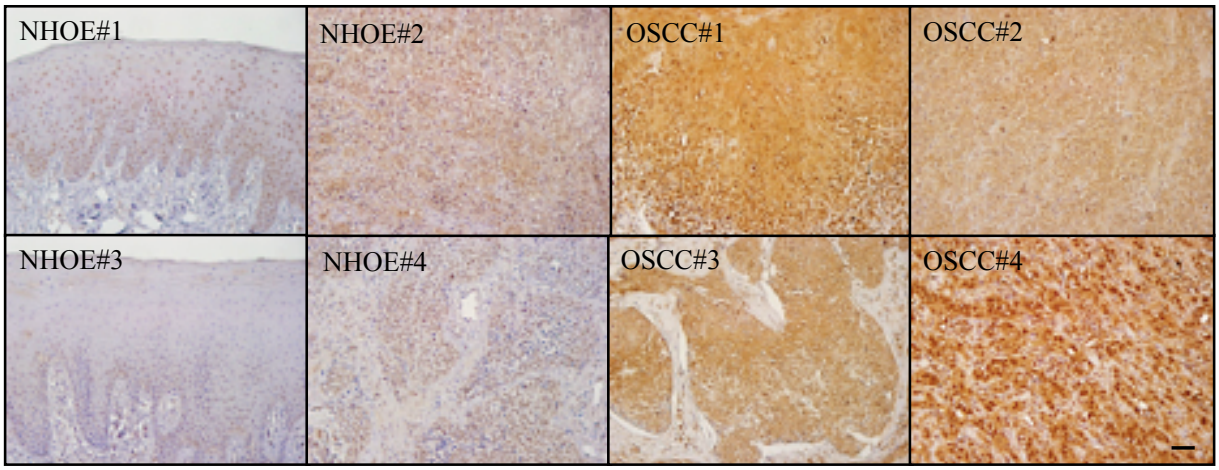

NHOE#1
NHOE#2
OSCC#1
OSCC#2
NHOE#3
NHOE#4
OSCC#3
OSCC#4

Figure 7. Effect of JMJD6 modulation on CSC factor expression

The relative mRNA levels of several CSC factors were measured to see if they are regulated by JMJD6. (A) mRNA expression of CSC factors in SCC4/EV and SCC4/JMJD6 were compared to determine statistical significance. (B) mRNA expression of CSC factors in SCC4/EV and SCC4/JMJD6shRNA were compared to determine statistical significance. (* $P<0.05$, unpaired two-tailed Student's t test. ** $P<0.01$, unpaired two-tailed Student's t test.)

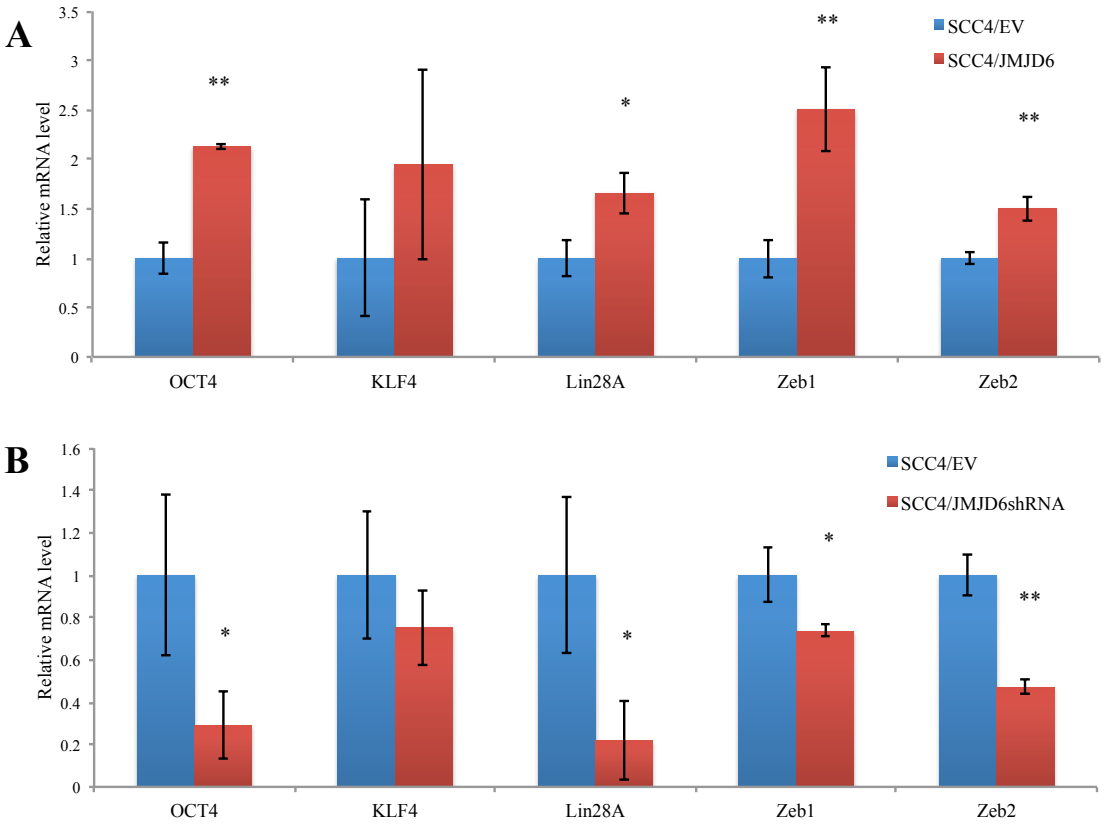
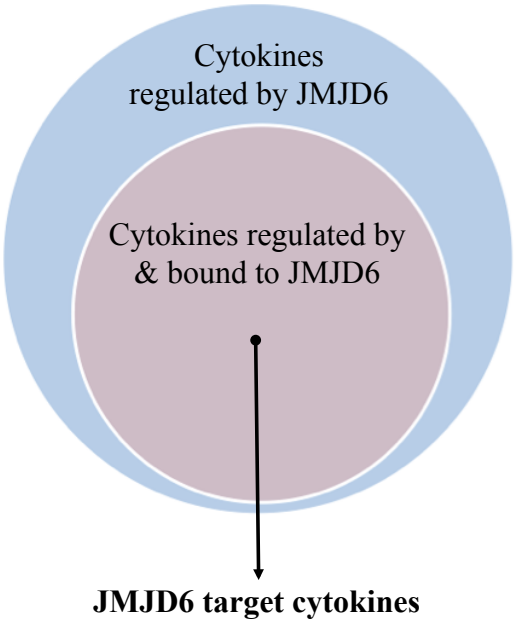

A
Relative mRNA level
3.5
3
2.5
2
1.5
1
0.5
0
SCC4/EV
SCC4/JMJD6
**
*
**
**
OCT4
KLF4
Lin28A
Zeb1
Zeb2
B
Relative mRNA level
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SCC4/EV
SCC4/JMJD6shRNA
*
*
*
**
OCT4
KLF4
Lin28A
Zeb1
Zeb2

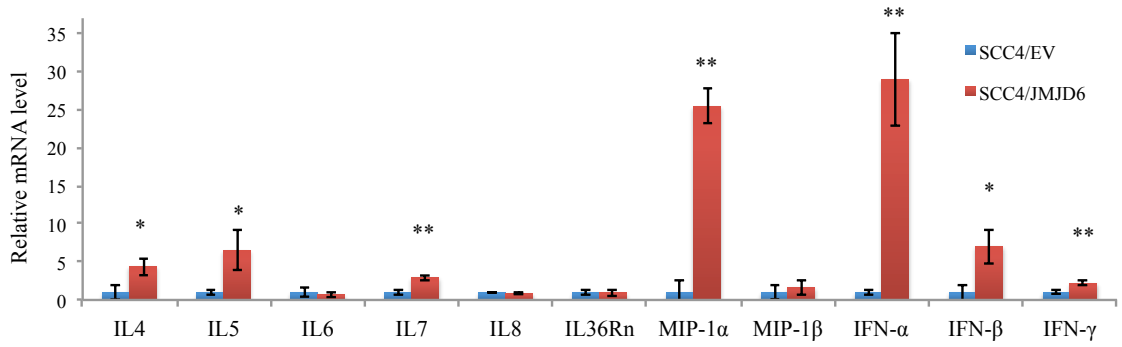
Figure 8. Identification of JMJD6 target cytokines.

(A) We expected JMJD6 target cytokines to be both regulated by and bound to JMJD6. Therefore, we measured the expression level of 22 different cytokines in SCC4 sphere as well as monolayer and identified 11 cytokines that were significantly up-regulated in SCC4 sphere. (Unpublished data in our lab) These CSC-specific cytokines are IL-4, IL-5, IL-6, IL-7, IL-8, IL36RN, IFNα, IFNβ, IFNγ, MIP-1α, and MIP-1β. (Data not shown) (B)(C) Out of these 11 CSC-specific cytokines, 8 cytokines (IL-4, IL-5, IL-7, MIP-1α, and MIP-1β, IFNα, IFNβ, IFNγ) were consistently up-regulated with JMJD6 overexpression and down-regulated with JMJD6 knockdown. mRNA expression of cytokines in SCC4/EV and SCC4/JMJD6 or SCC4/JMJD6shRNA were compared to determine statistical significance. (* $P<0.05$, unpaired two-tailed Student's t test. ** $P<0.01$, unpaired two-tailed Student's t test.)

A


Cytokines
regulated by JMJD6
Cytokines regulated by
& bound to JMJD6
JMJD6 target cytokines

B


Relative mRNA level
35
30
25
20
15
10
5
0
SCC4/EV
SCC4/JMJD6
IL4
IL5
IL6
IL7
IL8
IL36Rn
MIP-1α
MIP-1β
IFN-α
IFN-β
IFN-γ

C

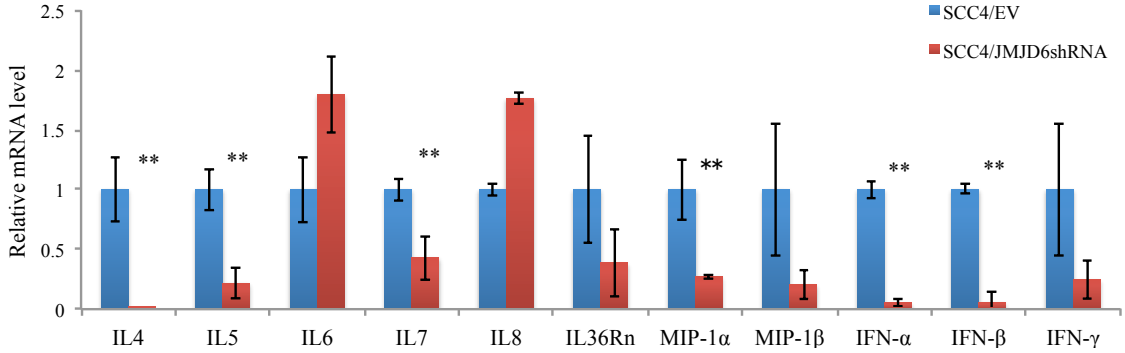
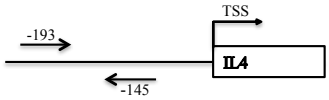
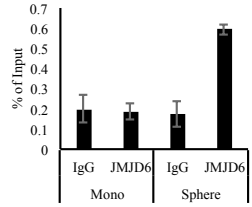

Relative mRNA level
2.5
2
1.5
1
0.5
0
SCC4/EV
SCC4/JMJD6shRNA
IL4
IL5
IL6
IL7
IL8
IL36Rn
MIP-1α
MIP-1β
IFN-α
IFN-β
IFN-γ

Figure 9. Binding of JMJD6 to the promoter region of cytokines.

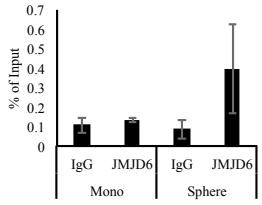
We performed Chromatin immunoprecipitation assay with these 8 cytokines that were regulated by JMJD6. IgG was included as a negative control. JMJD6 preferentially bound to the promoters of 6 cytokines (IL-4, IL-5, MIP-1α, MIP-1β, IFNα, and IFNβ) in SCC4 sphere cells compared to SCC4 monolayer cells.

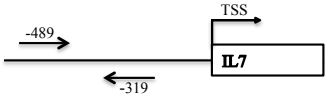


IL4

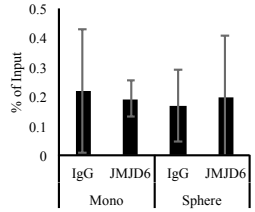


IL5

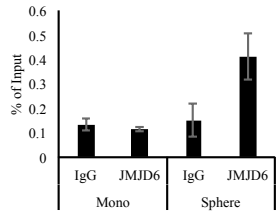


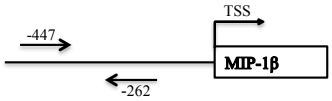


IL7

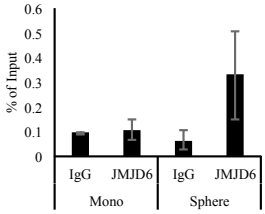


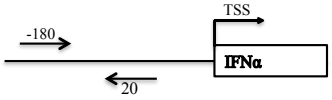
MIP-1α



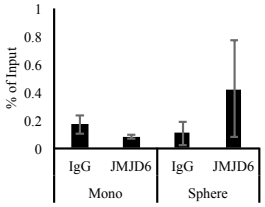


MIP-1β

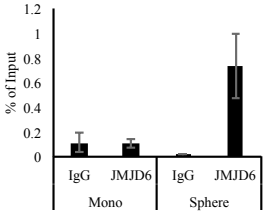


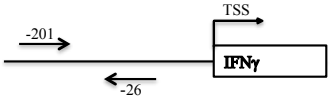


IFNα

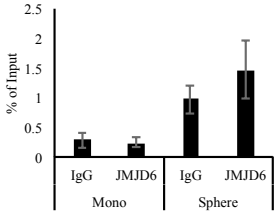


IFNβ





IFNγ



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