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The role of cytokines on oral cancer stem cells

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The role of cytokines on oral cancer stem cells

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in Oral Biology

By

Nicole Kristina Rigas

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ABSTRACT OF THE THESIS

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Nicole Kristina Rigas

Master of Science in Oral Biology

University of California, Los Angeles, 2014

Professor Ki-Hyuk Shin, Co-Chair

Professor Mo Kwan Kang, Co-Chair

Cancer stem cells (CSCs; also known as cancer initiating cells) are a small population of cancer cells located within a tumor that are highly tumorigenic, capable of tumor initiation and resistant to cancer therapies. Cytokines have been previously shown to be involved in tumorigenesis of many different cancers such as breast, lung, colorectal and ovarian cancers. In this study, to investigate potential involvement of cytokines in oral CSCs, we screened expression of 25 cytokines in CSC-enriched oral squamous cell carcinoma (OSCC) populations. Among them, chemokine (C-C motif) ligand 3 (CCL3)

was unequivocally overexpressed in all tested CSC-enriched OSCC populations. Subsequent functional analysis showed that CCL3 enhanced OSCC CSC phenotypes such as self-renewal capacity, migration, and CSC-related gene expression. Moreover, we found that CCL3 receptors were also significantly overexpressed in CSC compared to non-CSC population, and a CCL3 receptor antagonist suppressed the effect on CCL3 on CSC. Mechanistically, CCL3 increased SOX9 whose expression was highly overexpressed in CSC population. Knockdown of SOX9 suppressed CSC phenotypes in OSCC. These findings suggest that CCL3-SOX9 axis is a novel CSC-regulatory pathway in OSCC, and its inhibition may offer an effective means of selectively targeting the OSCC CSC population.

The thesis of Nicole Kristina Rigas is approved.

Reuben Kim

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2014

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. HNSCC form 1-4% of all cancers in western world (Joshi, 2014). HNSCC most commonly develops in the oral cavity, larynx, oropharynx and hypopharynx. The main two risk factors for HNSCC are alcohol and tobacco use. People who are diagnosed with HNSCC will undergo a combination of therapies such as radiotherapy, surgery, and systemic therapy. The major cause of death is relapse and even after treatment 40-60% of patients may succumb to relapse (Pascal, 2014). In addition, most often death is caused tumor metastasis. Furthermore, oral cancer stem cells may be an additional cause of recurrence and metastasis that results in spread of cancer to other vital or primary systems including lymph nodes, bone and lung.

Cancer stem cells (CSCs) are a small population of cells within a tumor that are self-sustaining and cause heterogeneous lineages of cancer cells. There are three distinct characteristics of CSC. The first of these three characteristic is tumor initiation, which is the ability to regenerate the tumor from a limited number of cells. Secondly, self-renewal demonstrates the ability of stem cells to increase the size of the stem cell pool while maintaining its undifferentiated state. The third of these characteristics is the ability to give rise to heterogeneous progeny that is a phenotype of the primary tumor (Major, 2013). The degree to which cancer stem cells differentiate into certain cell types and their ability to self-renew differs based on intrinsic properties of each cell individually. (Bjerkvig, 2005). In this study we are investigating the role of chemotactic cytokines, chemokines, and there role in increasing oral squamous cell carcinoma (OSCC) CSC

phenotypes such as self-renewal and migration. In addition we will be investigating the effect of chemotactic cytokines on the expression level of known CSC factors such as ALDH1, cMYC, EZH2, GLi-1,HES-1, KLF4, OCT-4, ZEB-1, ZEB2, HEY-1, SOX-9, Snail and their relationship with chemokines.

Chemokines are small chemotactic cytokines that have the ability to trigger migration of cells toward a gradient by binding of a chemokine to its receptor. Chemokines consist of a superfamily of 50 human ligands and 20 receptors that play an important role in regulation of cell migration. Tumor cells can express functional chemokines receptors to induce proliferation, survival, angiogenesis and organ specific metastasis (Aurer, 2008). A recent study has shown a critical role of various cytokines, such as IL-6, IL-8, CCL2, and TGF-β, in the tumor microenvironment and plays an important role in regulating CSC (Chin, 2013). Chemokines and their receptors can be further divided into four families based on the pattern of cysteine residues (CXC, CC, C and CX3C) found in the ligand. They can be functionally classified as inflammatory or homeostatic. The majority of cytokines are inflammatory chemokines that are inducible and control cell recruitment to sites of inflammation. Cytokines can signal to the microenvironment in an autocrine, paracrine or endocrine fashion to regulate tumor growth, migration/invasion and metastasis.

An inflammation response has a significant impact on the different stages of tumor development such as initiation, promotion, malignant conversion, invasion and metastasis. There is clear evidence showing that inflammation plays a key role in tumorigenisis. It has been clearly evident that the inflammatory microenvironment is

necessary for all tumor survival, proliferation and homeostasis (Mantovani, 2008). Most cancers are correlated to somatic mutations and/or environmental factors such as chronic inflammation. Roughly, 20% of cancers are linked to chronic infection (Aggarwal, 2009). Due to the different types of inflammation the tumor microenvironment contains a vast number of different cell types including the innate (neutrophils, macrophages, mast cells, dendritic cells, myeloid derived suppressor cells and natural killer cells) and adaptive immune cells (B and T lymphocytes) along with the surrounding stroma (fibroblasts, endothelial cells, pericytes and mesenchymal cells) and cancer cells.
(de Visser, 2006).

The different cytokines and chemokines expressed in the tumor microenvironment can significantly affect the tumor's ability to develop and progress. The cytokines and chemokines can either promote or inhibit tumor growth regardless of the source that is releasing them. The release of anti-tumor cytokines include IL-12 and IFN can activate downstream effectors such as AP-1, STAT, SMAD and NF-KB in addition to causing the release of IL-6, IL-7, IL-23. Meanwhile TNFα and TNFβ can enhance tumor progression and survival (Grivennikov, 2010). Many cytokines have been previously shown to activate transcription factors that are crucial for inflammation and tumor growth. As previously known, cytokines such as IL-1 and IL-6 can activate downstream transcription factors such as Zeb1 and STAT3 respectively that result in increased tumorigenesis in colorectal cancers (Li, 2012 and Grivennikov, 2009). Pharmacological intervention can alter cytokine signaling and decrease tumorigenesis in addition to cancer growth and development. These pharmacological interventions can

work to achieve preventative and therapeutic approaches to decrease tumorigenesis and promote cancer survival rates.

IL-4, a proinflammatory cytokine, has previously been demonstrated to initiate apoptosis resistance in chronic lymphocytic leukemia B-cells and to enhance the protein expression level of anti-apoptotic proteins in breast, prostate and bladder tumor cell lines. Moreover, there has been indication that IL-4 enhances the proliferation of human cancer pancreatic cells lines suggesting that IL-4 may upregulate antiapoptotic genes which help to promote the survival of cancer cells (Todara, 2007). IL-4 demonstrates the role of cytokines in cancer progression and is found to be associated with cancer development. In our previous studies, we found an increase in IL-4 expression in OSCC cell lines and increased CSC phenotypes via gene expression levels and CSC phenotypic assays.

Chemokines are 70-130 amino acids soluble proteins that contain three disulfide bonds. Chemokines develop a characteristic fold that consists of an N-terminal unstructured domain with a three-stranded β sheet connected by loops and turns and a C – terminus helix. The chemokine receptors are seven-transmembrane GPCRs (G-protein-coupled receptors). They most often signal through heterotrimeric G-proteins but can also signal through other G-protein subtypes or even non-G-protein mediated pathways. The G-protein is comprised of three major signaling subunits. The alpha (α) subunit inhibits the adenylate cyclase and transduce signals through tyrosine kinase or the α subunit induces phosphoinositide 3-kinase (O'Harye, 2008). The beta (β) and gamma (□) subunits play an important role in the activation of many chemokine-induced pathways including phosphoinositide 3-kinase □ (PI3K □), phospholipase C (PLC), Ca2+ induced

protein kinase C isoform activation, MAP kinase and JAK/STAT signaling cascade (Aurer, 2004). In this study, we will confirm the expression of the CCL3 receptor, CCR4 and CCR5, in the SCC4 non-CSC and CSC populations using qPCR. In addition we examined the effect of CCR5 antagonist on OSCC CSC properties.

The function of chemokines and their receptors in regards to cancer can be divided into three main categories. The first category is providing directional cues for migration/metastasis, secondly shaping the tumor microenvironment and lastly survival and/ or growth signals. The tumor microenvironment contains a large number of cells from the innate and adaptive immune system which become activated by a profile of chemokines, cytokines, growth factors and proteases. Chemokines and their receptors play an important role in modulating angiogenesis, cell recruitment, tumor survival and proliferation resulting in the progression of cancer (O'Hayre, 2008). We are interested in examining the effect of chemokines on OSCC and how it effects the progression of the cancer in addition to the effect on CSC phenotypes.

Cells that express chemokine receptors have the capability to migrate to cells that release a chemokine gradient. The interaction with the receptors initiates signal transduction pathways and activates downstream effector cells. G-protein coupled receptor downstream signaling induces actin polymerization causing chemotaxis and a change of the cell shape. CCR5 is well known for its involvement in HIV. There have been many studies involving CCR5 as an anti-HIV target, although less is known about its involvement in cancer progression. CCR5 is expressed solid tumors and hematologic

malignancies. It is also known to be associated with cancer metastasis and low survival rate (Aurer, 2008).

Macrophage inflammatory protein (MIP)-1α, also known as CCL3, is one of four members of the MIP family and is part of the chemokine subfamily CC. CCL3 is released by many types of immune cells including macrophages, dendritic cells and lymphocytes. CCL3 acts by signaling the G-protein-coupled cell surface receptors and is expressed on macrophages and lymphocytes which activate inflammatory and chemotactic responses. The CCL3 gene is inducible in mature hematopoietic cells (Baba, 2013). Lymphocytes, macrophages, dendritic cells, natural killer cells and neutrophils have the ability to produce mass amounts of MIP-1. In addition, CCL3 can be produced by other proinflammatory cytokines/substances such as lipopolysaccharide (LPS), substance P, viral infection, TNF α, IFN □, and IL-1 α and β.

CCL3 induces acute and chronic inflammatory host response by recruiting pro-inflammatory cells to the site of injury or infection. MIP-1α plays an important role in T-cells chemotaxis by inducing the movement of the T-cells from the circulation to the inflamed tissue. In addition, CCL3 is crucial for the regulation of the transendothelial migration of monocytes/macrophages, natural killer cells and dendritic cells (Aurer, 2004). CCL3 receptors are CCR1, CCR3, CCR4 and CCR5. Most notably, CCL3 ligand binds to CCR5 and plays a significant role in pro-inflammatory response. CCR5 protein is in the family of beta chemokine receptors and is an integral membrane protein. It is predominantly expressed on macrophages, T cells and dendritic cells (Mueller, 2004). Our investigation is focusing on the role of CCL3 in increasing CSC phenotypes and the

pathway that is being employed to increase CSC phenotypes. We observed that CCL3 induced an increases expression of SOX-9 mRNA expression level in the CSC population. This CCL3-SOX-9 axis caused further investigation into the role of SOX-9 in increased OSCC CSC.

The role of SOX-9 in cancer is unclear. SOX-9 (sex-determining region Y (SRY)-box 9) is member of the family of SOX transcription factors in the sex- determining region of the Y chromosome which is defined by a common HMG domain identified originally as SRY. SOX-9 belongs to the group E of the SOX transcription family which includes SOX-8, SOX-9 and SOX-10. SOX-9 proteins play an important role in stem cell property maintenance, linage restriction, terminal differentiation and developmental processes that need to be tightly controlled for normal embryogenesis (Ling, 2011). In addition, SOX-9 is a master regulator of chondrogenic expression which is activated by receptor tyrosine kinase signaling and activation of mitogen-activated protein kinase (Ling, 2011). SOX-9 is most often overexpressed in skin, prostate, lung and brain tumors. In some melanomas, SOX-9 acts as a tumor suppressor. Previous data has demonstrated that SOX-9 could possibly induce carcinogenesis by regulating stem cells, promoting cell survival and increasing cancer cell invasion and migration. It has been shown that SOX-9 has increased invasion and migration in urotheilial carcinomas (Matheu, 2012). In addition, it has been previously demonstrated that SOX-9 is a mediator in colorectal cancer, induces TCF4 expression via Wnt/β Catenin expression in breast cancer and plays an important role in differentiation processes of organ tissue development (Darido, 2008 and Song, 2014).

In order to better understand the interaction between the CCL3 ligand and its receptor, CCR5, we studied the effect of CCR5 antagonist on CSC phenotypes. Maraviroc, a CCR5 antagonist, has been used to block ligands to bind to the CCR5 receptor. Since its discovery the early 1990's, CCR5 receptor has been long studied due to its role in HIV as a coreceptor for the natural entry of HIV-1 or HIV-2 into the host cell (Martin-Gonzalez, 2012). Multiple brands of CCR5 antagonist are currently being used as therapeutics for HIV patients such as Maraviroc, Vicriviroc and Aplaviroc. Maraviroc is most often used as antiretroviral therapy because it blocks initial binding of HIV onto the cell (Latinovic, 2009). More recently CCR5 has been studied as a potential therapeutic for cancer. Research has been conducted on the effect of CCR5 antagonist on reducing basal breast cancer cells (Velasco-Velazques, 2012). By blocking the uptake of CCL3, a pro-inflammatory cytokine, it may prevent an increase in cancer stemness capacity of OSCC.

In this present study, we found that CCL3 increased CSC phenotypes, including self-renewal and migration. We examined the potential role of CCL3 tumor growth and development by focusing on the effect of CCL3 on cancer stemness. There is little known about the effect of chemokine CCL3 on solid tumors and OSCC. The aim of this study is to determine the effect of CCL3 on OSCC and the potential role of SOX-9 in increasing CSC phenotypes. Collectively, our findings indicate that the CCL3-SOX9 axis may offer therapeutic and preventative benefits in targeting the OSCC CSC population.

MATERIAL AND METHODS

Cell and cell culture and acute exposure of cytokine.

Three human OSCC cell lines were used in this study: SCC4, SCC9/TN-α and BapT cells were cultured in Dulbecco's modified Eagle's Medium (DMEM)/F12 (Invitrogen) + 10% SCS + 0.4 µg/mL hydrocortisone (Invitrogen) and gentamicin. The SCC-9 cells were treated with 5 ng/mL TNF-α for extended periods to make the SCC-9/ TNFα cell line (Lee, 2012). Both SCC4 and SCC9/TNFα are oral epithelial cancer cells and obtained from the tongues of males patients. For our studies we treated the cell lines with 5, 10 or 50 ng/mL CCL3 for varying lengths of time (24–96 hours). In addition, SCC4 cells were transfected with SOX-9 siRNA and collected for other experiments including migration assays, sphere formation assays and proliferation assays were conducted using the cultured cells. All cells were incubated at 36.8º C with 5% carbon dioxide.

RNA Extraction and Reverse Transcriptase.

Total RNA was isolated from cells using Trizol reagent RNA isolation method. After centrifugation the aqueous layer containing the RNA is removed meanwhile the layers containing the DNA and protein (organic layers) are discarded. Isopropanol (2-propynol) is added to the aqueous layer to form RNA precipitate and to wash the RNA. Lastly, the RNA is washed with 75% ethanol and dissolved in RNase-free H2O. Final RNA concentrations were measured by ND-1000 Nanodrop Spectrophotometer (Thermo Fisher Scientific). The purity of the RNA is around 1.85- 1.95 (260/280) and

concentration between 900-1000 ng/µL. A total of 5.0 µg of extracted RNA is used to synthesize cDNA using SuperScript first-strand synthesis system (Invitrogen).

Real Time Quantitative reverse transcriptase Polymerase Chain Reaction (qRT-PCR).

To measure the expression level of RNA in various cell types (SCC4, BapT, SCC9/TNFα) we used qRT-PCR. Of the selected RNA primers, we used 5µg cDNA obtained from reverse transcriptase (Roche Light Cycler® 480). The primers sequence was determined by using Universal Probe Library (Roche). The reactions were conducted by heat denaturing at 95ºC for 10 minutes, then 55 cycles of 95ºC for 10 seconds followed by 58ºC for 45 seconds and lastly 72ºC for 10 seconds. The expression of RNA was measured by the CT value. The second derivative of the CT value was determined by using the CT value determination method to compare fold-difference according to the manufactures instructions.

Table 1 qPCR Primer Sequence.

Primer name	Sequence
ALDH1	Forward: TTTGGTGGATTCAAGATGTCT Reverse: CACTGTGACTGTTTTGACCTC
c-MYC	Forward: AAGGCTCTCCTCTGCTTAG Reverse: CTCTCCTCGTCGCAGTAGAAA
EZH2	Forward: ATGGCACCTGCAGAAGGA Reverse: TTGGGAAGCCGTCCTCTT
HES-1	Forward: GTGAAGCACCTCCGGAAC Reverse: GTCACCTCGTTCATGCACTC

HEY-1	Forward: CGAGCTGGACGAGACCAT Reverse: GAGCCGAACTCAAGTTTCCA
KLF4	Forward: GGACATCCACGACCTGAGC Reverse: GACGCCTTCAGCACGAAC
NANOG	Forward: AGATGCCTCACACGGAGACT Reverse: TCTCTGCAGAAGTGGGTTGTT
OCT-4	Forward: GAAACCCACACTGCAGATCA Reverse: CGGTTACAGAACCACACTG
SNAIL	Forward: CGGCTCGAGATGCCGCGCTCT Reverse: CGGAAGCTTCAGCGGGGACAT
SOX-9	Forward: GTACCCGCACTTGCACAAC Reverse: TCGCTCTCGTTCAGAAGTCTC
ZEB-1	Forward: GGGAGGAGCAGTGAAAGAGA Reverse: TTTCTTGCCCTTCCTTTCTG
ZEB-2	Forward: AAGCCAGGGACAGATCAGC Reverse: CCACACTCTGTGCATTTGAAC
CCL3	Forward: TTGCTAAATGCAGAGCATGG Reverse: AGCCCTTCATCTTGGGAAAG
CCL4	Forward: AGCACCAAATTCCAAACCAA Reverse: CCTTCCCTGAAGACTTCCTGT
FGF2	Forward: GCTGGCGGCAGATGATA Reverse: CTGAGTCGGCAACAGCA
IL-1α	Forward: TGGCCAAAGTTCCAGACATGT Reverse: TCTTCTTCAGAACCTTCCCGTT
IL-2	Forward: GAGGTTTGAGTTCTTCTTCTACACAC Reverse: ACCAGGATGCTCACATTTAAGTTTT
IL-4	Forward: AACTGCTTCCCCCTCTGTTCT Reverse: TCTTCTGCTCTGTGAGGCTGT
IL-5	Forward: ATCCTTTTAACAAGTGGATTAGGC Reverse: GCT CTT TGG GGA AAA CT

IL-7	Forward: TGGCAATATAGAAACACGAACTTT Reverse: TTCCACTCTGAAAA CTGCATAAG
IL-8	Forward: CTCTCTTGGCAGCCTTCCTGA Reverse: AATTTGGGGTGGAAAGGTTTG
IL-15	Forward: ATTTTGGGCTGTTTCAGTGC Reverse: TTACTTTGCAACTGGGGTGA
IL-18	Forward: GCTTCCTCTCGCAACAAACT Reverse: TGATGCAATTGTCTTCTACTG
IL-36RN	Forward: CGGCATTGAAGGTGCTTTA Reverse: CCACGCTGATCTCTTCACCT
IFN-α	Forward: CTCGCCCTTTGCTTTACTGAT Reverse: CTCCTGTTATCCAGGCTGTGG
IFN-β	Forward: ATGACCAACAAGTGTCTCCTC Reverse: TGCCACAGGAGCTTCTGACAC
IFN-□	Forward: ACAAGTTATATCTTGGCTTTTC Reverse: TCCGCTACATGTGAATGACCT
MMP-3	Forward: GGTGAGGACACCAGCATGA Reverse: ATCCCTGGAAAGTCTTCAGC
RANTES	Forward: TGCCCACATCAAGGAGTATTT Reverse: TTTCGGGTGACAAAGACG
SDF-1	Forward: TGACCCGAAGCTAAAGTGGA Reverse: CTCTGCGCCCCTTGTTTA
TGF-β	Forward: CCCAGCATCTGCAAAGTCC Reverse: GTCAATGTACAGCTGCCGCA
VEGF	Forward: TCCAGGAGTACCCTGATGAGA Reverse: ATCTGCATGGTGATGTTGGAC
CCR5	Forward: ACTGCAATTATTCAGGCCAAA Reverse: TTTCTCTTCTGGGCTCCCTAC

Western Blot.

Whole cell extracts were isolated with 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). The isolated extracts were fractionated using SDS-PAGE and transferred to polyvinyl difluoride protein membrane (Millpore). The membrane was incubated with a primary antibody overnight at 4^0C, secondary antibody was incubated at room temperature for two hours and both were exposed using chemiluminescence reagent (Bio-Rad) for protein detection. The antibodies detected were SOX-9 (Santa Cruz Biotechnology), GAPDH (Santa Cruz Biotechnology). Horse radish peroxidase conjugate was used for secondary antibody (Santa Cruz Biotechnology).

Tumor Sphere Formation Assay.

The tumor sphere formation assay is an in vitro technique to assay the clonogenic growth potential and differentiation of normal and neoplastic cells. The original sphere formation assay used neural cells to form neural spheroids. The OSCC spheres have shown the capability of self-renewal and dedifferentiation which indicates they contain cancer stem cells (Wang, 2013 and Hu, 2014). The OSCC cells were grown in DMEM/F12 (Invitrogen) media with 1:100 ratios of N2 Supplement (Invitrogen), 10ng/mL human recombinant BFGF (PeproTech), 10ng/mL EGF and 50μg/mL Gentamicin (Invitrogen). N2 supplement is a serum-free supplement that is recommended for growth and expression of neuroblastomas in addition to post-mitotic neurons in

primary cultures but can also be used in sphere formation assays. The growth factors, BFGF and EGF, enhance growth and differentiation of the spheres. Cells were plated in a 6-well plate at a density of 3000 cells/well and incubated for 6 days. The number of spheroids formed were observed and counted under a microscope. The assay was completed in triplets for each cell type and average number of spheres was determined for each cell line. For experimental groups each individual well was treated with 5, 10 or 50 ng/mL of CCL3 at day 0.

Knockdown exogenous SOX-9 by siRNA.

Exogenuous SOX-9 expression was knocked down using either Sox-9 siRNA or control siRNA scramble (Santa Cruz Biotechnology) that was introduced by Lipofectamine RNAimax. siRNA, also known as small interfering RNA, is a class of double stranded RNA molecules that are 20-25 base pairs in length. siRNA interferes with the expression of a specific gene sequence with a complementary gene sequence. siRNA alters the mRNA by degrading it causing the mRNA to be broken down and no translation can occur. The siRNA is used to knockdown the expression of the specific gene of interest, SOX-9. The OSCC cell lines were grown to be 80% confluent in a 60 mm dishes and transfected with 15 µg siRNA. SOX-9 or the control siRNA, Liptofectamine and serum free cell culture medium were added to the cells and grown for 3 days. After 3 days the cells were harvested.

Transwell Migration Assay.

Transwell migration assay is an in vitro assay used to measure chemotactic ability of cells. The chemotactic assay is used to determine wound repair, cell differentiation, embryonic development and tumor metastasis behavior. Increased cell migration is a hallmark of CSC phenotypes and malignant tumor progression. Cell migration has been well documented as CSC phenotypes in many cancer types but most notably in colorectal CSC (Brabletz, 2005). In our study, we were trying to examine the effect of cell migration on OSCC CSC phenotypes. SCC4 cells were resuspended in 100µL of DMEM/F12 and then added to Transwell inserts. DMEM/F12 and 1% SCS was added to the well. The low concentration of serum serves a chemoattractant for the cells to migrate toward it. The CSCs will migrate more due increased migratory phenotype (Brabletz, 2005). After seeding the migration assay was incubated at 36.8º C with 5% carbon dioxide for 96 hours. The pore size of the Transwell membrane is dependent on the size of the individual cells in the sample. The pores sizes ranged from 3-8µM. In these experiments we used size 6.5µM (Justus, 2014). The cells were fixed with 600µL 10% formalin then stained with 600µL 0.25% crystal violet. The cell lines used were SCC4, SCC9/TNF-α and BapT. The cell density was confirmed by examining the cells under the microscope. The cell density needs to be similar between the control and experimental group in order to determine if more cells migrated or not. The non-migrated cells were wiped out with cotton and the migrated cells remain on the outer portion of the insert. The migrated cells were photographed using 4x magnification.

Proliferation Assay.

Cell-Proliferation assay is an in vitro assay used to determine the growth of cells over a period of time. We investigated the proliferation rate of SCC4 cells that were transfected with SOX-9 siRNA and negative control in order to confirm the effect of the knockdown. Control and SOX-9 siRNA transfected SCC4 cells were seeded with 10000 cells per well in a 6-well culturing plates in triplicate for each cell type (control and SOX-9 siRNA). Cells were incubated at 36.8 ºC with 5% carbon dioxide for either 3 or 6 days. The cells were counted under a microscope using the hemocyotmeter at day 3 and 6. The cells were trypsinized with 1.0 mL trypsin for 5 minutes in the incubator then neutralized with 3.0 mL DMEM/F12. A single suspension was obtained by pipetting the cells from each well into one single centrifuge tube. A 10µL sample was taken from the single suspension and counted using the hemocytometer. The average number of cells per set of three wells were calculated at day 3 and 6.

CCR5 Anatagonist (Maraviroc)

Maraviroc (CCR5 antagonist) was used to determine the effect of the CCL3/CCR5 interaction on OSCC (Sigma Aldrich). A sphere assay was set up with 3000 SCC4 cells per well and 0µM, 10µM, 50µM or 100µM ofMaraviroc was added to each well. The Maraviroc was reconstituted in DMSO (Dimethyl Sulfoxide) at a concentration of 10mM. Due to this, a corresponding control was specified for each dosage of Maraviroc treated to the cells. After six days, the number of spheres were counted and compared against the corresponding control set.

MTT Cell Proliferation Assay

MTT assay is used to measure cell viability and cell proliferation rate. The yellow tetrazolium MTT (3-(4,5-dimethylhiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced metabolically by active cells to generate reducing equivalents NADH and NADPH. This results in intracellular purple formazan that form a precipitate that can solubilized and quantified using a spectrophotometer. The MTT assay will yield low background absorbance values when there is an absence of viable cells. 2000-4000 cells were plates per well in a 96 well plate. The cells were treated with multiple concentrations including 0µM, 10µM, 50µM, 100µM and250µM of Maraviroc. For each concentration there was a corresponding control set treated with only DMSO. The assay was terminated at days 4 and day 6. The data was quantified using the spectrophotometer.

RESULTS

Cytokine profiling in non-CSC vs. CSC.

To begin investigating the role of cytokines in OSCC, we wanted to examine the RNA expression level of cytokines in non-CSC and CSC populations of OSCC cell lines. The connection between cytokines and increased cancer stemness has been correlated in other epithelial cancers such as breast cancer and colorectal cancers (Korkaya, 2011 and Li, 2012). In our study, we investigated the role of pro-inflammatory cytokines and chemokines on CSC properties. Our aim was to determine the increased expression level of various cytokine and chemotactic cytokine, chemokines, on CSC populations in OSCC cells lines. To initially investigate the difference in cytokine and chemokine expression levels of OSCC non- CSC and CSC populations, we examined SCC4 monolayer and SCC4 sphere populations. We examined the difference of relative mRNA expression levels of non-CSC vs. CSC by comparing the cytokine expression of various known pro-inflammatory cytokines or chemokines such as IL-1β, IL-1 α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-13, IL-15, IL-18, IL-36 RN, IFN α, IFN β, IFN □, TGF- β, BRAK, CCL3, CCL4, MMP3, VEGF, RANTES, SDF-1, and FGF2 (Figure 1).

The qPCR analysis of the cytokine expression in non-CSC and CSC populations indicated that there were thirteen cytokines or chemokines that were increased in the SCC4 CSC population compared to the SCC4 non-CSC population (control group). There was increased cytokine expression level shown in the following cytokines: IL-2, IL-4, IL-5, IL-7, IL-8, IL-36RN, IFN-α, IFN-β, IFN-□, CCL3, CCL4, Rantes (CCL5)

and SDF-1. Next, we wanted to examine the effect of the cytokine and/or chemokine on known CSC phenotypes such as sphere formation ability. We analyzed six of the cytokines that we had previously screened to further investigate there role in cancer stemness. Based on the data from the OSCC cell lines we narrowed our focus to six cytokines of interest. The six cytokines of focus were IL-4, IL-5, IL-36 RN, CCL3, IFN-α, IFN-β. These cytokines and/or chemokines had shown an increase in relative mRNA expression level in SCC4 and have been previously linked to tumorigenesis in other cancers. Next, we used two other cell lines, SCC9/TNFα and BapT, to confirm the results (Figure 2). These findings suggest that IL-4, IL-5, IL-36- RN, CCL3, IFN-α, IFN-β could possibly play a role in increasing CSC phenotypes in OSCC. From these results, we identified six putative oral CSC specific cytokines including IL-4, IL-5, IL-36 RN, CCL3, IFN-α, IFN-β that will be further studied in the following experiments. .

Effect of cytokines on CSC self-renewal capacity.

In order to examine the effect of cytokines on OSCC CSC populations, we decided to focus our studies on the effects of IL-4 and CCL3 on CSC phenotypes for two main reasons. First, IL-4 has been shown in previous studies to increase CSC properties in colon cancer (Francipane, 2008). In addition, the IFN family is already known to be used as a cancer therapeutic. This further strengthens our results because IFN-α and IFN-β showed increased expression in the CSC population as we expected from the previous knowledge regarding IFNs affect in cancer therapies. Secondly, to our knowledge, there have been no studies correlating CCL3 with an increase in CSC phenotypes and its effect on tumorigenesis. We aimed to further examine the effect of this cytokine and chemokine

on self-renewal capacity by analyzing the sphere formation potential. There were various OSCC cell lines selected to be treated with IL-4 and/or CCL3. We found that IL-4 and CCL3 increased sphere formation in BapT, UM5, UM17B, SCC4 and SCC9/TNFα. The OSCC cells were treated at varying concentrations ranging from 0ng/mL, 5ng/mL, 10ng/mL, and 50ng/mL. The optimal concentration for sphere formation assay in all cell lines was 10ng/mL. There was a significant increase in sphere formation capacity in the SCC4 cell line when treated with IL-4 (Figure 3) and CCL3 (Figure 4). Although, both IL-4 and CCL3 increased sphere formation ability, CCL3 had a more significant increase in self-renewal capacity. We focused on the effect of CCL3 on oral cancer stemness throughout the rest of the study due to the novelty of studying CCL3 in regards to OSCC CSC phenotypes. It has been previously demonstrated that a CCL3-CCR5 axis increases angiogenesis leading to increased tumorigenesis in a murine lung cancer model but the effect of CCL3 in OSCC has not be previously studied to our knowledge (Wu, 2008). Furthermore, in our preliminary data CCL3 notably increased the size of the spheres compared to the control (Figure 4B). These results suggest that CCL3 increases the self-renewal capacity of OSCC.

Effect of cytokine on migration of OSCC.

Next, to analyze the effect of CCL3 on other CSC phenotypes such as cell migration, we performed an in vitro cell migration assay. Due to our previous findings, we wanted to further investigate the effect of CCL3 on known CSC phenotypes such as self-renewal capacity and migration. Therefore, we next examined the effect of CCL3 on

SCC4 and BapT's potential for increased cell migration. We treated the SCC4 and BapT cells with 10ng/mL CCL3 and terminated the experiment after 96 or 72 hours, respectively. SCC4 cells migrate more slowly meanwhile the BapT cells migrate faster which results in an varying incubation times for the two different OSCC cell lines. CCL3 increased the migration of SCC4 compared to the control after 96 hours and CCL3 increased the migration of BapT compared to the control after 72 hours (Figure 5). As we expected from the previous sphere formation data, CCL3 increased the CSC properties of the OSCC. In addition, we performed a wound healing assay to further validate the data suggesting that CCL3 increased cell migration. SCC4 treated with CCL3 had an increase migratory ability after 48 hours which led to a significant reduction in the width of the wound compared to the control (Figure not shown). This data suggests that CCL3 can increase OSCC CSC phenotypes such as cell migration in addition to sphere formation ability. This further validates the role of CCL3 in increasing OSCC CSC phenotypes.

Effect of CCL3 on CSC factors.

There are many CSC factors that are overexpressed in CSC populations. In order to further determine the effect of CCL3 on OSCC, we analyzed the mRNA expression of various CSC factors. This was a critical step in our study because we wanted to correlate the increase in cancer stemness, due to the presence of CCL3, with a CSC factor in order to further understand the mechanism of action for CCL3 in OSCC. We examined the mRNA expression level of many different known CSC factors to investigate the effect of CCL3 on cancer stemness using qPCR. Our goal was to correlate CCL3 with a specific

dosage and time dependent expression trend of various CSC transcription factors. This would provide possible explanation that could be further explored for the observed increase in cancer stemness when OSCC are in the presence of CCL3. After treatment of SCC4 cells with 10ng/mL of CCL3 at 12 hours, 24 hours, 48 hours and 72 hours, we found an increase in mRNA relative expression level of two CSC factors, HES-1 and SOX-9, after 24 hours of treatment with CCL3 (Figure 6). This suggested that HES-1 and/or SOX-9 could be potential downstream targets of CCL3. Hes-1 is a known transcription factor involved in Notch signaling target and has been demonstrated to be involved in colon cancer self-renewal and tumorigenesis. SOX-9 is also a known transcription factor that is involved in downstream effectors and regulators of the WNT pathway as means to regulate intestinal epithelium homeostasis and can be a target of BMP, Hedgehog and Notch in regulation of stem cells from various tissue types (Matheu, 2012). SOX-9 is overexpressed in a wide range of cancers such as colorectal, lung, breast and prostate cancer (Shi, 2013). This data suggests that HES-1 and/or SOX-9 could be downstream signaling targets of CCL3.

Expression levels of HES-1 and SOX-9 in OSCC CSC and non-CSC populations.

Due to the previous data, we wanted to further examine the effect of HES-1 and SOX-9 expression levels in non-CSC populations compared to CSC populations of different cell lines. There had been a previous report stating that SOX-9 and HES-1 form a signaling axis whereby SOX-9 is an upstream initiator of HES-1. SOX-9 will be stimulated causing SOX-9 to bind to the HES-1 enhancer site initiating HES-1 expression

(Muller, 2010). We wanted to test if this was possible in our system because we found there to be increased expression level of SOX-9 and HES-1 in our previous data after 24 hours of CCL3 treatment. To test if this was possible in our system, we investigated the expression of HES-1 and SOX-9 in non-CSC and CSC populations sets of OSCC (SCC4, SCC9/TNFα, BapT and YD38) to confirm the expression of SOX-9 and HES-1 (Figure 7). We found increased expression of HES-1 and SOX-9 all OSCC sets, although, it was noted that SCC4 did not show an increase in HES-1 expression level. This suggests a potential role of HES-1 and SOX-9 in CSC phenotypes.

Effect of SOX-9 on self-renewal capacity.

After, discovering the increased expression of SOX-9 in the CSC populations of multiple OSCC cell lines, we investigated the effect of SOX-9 on self-renewal by using a transient siRNA for SOX-9 (SOX-9i) that would knockdown the expression of SOX-9. Knocking down the expression of SOX-9 would give insight to the effect of CCL3 on OSCC. In order to complete the knockdown of SOX-9, we performed a transfection with SOX-9i for 3 days then harvested the cells to be used for sphere formation assay. When we treated SCC4 with SOX-9i, there was a significant decrease in sphere formation ability and SOX-9 mRNA expression level was decreased (Figure 8). The SCC4 treated with SOX-9i in the sphere formation assay showed a decrease in number and significant decrease in the size of the spheroids compared to the control. The SOX-9i spheres had more spheroids that were less than 1.0 mm compared to the control (CTLi) indicating SOX-9 expression is crucial for sphere formation ability. When CCL3 was added to the

SOX-9i cells, there were more spheres and some larger spheres but the majority of the spheres were less than 1.0 mm in size. In order to validate this data we repeated two more trials which gave similar results. This indicated that in the absence of SOX-9 there was a decrease in self-renewal capacity of SCC4. This suggests that SOX-9 could have a potential role of increasing the self-renewal capacity of the SCC4 and other cell lines.

To confirm effect of SOX-9 on self-renewal capacity, we examined the effects of SOX-9i on two other cell lines, YD38 and BapT. YD38 and BapT showed a decrease in self-renewal capacity when treated with SOX-9i compared to the control. SOX-9i had a more significant effect on BapT sphere formation ability compared to YD38, although both cell lines showed a decrease in sphere formation (Figure 9). We validated the siRNA SOX-9 expression in both qPCR and western blot analysis to confirm the SOX-9 knockdown. This data suggests SOX-9 plays a pivotal role self-renewal capacity of OSCC.

Effect of SOX-9 on migration of SCC4.

Due to the previous findings, we wanted to further investigate the effect and role of SOX-9 on CSC properties. We wanted examined the effect of the transient SOX-9 expression on cell migration due to the previous data showing that CCL3 increased migration of SCC4. In addition, our previous sphere formation assay suggested that SOX-9 plays an important role in OSCC stemness and suggests that SOX-9 could be a downstream target of CCL3. Due to our previous observation we expect that SOX-9i there will decrease cell migration. Using the in vitro Transwell migration assay, we

examined the effect of SOX-9i on cell migration after 96 hours of incubation (Figure 10). Our data shows that the SOX-9i treated SCC4 had significantly decreased migration compared to the control. This supports our model that CCL3 activates the SOX-9 transcription factor which results in increased CSC phenotypes such as sphere formation assay and increased cell migration. Further studies need to be conducted in order to thoroughly understand the interaction between CCL3 and SOX-9 axis.

Effect of SOX-9i on cell proliferation.

In order to validate the effect of SOX-9 on self- renewal capacity of OSCC, we performed a cell proliferation assay to ensure the effect SOX-9i on self-renewal was not due to a decrease in cell proliferation. We incubated SCC4 CTLi and SOX-9i cells for 3 and 6 days in a six well plate then harvested and counted the number of cells (Figure 11). We found that there was a slight decrease in cell proliferation in the SOX-9i cell line when compared to the control. The decrease in proliferation was not significant enough to cause the dramatic decrease we observed in both the size and number of spheres in the sphere formation assays and the decrease in cell migration observed in the SOX-9i expressed SCC4. This data implies that the transient expression of SOX-9 doesn't have a significant effect on the proliferation of the SCC4. Therefore, this data suggests that the decrease in CSC phenotypes such as sphere formation and cell migration in the presence of SOX-9i were due to the knockdown of SOX-9 and weren't due to a decrease in cell proliferation.

CCR4/5 Expression in SCC4 cells.

In order to further investigate the role of the chemokine CCL3 in OSCC, we examined the expression of CCL3 receptors on SCC4 non-CSC and CSC populations using qPCR (Figure 11). It was crucial to correlate an increase in CCL3 receptors with an increase of CSC phenotypes in the SCC4 CSC populations. Our data showed that CSC population had increased expression of cell surface receptors CCR4/5 compared to the non-CSC populations (Figure 12). CCL3 ligand binds to CCR1, CCR4 and CCR5 but has a high- affinity for CCR5 and CCR1 meanwhile CCL3 binds with a low-affinity to CCR4 (Blainpain, 2001). The increased relative mRNA expression level of CCR5 indicates that there are more CCR5 cell surface receptors on the SCC4 CSC population compared to the non-CSC population. We expected this trend because the CCL3 ligand will bind the CCR5 receptor with high affinity on the cell surface. This data further suggest that CCL3 is present and interacting with its known CCR4/5 receptors on the cell surface of OSCC. A future study needs to be conducted to analysis the mRNA expression level of CCR1 on the surface of SCC4 cells. We would expect that CCR5 and CCR1 will show a similar expression trend because they both bind the CCL3 ligand with a high affinity.

Effect of CCR5 antagonist, Maraviric, on the CCL3-induced self-renewal capacity

In order to further investigate the role of the CCL3/CCR5 interaction in cancer stemness we wanted to study the effect of a CCR5 antagonist, Maraviroc, on the CCL3-induced self-renewal capacity. First, to analyze the effect of the CCR5 antagonist, we set

up a sphere formation assay with increasing concentrations of Maraviroc (0µM, 10µM, 50µM, 100µM). After 6 days of incubation there was a decrease in sphere formation ability at 50µM and 100µM Maraviroc, although, 100µM had the most significant effect on the sphere formation ability (Figure 13). In addition, when 10ng/mL of CCL3 was added along with the Maraviroc there was a decrease in sphere formation ability compared to the CCL3-treated cells, indicating that the CCR5 antagonist blocked the effect of CCL3 on self-renewal capacity (Figure 14). Furthermore, a MTT assay was conducted to examine the cytotoxic effect of Maraviroc on OSCC and showed no cytotoxic effect of Maraviroc on the cells. Taken together, this suggests that the CCL3-induced self-renewal is dependent on CCR5 receptor.

DISCUSSION

In the present study, we aimed to identify cytokines and chemokines that increase cancer stem cell (CSC) phenotypes in oral squamous cell carcinoma (OSCC). More recently, there has been more scientific evidence suggesting the significant role the immune system plays in cancer development and progression (Blair, 2008). During the immune response, cytokines and/or chemokines are released to help ward off infection or destroy abnormal cell growth via ligand-receptor interactions which have oncogenic potential (Sriuranpong, 2003). Most often cytokines can be secreted either in an autocrine, paracrine or endocrine fashion. Although, not all cytokines need receptors to enter the cell; some cytokines are membrane soluble and will diffuse into the cell. In the last decade, there has been significant evidence demonstrating the role of cytokines in tumorigenesis in a wide variety of cancers. Most notably IL-1, IL-4, IL-6, IL-10, IL-12, IL-23, TGF-β, TNF-α and TRAIL which have been shown to signal through the NF-Kβ, STAT and caspases (Lin, 2007).

In our present study, we found that two cytokines IL-4 and, more notably, CCL3 increased the self-renewal capacity and migration of OSCC cell lines when compared to the control. IL-4 has been previously shown to increase cancer stemness in other epithelial cancers such as colorectal cancer (Francipane, 2008). Therefore, we focused the remainder of the studies on CCL3 due to the novel involvement of CCL3 in cancer and OSCC. Although, our results were consistent with other evidence that IL-4 increased CSC phenotypes such as migration, self-renewal and cell survival (Todaro, 2008 and Francipane, 2008). We found that CCL3 treated cell lines showed an increase in CSC

factors when compared to the control group. Furthermore, SOX-9 plays a critical role and forms a CCL3-SOX-9 axis that increases CSC phenotypes such as migration, self-renewal and CSC transcription factors expression levels.

When conducting the initial cytokine profiling, there were fifteen cytokines that were increased in the SCC4 CSC population compared to the control (non-CSC population). IL-2 and CCL4 were immediately excluded any future study because the basal level of these two cytokines were very low. There were six other putative cytokines (IL-4, IL-5, CCL3, IL-36RN, IFN-α, IFN-□) that were further analyzed in two other cell lines: BapT and SCC9/TNFα. Further investigation should be conducted on the effect of the IFN family on OSCC due to the previously known effect IFN-α has on pancreatic CSCs. In addition, the IFN family has been known to decrease tumorigenesis and is currently used as an anti-tumor drug (Ferrantini, 2008). According to our data, IL-4 and CCL3 showed an increase in sphere formation ability. Due to the previous known role of IL-4 in cancer stemness, we expected to find an increase of IL-4 in OSCC. We confirmed the observed CSC phenotype, increased sphere formation, by examining the sphere formation ability on other OSCC cells lines such as BapT, UM5, UM17B, SCC4, SCC9/TNFα. CCL3 increased the size of the spheres significantly after 6 days of incubation. We repeated the sphere assays multiple times to confirm the results and we found a similar trend although, BapT, SCC4 and SCC9/TNFα had a more significant increase in cancer stemness compared to UM5 and UM17B. Taken together this data suggests that CCL3 plays an important role in increasing CSC phenotypes.

To further explore the effect of CCL3 on CSC phenotypes, we examined the effect of CCL3 on cell migration. It has been previously shown that chemokines have influenced the development of primary tumors and metastasis (Balkwill, 2004). It has been suggested that the role of cytokines in host-tumor interactions determine the site-specific spread of cancer cells and the metastatic ability of the tumor progression. Understanding the metastatic behavior and migration pattern of the tumor cells can lead to a better global understanding of tumor growth and development. Our in vitro Transwell migration assay data suggested that CCL3 increased the migration of the SCC4 compared to the control (non-treated) after 96 hours of incubation. We also performed a cell migration assay with another cell line, BapT. We obtained similar results for BapT migration assay, the BapT treated with CCL3 had increased migration after 72 hours compared to the control. This further suggests that CCL3 increases CSC phenotypes such as migration and sphere formation ability.

In order to explore the method that CCL3 is employing to increase CSC phenotypes, we screened for known expression of CSC factors that were increased in the CCL3 treated SCC4. We found that HES-1 and SOX-9 both were increased in the CCL3 treated SCC4. It has been previously shown that there is a SOX-9 and HES-1 axis in human breast cancer cells. It was shown that SOX-9 regulates HES-1 expression by a regulatory element upstream of HES-1 (Muller, 2009). HES-1 and SOX-9 are both known CSC transcription factors and are involved in various signaling pathways in cancer. HES-1 is known to be involved with Notch signaling pathway. It has been previously demonstrated that in pancreatic cancer cells, Notch activation leads to

increased expression of Notch target transcription factors Hes-1 and c-MYC (Tremblay, 2013). In addition, MEK/ERK pathway will also promote Hes-1 expression and expression of other Notch target genes (Tremblay, 2013).

HES-1 has also been shown to be linked to increased self-renewal and tumorigenicity in colon cancer. The expression of HES-1 in colon cancer indicates a pivotal role for HES-1 in progression of colon cancer via the induction of stem-like cancer cells (Gao, 2015). Meanwhile, less is known about the role of SOX-9 in cancer and CSC populations. SOX-9 has been shown to be involved in Sonic Hedgehog (SHH) pathway and WNT signaling (Shi, 2013). More recently, it has been suggested that SOX-9 is stimulated through the activation of mitogen-activated protein kinase (P44/P42 MAPK and ERK 1/2) (Ling, 2011). In addition, SOX-9 has been demonstrated to have a role in the Hippo pathway and is regulated by the upstream YAP1 binding to the TEAD sequence on the SOX-9 promoter. It has been shown that YAP1-SOX-9 axis increases tumorigenesis and increases CSC phenotypes including sphere formation ability and propagation (Song, 2014)

We sought out to investigate the expression of known chemokine receptors on the different cell types. We analyzed the expression of CCR4 and 5 on SCC4 non-CSC and CSC populations in order to confirm that the cytokines were increasing the expression of CCL3 specific receptors on the surface of the cancer cells. This also allowed us to validate that the chemokine we were treating the cells with was able to interact with the cell surface receptors and that the chemokine was in fact responsible for the change in CSC transcription expression as we had previously seen.

In order to investigate the potential role of HES-1 and/or SOX-9 in our system we employed transient siRNA via transfection to examine the effects of HES-1 and SOX-9 on CCL3 induced CSC phenotypes such as self-renewal and migration capacity. We found a significant decrease in self-renewal capacity when SCC4, BapT were treated with SOX-9 siRNA. SCC4 and BapT had a decrease in both size and number. YD38 showed a decrease in sphere number, but it was not as significant as SCC4 and BapT. We also investigated the effect of SOX-9i on migration. As we expected, when SOX-9 was knocked down we saw a decrease in migration. Taken together this data further suggests the crucial role of SOX-9 in inducing CSC phenotypes.

This data indicates that CCL3 plays a significant role in increasing cancer stemness in OSCC. We demonstrate this by showing increase in self-renewal, migration and an increase in CSC factors. We found that there was an increase in CCL3 specific cell surface receptors such as CCR4/5 on the SCC4 non-CSCs compared to the CSCs population. This study suggests that CCL3 is an upstream regulator of SOX-9 which regulates CSC phenotypes such as self-renewal and migration. Further studies need to be conducted in order to determine the mechanism of activation for signaling from CCL3 to SOX-9. There are many major signaling pathways involved in chemokine signaling and specific pathway for CCL3 needs to still be determined.

FIGURE LEGENGS AND FIGURES

Figure 1. **Cytokine Profiling in SCC4 non-CSC populations and CSC populations.**

The relative mRNA expression level of pro-inflammatory cytokines and chemokines in non-CSC population (monolayer) and CSC populations (spheroid cells) was determined by using qPCR for SCC4. Of the twenty-five cytokines, fourteen of the cytokines (IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-36RN, IFNα, IFNβ, IFN□, CCL3, CCL4, RANTES, SFD-1) showed elevated expression level in the spheroid cells compared to the control. SCC4 monolayer was grown in normal medium whereas the SCC4 spheroidal cells were grown in sphere forming medium.

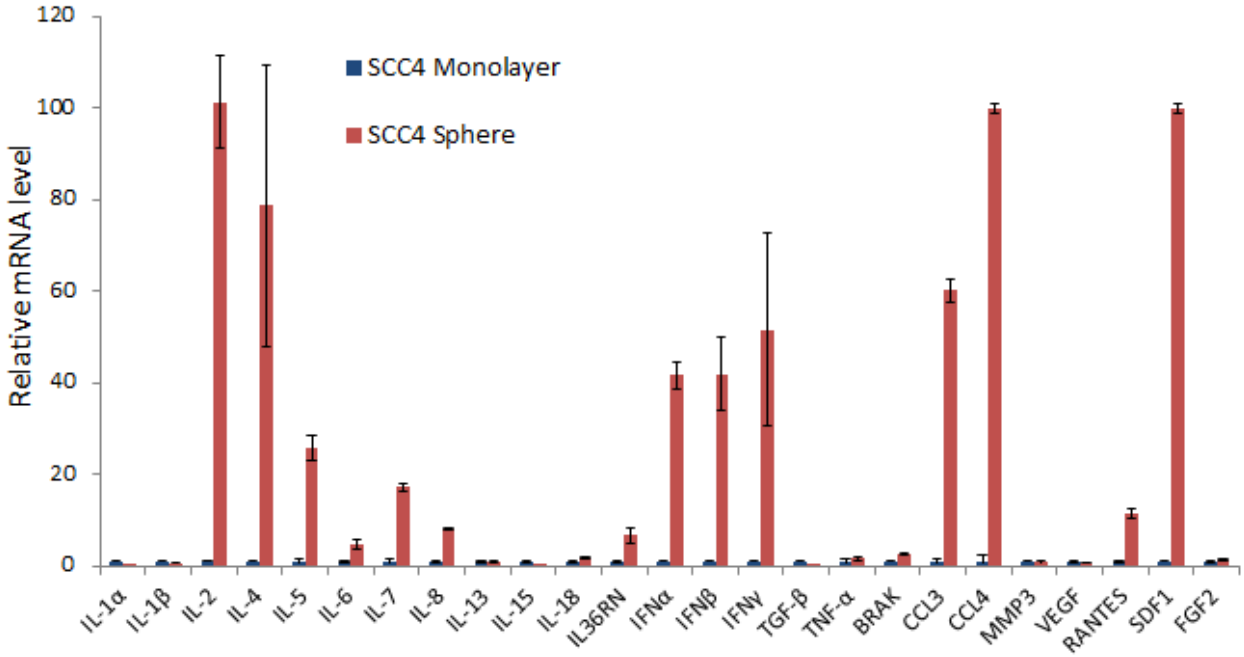
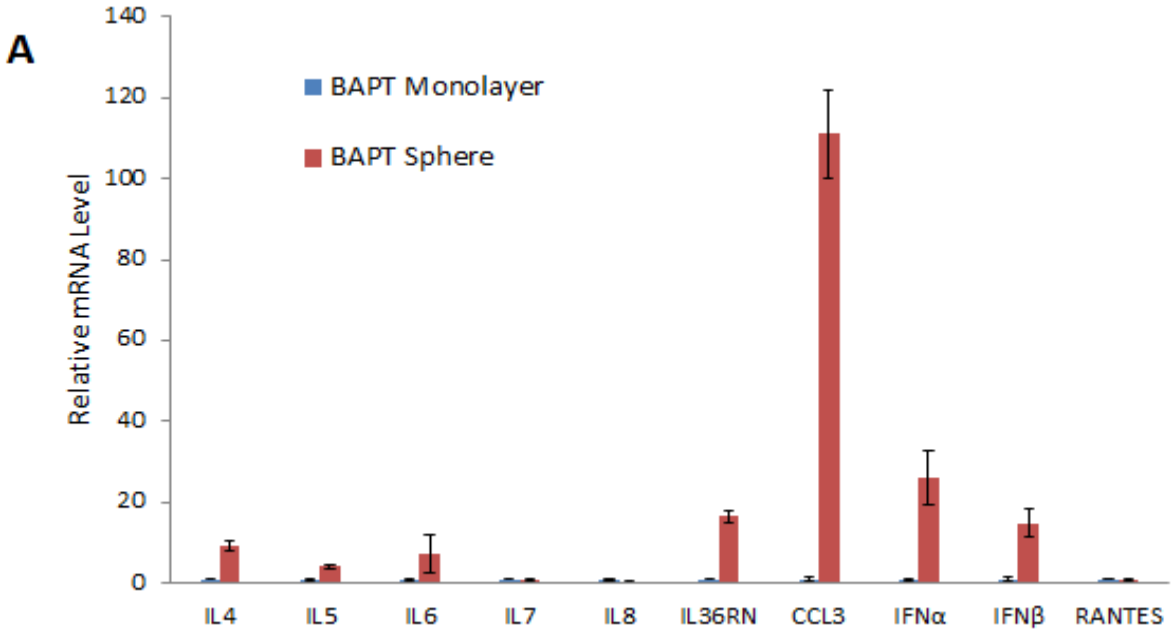

120
100
80
60
40
20
0
Relative mRNA level
SCC4 Monolayer
SCC4 Sphere
IL-1α
IL-1β
IL-2
IL-4
IL-5
IL-6
IL-7
IL-8
IL-13
IL-15
IL-18
IL36RN
IFNα
IFNβ
IFNγ
TGF-β
TNF-α
BRAK
CCL3
CCL4
MMP3
VEGF
RANTES
SDF1
FGF2

Figure 2. Confirmation of CSC-specfic cytokine in BapT and SCC9-TNFα.

The relative mRNA expression levels of the CSC-specific cytokines were determined in monolayer and spheroid of BapT and SCC9/TNFα cells. Of the ten cytokines, six cytokines (IL-4, IL-5, IL-36RN, CCL3, IFNα, IFNβ) had elevated expression level in both OSCC cell line. The OSCC cells monolayer was cultured in normal medium for each cell type and the spheroid cells were grown in sphere formation medium. (A) The relative mRNA expression level of CSC-specific cytokines in Bapt non-CSC population (monolayer) and CSC population (spheroid cells) of BapT cells was determined by qPCR. The Bapt monolayer was grown in normal cell culture medium. The BapT spheroid cell were grown for 7 days in sphere formation medium then sphere were collected to make cDNA for qPCR analysis. (B) The relative mRNA expression level of CSC-specific cytokines in SCC9/TNFα non-CSC population (monolayer) and CSC population (spheroid cells) of SCC9/TNFα cells was determined by qPCR. The cells were cultured and spheres collected as mentioned above. Six OSCC CSC specific cytokines were identified: IL 4, IL-5, IL-36RN, CCL3, IFN-α, IFN-β


A
140
120
100
80
60
40
20
0
Relative mRNA Level
BAPT Monolayer
BAPT Sphere
IL4
IL5
IL6
IL7
IL8
IL36RN
CCL3
IFNα
IFNβ
RANTES

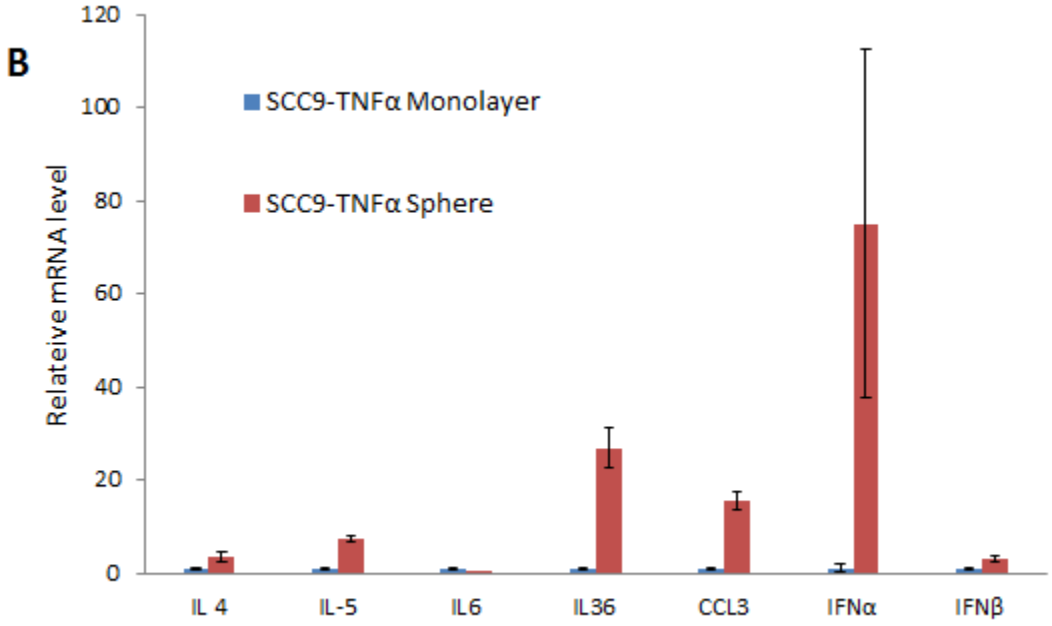

B
120
100
80
60
40
20
0
Relateive mRNA level
SCC9-TNFα Monolayer
SCC9-TNFα Sphere
IL 4
IL-5
IL6
IL36
CCL3
IFNα
IFNβ

Figure 3. Effect of IL-4 on self-renewal of OSCC.

The effect of pro-inflammatory cytokine, IL-4, on self-renewal capacity was determined in five cell lines: BapT, UM5, UM17B, SCC4, SCC9-TNFα via sphere formation assay. The effect of IL-4 was compared to the control (CTL) was quantitated for all five sets of cell lines. The fold induction was elevated in all OSCC cells that were treated with IL-4 compared to the control. The OSCC cells were cultured in sphere formation medium and treated with 10 ng/mL of IL-4. The spheres were grown for six days then analyzed. Relative number of spheroids formed by CTL and IL-4 were compared to determine statistical significance. (** $P<0.01$, unpaired two-tailed Student's t test.)

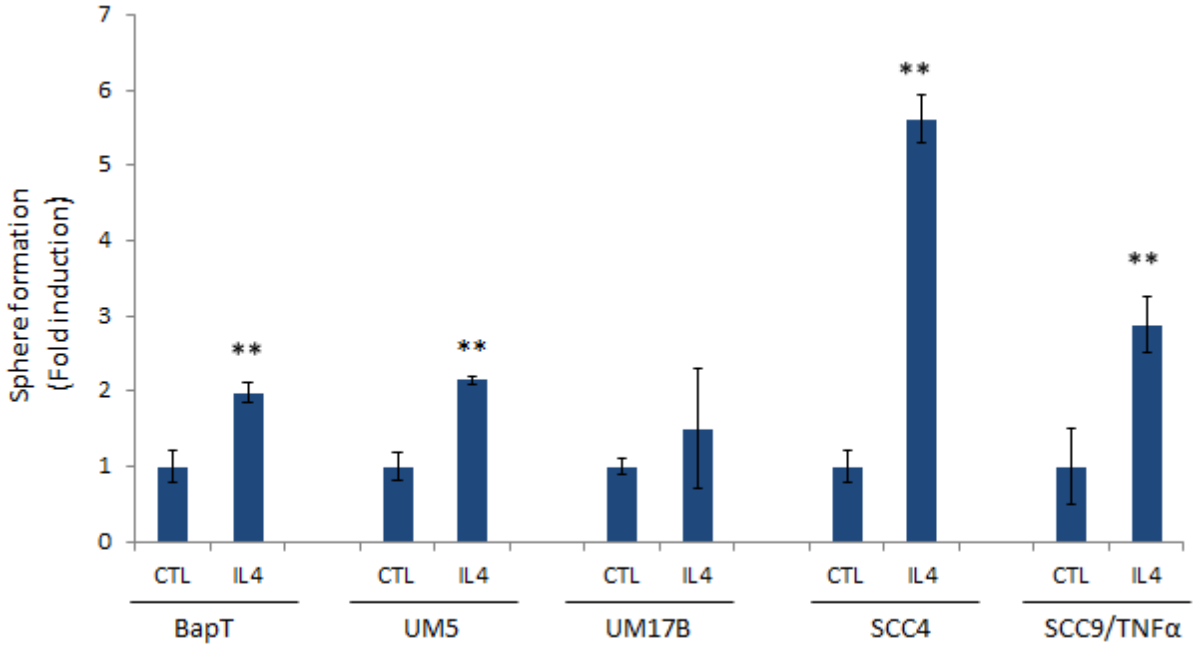
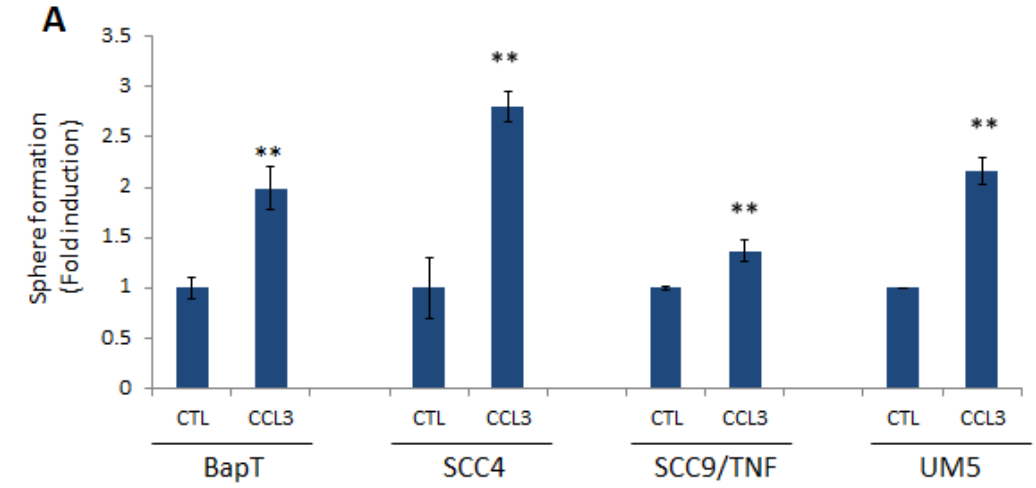

Sphere formation
(Fold induction)
7
6
5
4
3
2
1
0
**
**
**
**
CTL
IL4
CTL
IL4
CTL
IL4
CTL
IL4
CTL
IL4
BapT
UM5
UM17B
SCC4
SCC9/TNFα

Figure 4. Effect of CCL3 on self-renewal of OSCC.

The effect of pro-inflammatory cytokine, CCL3, on self-renewal capacity was determined in four cells: BapT, SCC4, SCC9-TNFα, UM5 via sphere formation assay. The effect of CCL3 was compared to the control in all four cell lines. (A) CCL3 treated cells had elevated sphere formation capacity (fold induction) compared to the controls for each cell set. The OSCC cells were cultured in sphere formation medium and treated with 10 ng/mL of CCL3. The spheres were grown for six days then the number of spheres was analyzed. (B) Representative image of tumor sphere formed by SCC4 control (CTL) and SCC4 treated with CCL3. The photographs were taken with a magnification of 4X (Scale bar= 0.1 mm). (C) Quantitative analysis of the number of sphere size greater than 1 mm was determined for the SCC4 CTL and SCC4 CCL3 treated spheres. The SCC4 CCL3 spheres had more spheres greater than 1 mm in size compared to the SCC4 CTL spheres. Relative number of spheres formed by SCC4 CTL and SCC4 CCL3 were compared to determine statistical significance. (** $P<0.01$, unpaired two-tailed Student's t test.)


A
Sphere formation (Fold induction)
3.5
3
2.5
2
1.5
1
0.5
0
**
**
**
**
CTL
CCL3
CTL
CCL3
CTL
CCL3
CTL
CCL3
BapT
SCC4
SCC9/TNF
UM5

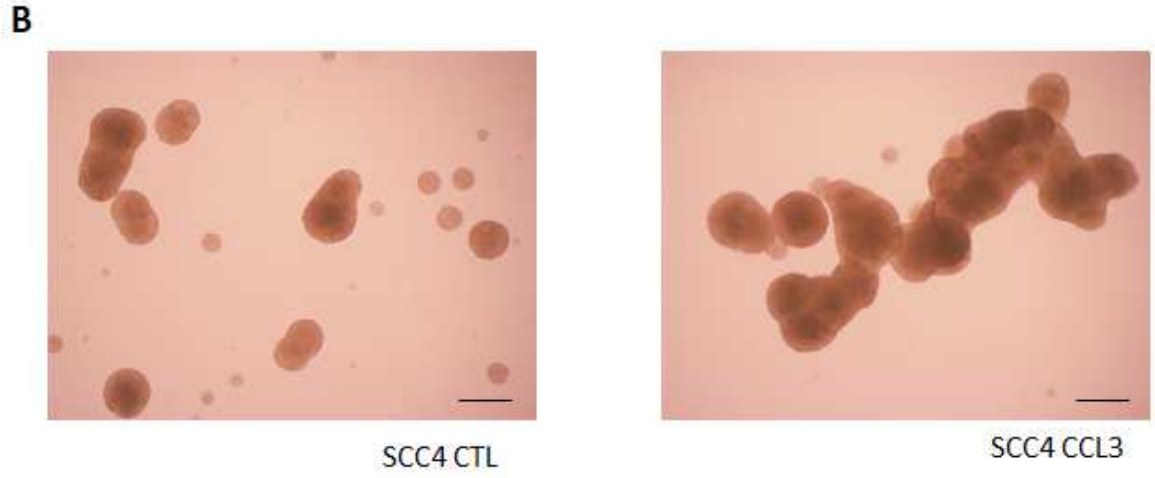

B
SCC4 CTL
SCC4 CCL3

Figure 5. Effect of CCL3 on migration of OSCC.

CCL3 increases the migratory capacity of OSCC. Transwell migration assay was performed and the number of migrated cells was counted. Representative images were taken with a magnification of 4X. (A) Treatment with CCL3 increased migratory capacity of SCC4 compared to the non-treated cells after 96 hours (Scale bar = 0.1 mm). (B) Treatment with CCL3 increased the migratory capacity of BapT compared to non-treated cells after 72 hours (Scale bar = 0.1 mm). Relative number of SCC4 CTL and/or BapT CTL and SCC4 CCl3 and/or BapT CCL3 migrated cells were compared to determine statistical significance. (** $P<0.01$, unpaired two-tailed Student's t test.)

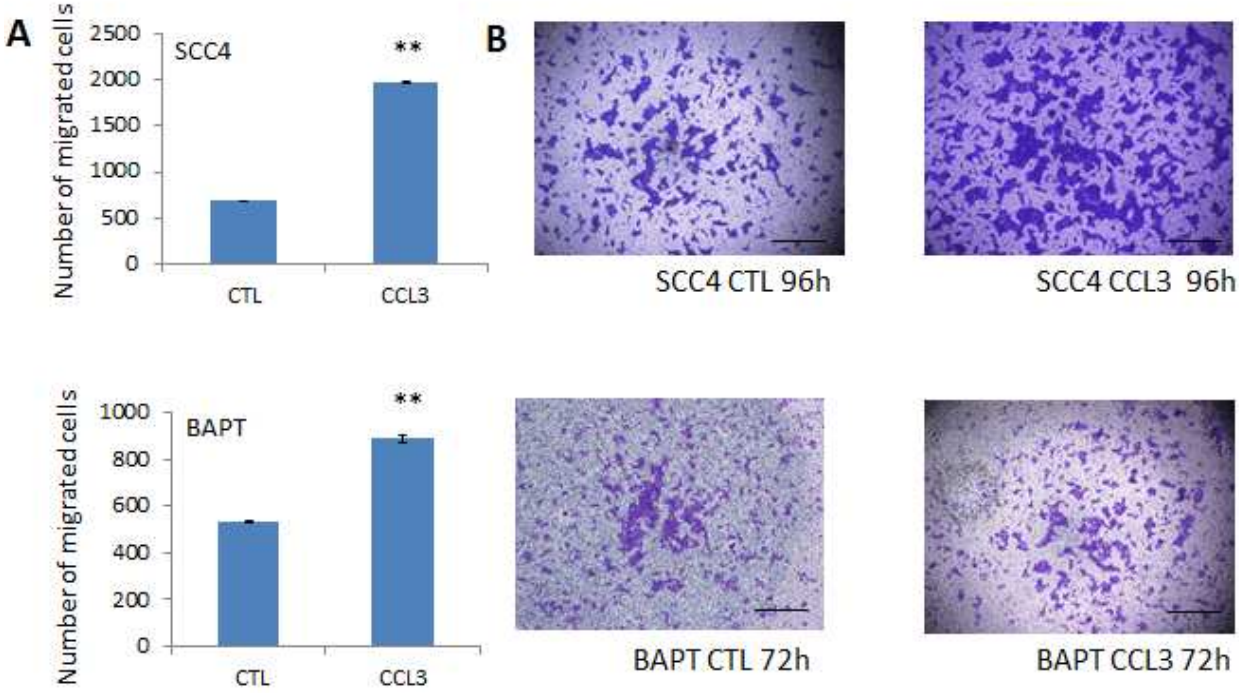

A
B
SCC4
Number of migrated cells
2500
2000
1500
1000
500
0
**
CTL
CCL3
SCC4 CTL 96h
SCC4 CCL3 96h
BAPT
Number of migrated cells
1000
800
600
400
200
0
**
CTL
CCL3
BAPT CTL 72h
BAPT CCL3 72h

Figure 6. Effect of CCL3 on CSC factors.

The relative mRNA expression level of CSC transcription factors was determined by using qPCR for SCC4 control (CTL) and SCC4 CCL3 treated cells. Of the thirteen CSC transcription factors, two of the CSC transcription factors (HES-1 and SOX-9) had elevated expression in the SCC4 CCL3 compared to the SCC4 CTL. SCC4 CCL3 cells were treated with 10 ng/mL of CCL3 for 24 hours.

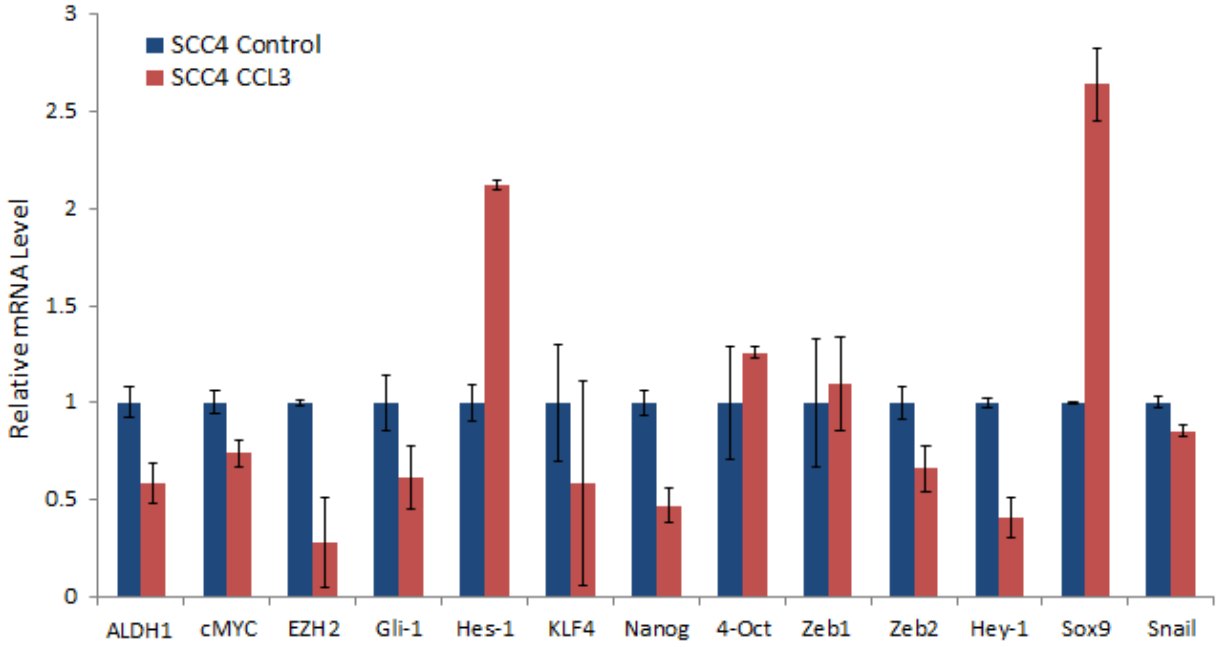
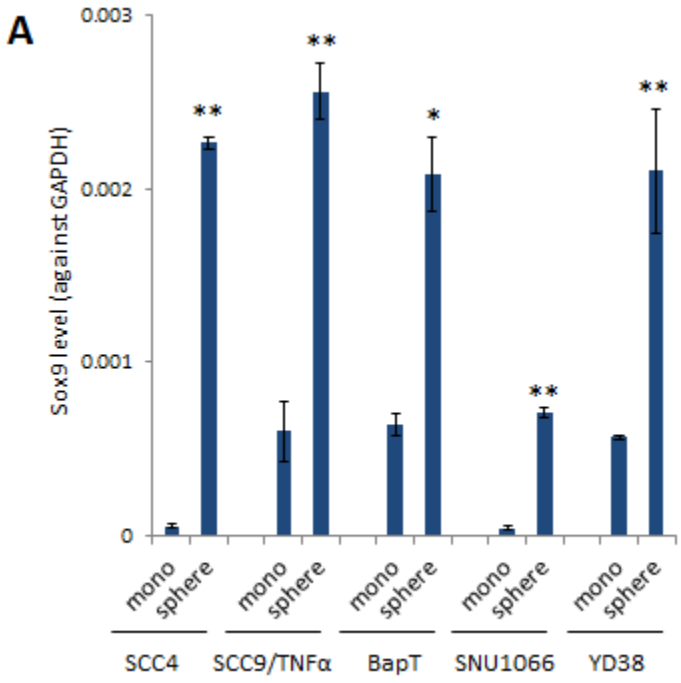

SCC4 Control
SCC4 CCL3
Relative mRNA Level
3
2.5
2
1.5
1
0.5
0
ALDH1
cMYC
EZH2
Gli-1
Hes-1
KLF4
Nanog
4-Oct
Zeb1
Zeb2
Hey-1
Sox9
Snail

Figure 7. Expression of HES-1 and SOX-9 in non-CSC and CSC populations.

The relative mRNA expression of HES-1 and SOX-9 was determined in five cell lines (SCC4, SCC9/TNFα, BapT, SNU1066, YD38) using qPCR. The OSCC monolayers (mono) were grown in normal cell culture medium. The OSCC spheres were grown in sphere formation medium for 6 days. (A) The relative mRNA expression of SOX-9 was determined in five cell line's non-CSC population (monolayer) and CSC population (sphere). The transcriptional expression of SOX-9 was increased in all five of the OSCC cell lines (SCC4, SCC9/TNFα, BapT, SNU1066, YD38). (B) The relative mRNA expression of HES-1 was determined in five cell line's non-CSC population (monolayer) and CSC population (sphere). The transcriptional expression of HES-1 was increased in four of the OSCC cell lines (SCC9/TNFα, BapT, SNU1066, YD38). Relative mRNA expression levels of monolayers and CCL3 treated OSCC were compared for each cell line 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
0.003
0.002
0.001
0
Sox9 level (against GAPDH)
**
**
*
**
**
mono
sphere
SCC4
SCC9/TNFα
BapT
SNU1066
YD38

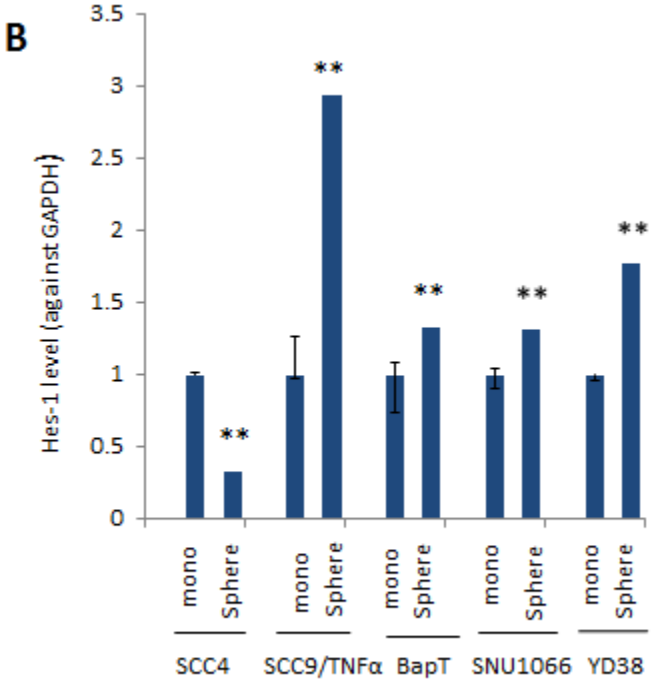
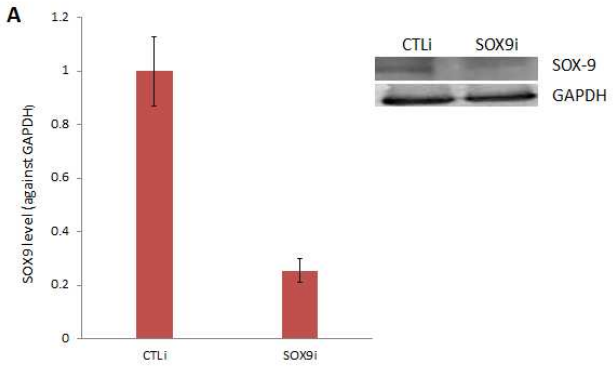
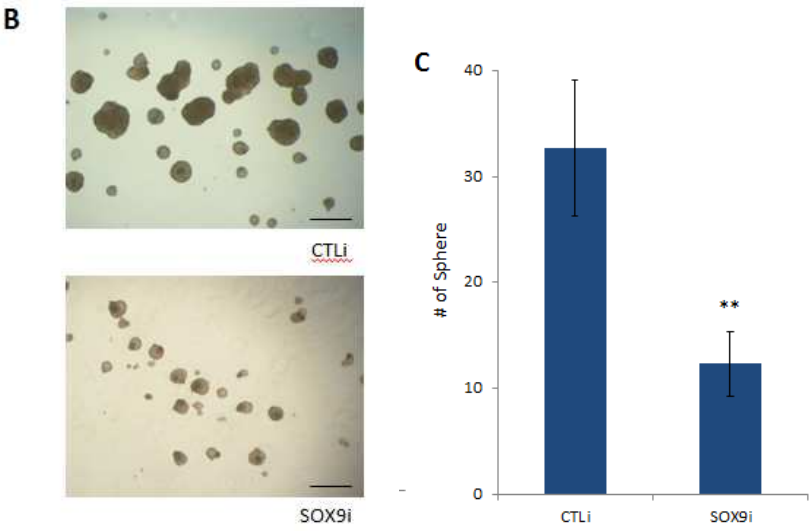

B
3.5
3
2.5
2
1.5
1
0.5
0
Hes-1 level (against GAPDH)
**
**
**
**
**
mono
Sphere
SCC4
SCC9/TNFα
BapT
SNU1066
YD38

Figure 8. Effect of SOX-9 on self-renewal of OSCC.

SCC4 was transfected with siRNA that transiently knockdowns endogenous SOX-9. (A) The relative mRNA expression of SOX-9 was determined in SCC4 transfected with control siRNA (CTLi) and SCC4 transfected with SOX-9 siRNA (SOX9i). The SCC4 with SOX9i had decreased expression of SOX-9 compared to CTLi. Protein analysis confirms the knock down of SOX9i (B) Sphere formation assay was performed to determine the effect of SOX9i on self-renewal capacity. CTLi and SOX9i cells were cultured in sphere medium for 6 days. Representative image of tumor spheres formed by CTLi and SOX9i. Image was taken with a magnification of 4x (Scale bar = 0.1 mm). (C) Quantitative analysis of the number of spheres in CTLi and SOX9i. (D) The size of the spheres formed in CTLi and SOX9i were analyzed. For CTLi and SOX9i, spheres less than or greater than 0.1 mm in size were counted and percentages (out of 100%) were determined for CTLi and SOX9i. Relative mRNA expression level of SOX-9 in CTLi and SOX9i were compared to determine statistical significance. Relative number of spheres in CTLi and SOX9i were compared to determine statistical significance. (** $P<0.01$, unpaired two-tailed Student's t test.)


A
1.2
1
0.8
0.6
0.4
0.2
0
SOX9I level (against GAPDH)
CTLi
SOX9i
CTLi
SOX9i
SOX-9
GAPDH


B
CTLi
SOX9i
C
40
30
20
10
0
# of Sphere
**
CTLi
SOX9i

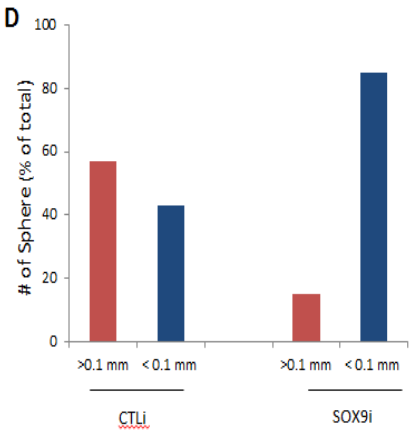

D
100
80
60
40
20
0
# of Sphere (% of total)
>0.1 mm
< 0.1 mm
>0.1 mm
< 0.1 mm
CTLi
SOX9i

Figure 9 . Effect of SOX-9 on self-renewal on BapT.

BapT was transfected with siRNA that transiently knockdowns endogenous SOX-9. (A) BapT CTLi and SOX9i were cultured in sphere formation medium for 6 days. Representative image of tumor sphere formed by CTLi and SOX9i. Image was taken with a magnification of 4x (Scale bar = 0.1 mm). (B) Quantitative analysis of the number of spheres in CTLi and SOX9i. (C) The size of the spheres formed in CTLi and SOX9i were analyzed. For CTLi and SOX9i, spheres less than or greater than 0.1 mm in size were counted and percentages (out of 100%) were determined for CTLi and SOX9i.

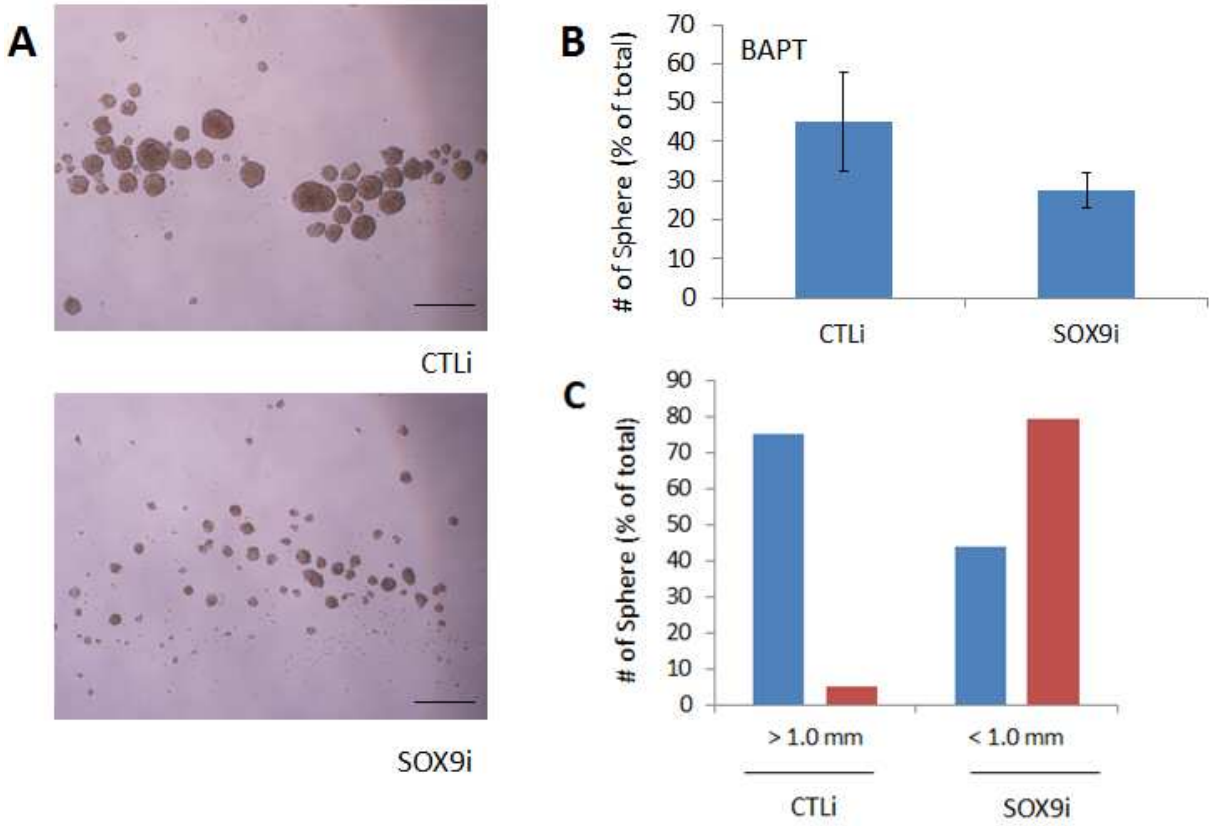

A
CTLi
SOX9i
B
BAPT
# of Sphere (% of total)
70
60
50
40
30
20
10
0
CTLi
SOX9i
C
# of Sphere (% of total)
90
80
70
60
50
40
30
20
10
0
> 1.0 mm
< 1.0 mm
CTLi
SOX9i

Figure 10. Effect of SOX-9 on migration of SCC4.

SCC4 was transfected with siRNA that transiently knockdowns endogenous SOX-9. (A) Transwell migration assay was performed with control siRNA (CTLi) and SOX-9 siRNA (SOXi). After 94 hours of incubation, the SOXi decreased migratory capacity compared to CTLi (B) Representative image of the stained CTLi and SOX9i migrated cells 94 hours of incubation. The image was taken with a magnification of 4x (Scale bar = 0.1 mm) SCC4 cells were treated with SOXi via transfection and incubated for 96 hours compared to the control. (** $P<0.01$, unpaired two-tailed Student's t test.)

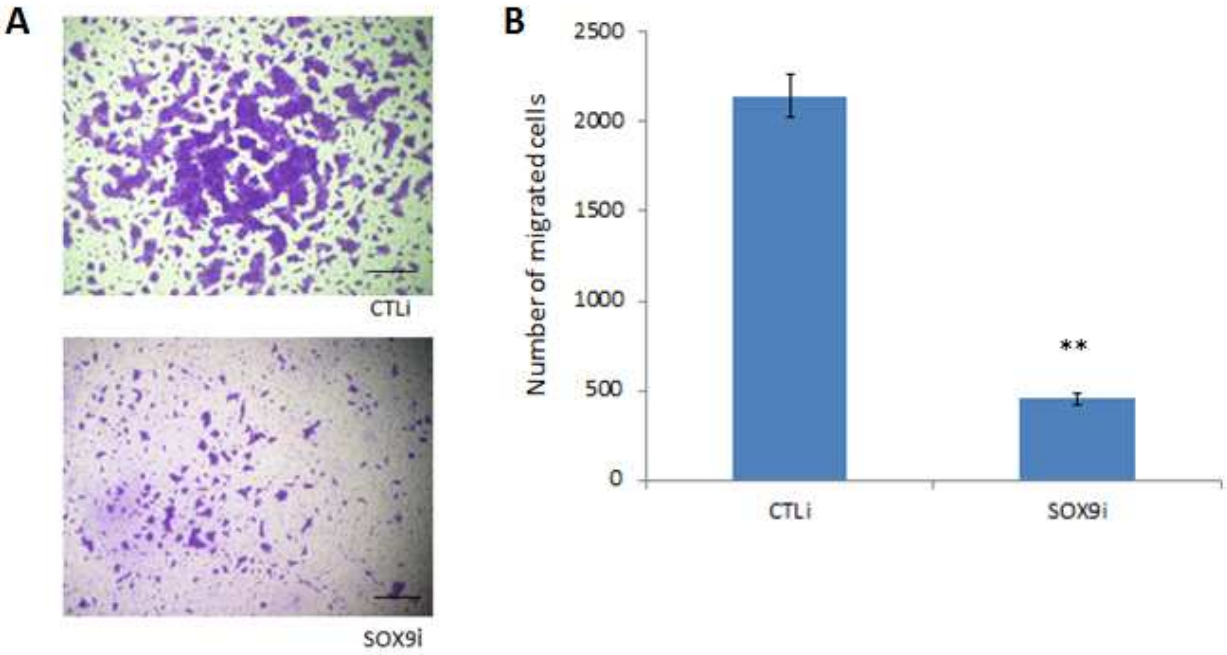

A
CTLi
SOX9i
B
Number of migrated cells
2500
2000
1500
1000
500
0
**
CTLi
SOX9i

Figure 11. Effect of SOX-9 on cell proliferation of SCC4.

Cell proliferation assay was performed to determine the effect of SOX9i on the cell proliferation rate of SCC4. The rate of proliferation was measure on day 0,3,6. The CTLi and SOX9i cells were plated in triplicate and the cell number was determined by counting the cells then taking the average of the total number of cells from each of the three wells. The rate of proliferation decreased for SOX9i compared to the CTLi.

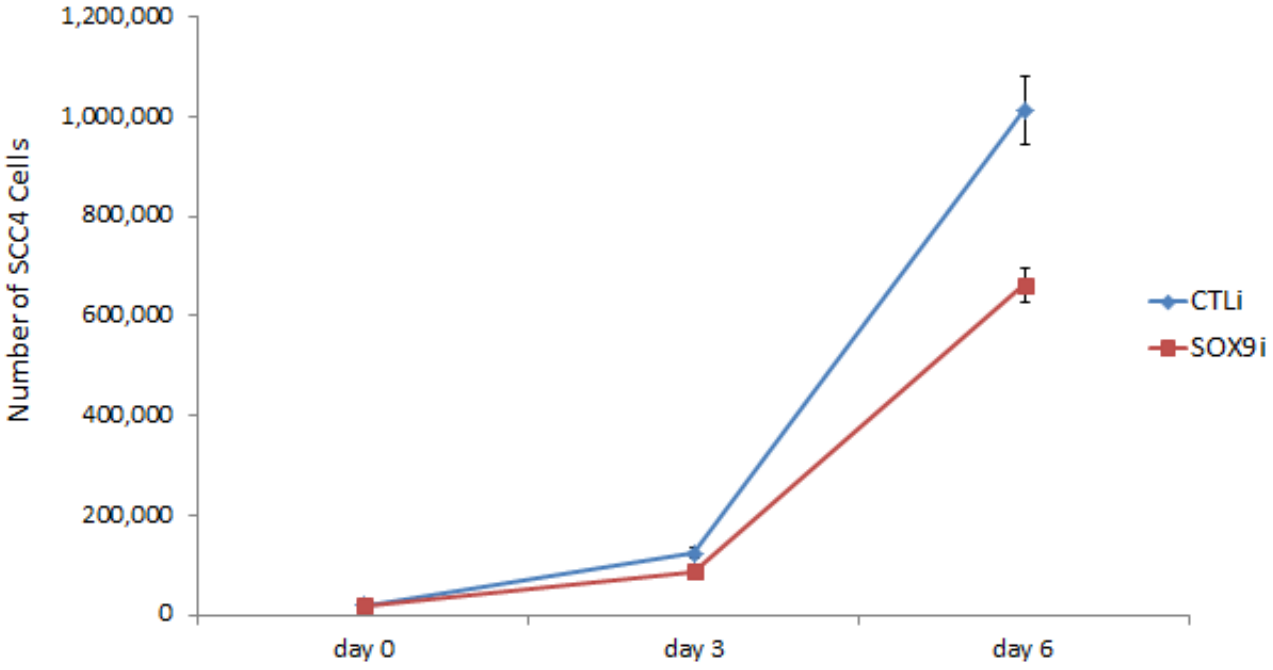

1,200,000
1,000,000
800,000
600,000
400,000
200,000
0
Number of SCC4 Cells
day 0
day 3
day 6
CTLi
SOX9i

Figure 12. Expression of CCL3 receptors (CCR4 and CCR5) in SCC4.

The relative mRNA expression level of CCR4 and CCR5 was determined in SCC4 non-CSC population (monolayer) and CSC population (spheroids). The SCC4 monolayer was grown in normal medium meanwhile the spheroids were obtained by growing SCC4 cells in sphere formation medium. There was elevated expression of CCR4 and CCR5 in CSC population compared to the non-CSC population.

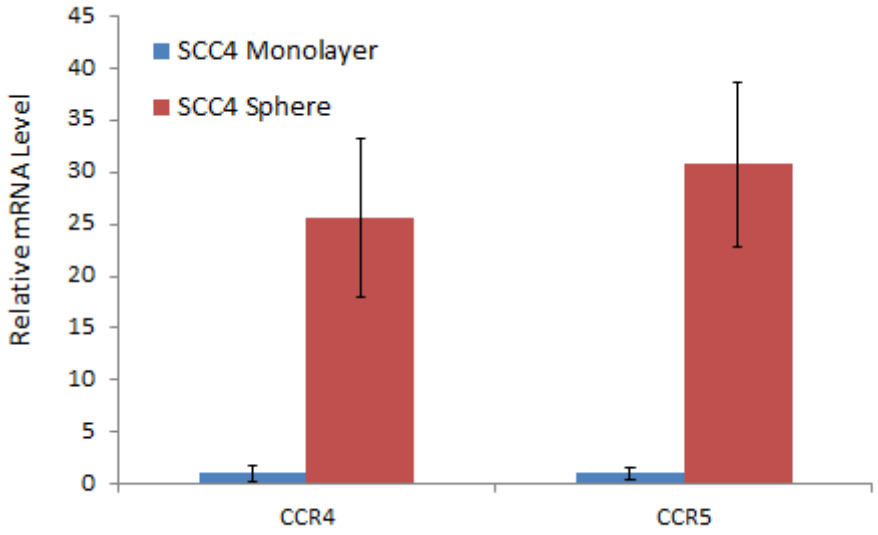

45
40
35
30
25
20
15
10
5
0
Relative mRNA Level
SCC4 Monolayer
SCC4 Sphere
CCR4
CCR5

Figure 13. Effect of CCR5 antagonist, Maraviroc, on self- renewal capacity.

SCC4 was treated with CCR5 antagonist, Maraviroc, to determine the effect on sphere formation and self-renewal capacity. SCC4 was cultured in sphere formation medium with 100µM of Maraviroc. The control (CTL) was treated with DMSO (0.1% of total volume in well). The SCC4 treated with Maraviroc had decreased spheroid formation compared to the SCC4 CTL DMSO. (A) Representative image of SCC4 in CTL DMSO and Maraviroc (Scale bar = 0.1 mm). (B) Quantitative representation of the number of spheres in SCC4 DMSO compared to Maraviroc.

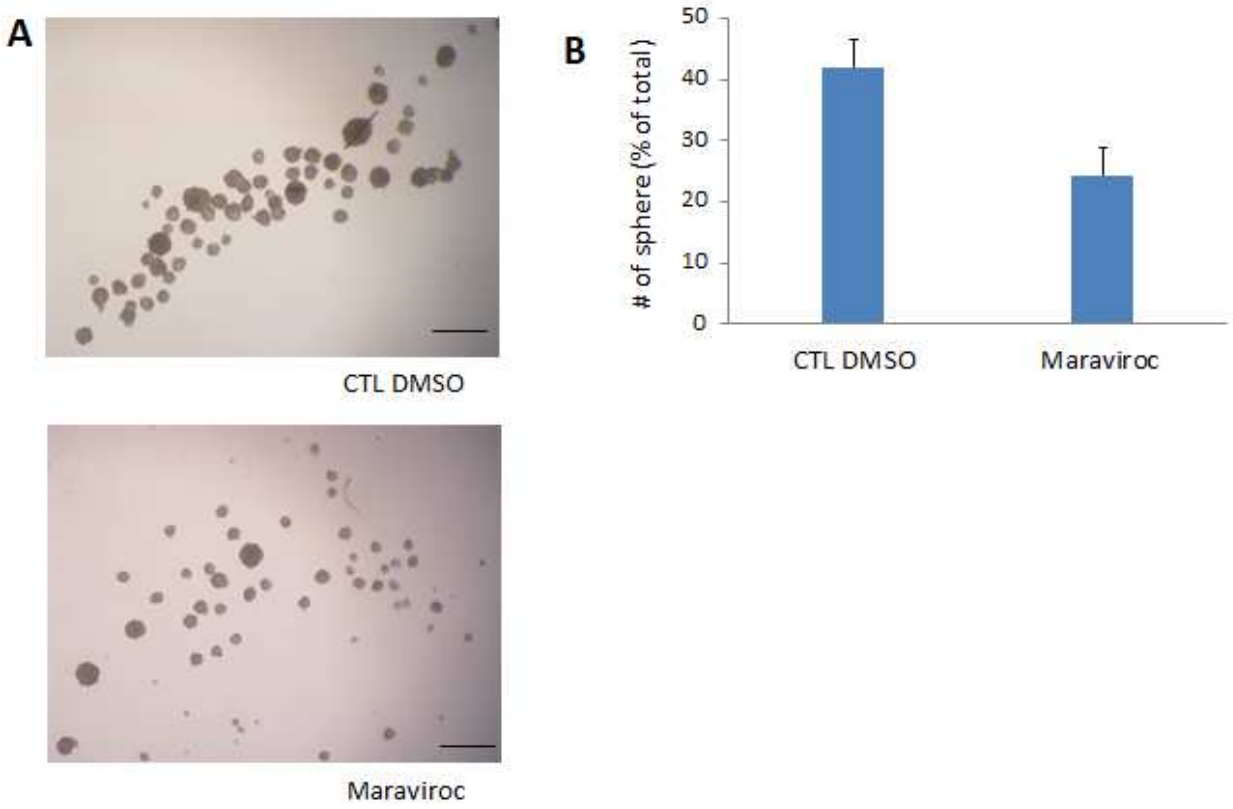
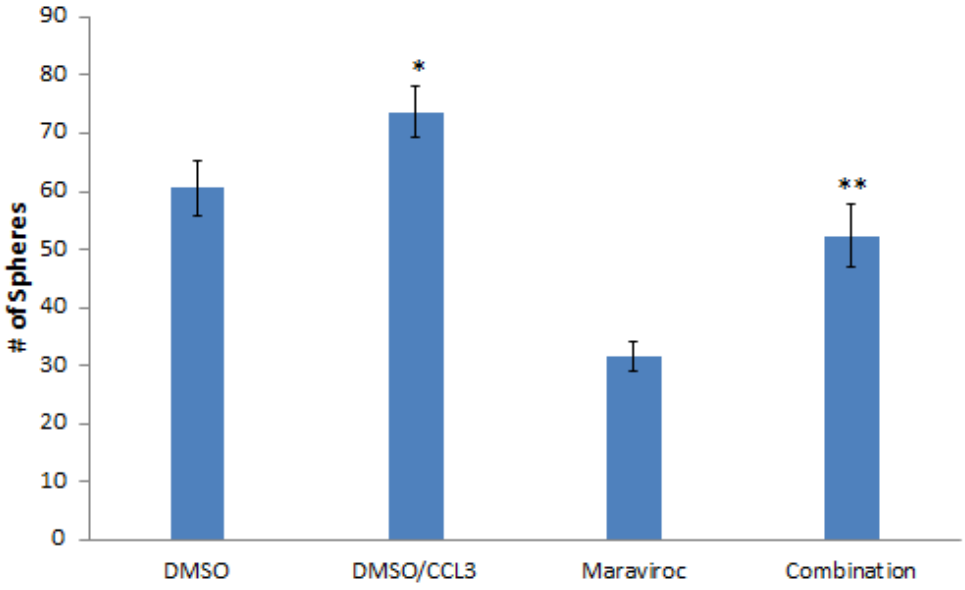

A
CTL DMSO
Maraviroc
B
# of sphere (% of total)
50
40
30
20
10
0
CTL DMSO
Maraviroc

Figure 14. Effect of Maraviroc on the CCL3-induced self-renewal capacity.

SCC4 was treated with CCR5 antagonist, Maraviroc, and CCL3 combination to determine the effect on sphere formation and self-renewal capacity. SCC4 was cultured in sphere formation medium with 100μM of Maraviroc and 10ng/mL of CCL3. The DMSO treated SCC4 were treated with DMSO at a concentration of 0.1% of total volume in well. The combination therapy included 100μM of Marviroc and 10ng/mL of CCL3. The SCC4 treated with Maraviroc had decreased spheroid formation compared to the SCC4 DMSO. The SCC4 treated with the combination had increased spheroids compared to the Maraviroc treated but didn't show as many spheres as the DMSO or DMSO/CCL3 treated. The quantitative representation is showing the number of spheres in SCC4 DMSO, DMSO/CCL3, Maraviroc and combination (Maraviroc and CCL3).
(* $P<0.05$, unpaired two-tailed Student's t test. ** $P<0.01$, unpaired two-tailed Student's t test.)


90
80
70
60
50
40
30
20
10
0
# of Spheres
*
**
DMSO
DMSO/CCL3
Maraviroc
Combination

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