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LOS ANGELES

CHRONIC ALCOHOL EXPOSURE INCREASES GLYCOLYSIS AND CANCER STEMNESS

OF OSCC THROUGH NFAT ACTIVATION

A thesis submitted in partial satisfaction

of the requirement for the degree Master of Science

in Oral Biology

by

Anthony Nguyen

2021

ABSTRACT OF THE THESIS

CHRONIC ALCOHOL EXPOSURE INCREASES GLYCOLYSIS AND CANCER STEMNESS

OF OSCC THROUGH NFAT ACTIVATION

by

Anthony Nguyen

Master of Science in Oral Biology

University of California, Los Angeles, 2021

Professor Ki-Hyuk Shin, Co-Chair

Professor Yong Kim, Co-Chair

Chronic alcohol consumption is associated with cancer development and progression. Previous reports have demonstrated that cancer cell metabolism is heavily involved in the development of cancer, including a side population know as cancer stem cells (CSCs). Therefore, it is generally accepted targeting cancer cell metabolism may provide a new and effective method for the treatment of tumors. There are emerging reports of the effects of alcohol on glucose metabolism, however the effects of alcohol and cancer metabolism and its underlying mechanisms are not fully understood in cancers including OSCC. In this study, we investigated the local effect of alcohol on cancer cell metabolism and its underlying mechanism by which alcohol promotes oral cancer progression. We report chronic alcohol promotes

malignant growth of OSCC. In addition, chronic alcohol treatment increased the rate of aerobic glycolysis. Increased aerobic glycolysis was required for the maintenance of CSC population as glycolysis inhibition suppressed CSC properties. In order to explore the molecular mechanism by which chronic alcohol induces glycolysis and stemness, we investigate the role of NFAT signaling as NFAT is known to be involved in glycolysis and malignant cancer properties. NFATc2 is upregulated in chronic EtOH treatment OSCC and is required for for EtOH mediated glycolysis and maintenance of CSC population. Our study suggests that NFATc2 may be a potential therapeutic target for eradicating CSCs in OSCC by suppressing glycolysis in alcohol related cancer patients.

The thesis of Anthony Nguyen is approved.

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2021

Contents

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a significant cause of cancer morbidity and mortality in the world. It is the sixth most common cancer worldwide with over 50,000 new cases and 10,000 deaths in the United States alone (Siegel, Miller, & Jemal, 2018). The major risk factors of oral cancer include tobacco use, alcohol consumption and human papillomavirus (HPV) (Shin & Kim, 2018) (Rivera, 2015). Cancer is caused by a build-up of genetic, epigenetic, and transcriptional modifications leading to the advancement of key properties of cancer such as metastasis and drug resistance (Nassar & Blanpain, 2016). Early-stage treatment can be managed through surgery and radiotherapy, the main form of treatment. However, recurrence and distant metastasis following treatment is common in OSCC. Therefore, identifying various mechanisms which drive OSCC development and metastasis is critical.

According to the 2019 National Survey on Drug use and Health (NSDUH), 85.6% of respondents drank alcohol at some point in their lifetime. Due to alcohol abuse, 88,000 of people die annually, making alcohol the third-leading preventable cause of death behind tobacco (first) and poor diet (second) (National Institute on Alcohol Abuse and Alcoholism , 2020). Alcohol-related deaths can be attributed to lowered inhibitions and loss of critical judgment. However, one of the most significant diseases linked to chronic alcohol consumption is cancer.

Studies have shown that alcohol induces carcinogenesis in a number of organs such as the breast, colon, and mouth. Ethanol, the distinguishing ingredient in alcohol beverages and its metabolite, acetaldehyde, are classified as carcinogenic to humans (Tabea, 2016). The majority of acetaldehyde in the human body is created during ethanol metabolism. During alcohol

metabolism alcohol dehydrogenase (ADH) processes ethanol into acetaldehyde. Acetaldehyde prevents accurate DNA synthesis and repair, resulting in tumorigenesis (Acetaldehyde, 1985) (IARC, 2009). For instance, chronic administration of acetaldehyde in drinking water results in changes indicated by excessive cell proliferation of the mucosa cells of the upper digestive tract (Re-evaluation of some organic chemicals, hydrazine and hydrogen peroxide., 1999). One mechanism from which acetaldehyde acts as a carcinogen is when acetaldehyde binds rapidly to cellular proteins and DNA which causes morphological and functional impairment of the cell (Seitz, 2010). The formation of stable adducts triggers the incidence of replication errors and mutations in tumor suppressor genes and oncogenes (Fang, 1995). Alcohol was discovered to induce mutations in DNA including hypomethylation and promoter region hypermethylation (Varela-Rey, Woodhoo, Martinez-Chantar, Mato, & Lu, 2013). The regulation of methylation affects the expression of genes involved in cell cycle, DNA repair, and tumor suppression. It is predicted that 3.5% of all cancers within the United States are related to alcohol consumption (Cao & Giovannucci, 2016) (Boffetta, Hashibe, & La Vecchia, 2006). Alcohol consumption, especially in association with tobacco, has been seen as an important risk factor for squamous-cell carcinoma in the oral cavity since the mid-1950s.

About 75% of all oral cancers are associated with some type of alcohol or tobacco use. Regardless of this information, there is very little evidence that humans have adjusted their consumption of alcohol and on the contrary have even increased their alcohol consumption. Alcohol can act as both a local and systemic risk factor in the oral cavity. Consumption of alcohol can increase permeability of oral mucosa and cause epithelial atrophy by dissolving the lipid component of the epithelium (Rivera, 2015).

Cancer cells have exceptional increased metabolic requirements compared to their

noncancerous counterparts. In order to satisfy the energy demanded for rapid growth and

division, the glucose uptake and lactate secretion is dramatically increased. This phenomenon is

known as the "Warburg effect" (Lu, Tan, & Cai, 2015). Compared to mitochondrial respiration,

aerobic glycolysis is an inefficient means of generating ATP. However, the rate of glucose

metabolism through aerobic glycolysis is at such a level higher such that the production of

lactate from glucose occurs 10-100 times faster than the complete oxidation of glucose in the

mitochondria. As a survival technique this makes sense as the tumor microenvironment may

have limited availability of glucose and thus undergo competition for nutrients with stromal

cells and the immune compartment (Liberti & Localsale, 2016). Therefore, understanding

cancer cell metabolism is crucial in order to develop therapies against cancer.

Alcohol has been known to be involved in the process of glycolysis. For example, ethanol

consumption suppressed the activity of glycolytic enzymes in the liver of rats (Duruibe &

Tejwani, 1981). In addition, Ethanol exposure significantly suppressed glycolytic activity as well

as HK1 levels in mouse auditory cells *in vitro* (Kang, Choi, Prk, Lee, & Moon, 2020)*.* Furthermore,

ethanol has been reported to increase glycolysis in rats (Lelevich, 1987). These studies indicate

a role of ethanol on glycolysis. However, the effects of ethanol on glycolysis in human cells,

especially OSCC cells has yet to be investigated.

The effects of alcohol on carbohydrate metabolism are not fully understood. Chronic

alcohol exposure has been demonstrated to disrupt lactate and glucose (Lindberg, Ho, Peyton,

& Choi, 2019). A measurement of glycolysis is low pH due to the acidity of lactic acid secreted

by the cell. In addition, alcohol exposure decreases acidity levels and HK1 expression levels, an enzyme involved in glycolysis, in mouse auditory cells in a dose-dependent manner (Kang, Choi, Prk, Lee, & Moon, 2020). This data indicates that alcohol inhibits glycolysis. However, alcohol has been demonstrated to increase glucose and lactate content in the brain (Lelevich, 1987). In addition, phosphofructokinase and pyruvate kinase activity was elevated within the cortex (Lelevich, 1987). These data indicate that alcohol increases glycolytic activity. Collectively, previous studies indicate a role of ethanol on glycolysis. In order to understand alcohol and its effects on metabolism we must understand the role of alcohol in glycolysis.

Many cancer patients develop cancer recurrence and resistance to therapy. Studies have validated the role of cancer stem cells (CSCs) in OSCC development and maintenance. CSCs are a small population of cells within a tumor, known as "tumor initiating cells." Like normal stem cells, CSCs have the capacity to self-renew, can give rise to different progeny, and utilize common signaling pathways (Shin & Kim, 2018)**.** A popular *in vitro* model to study tumor forming ability of potential cancer stem cells is a tumor-sphere formation assay (Johnson, 2013). Cancer stem cells contain the ability to survive and self-renew in harsh conditions. Thus, cancer cells are grown in serum-free, non-adherent conditions in order to isolate the cancer stem cell population. Size and number of spheroids in culture can be measured in order to determine the cancer stem cells from the overall cell population (Dontu, 2003). They differ from normal stem cells in that their properties include tumorigenicity and chemoresistance. Furthermore, CSCs are thought to generate metastasis (Shiozawa, 2013). CSC-rich injection of human pancreatic cancer cells in mice developed liver metastasis (Hermann, 2007). Many have successfully isolated CSCs using various biomarkers. Studies have shown a distinct difference of

expression of CD44 in CSCs versus their differentiated counterparts (Liang Wang, 2018). In addition, the activity of aldehyde dehydrogenase 1 (ALDH1) can has been used as a CSC marker for many cancers (Clay MR, 2010) (Prince ME, 2007). For example, ALDH1^{HIGH} cancer cells display higher CSC properties compared to ALDH1^{LOW} (Prince MEP, 2016). The combination of CSC marker and metastasis have shown to be linked to poor prognosis and survival. These properties also make help isolate cancer stem cells responsible for the recurrence of tumors. Therefore, it is crucial for understanding and developing effective therapies for cancer.

Alcohol has been known to increase CSC phenotype in cancer. For example, studies have demonstrated that alcohol increased the mammary CSC population both *in vitro* and *in vivo* (Xu, et al., 2016). In addition, alcohol has been reported to increase migration ability in breast cancer cells (Xu, et al., 2016). Furthermore, alcohol-mediated induction of hepatic cancer stem cells has been associated with NANOG, a pluripotent (Keigo Machida 1, 2012). Therapy directed at targeting cancer stem cells may represent a significant strategy to improve cancer therapy. Thus, expanded knowledge of how cancer stem cell biology is regulated by alcohol can prove important for reducing the risk of ethanol-induced tumors.

Studies have shown that CSCs exhibit a higher glycolytic phenotype compared to their differentiated offsprings (Feng, et al., 2014). In CSCs, glucose uptake activates the expression of genes involved in glycolysis such as c-Myc, Glut-1, HK-1, HK-2, and PDK-1, contributing to the CSC population (Liu, Liao, & Tang, 2014). CSC population within ovarian cancer, breast cancer, and lung cancer rely mainly on glycolysis as their preferred metabolic state (Zhou, et al., 2011) (Ciavardelli, 2014) (Liao, Qian, & Tchabo, 2014). Furthermore, glycolysis was utilized more in

CD133 + stem cells in HCC (Song, et al., 2015). In addition, the abnormal dependence on aerobic glycolysis has been linked to drug resistance in cancer therapy. For example, treatment with the glycolytic inhibitor, 2-Deoxy-D-glucose (2-DG) significantly decreased the proportion of cells with CSC phenotype in breast cancer cells (O'Neill, Porter, McNamee, Marrtinez, & O'Driscoll, 2019). Consequently, glycolytic reprogramming in CSC is critical for the maintenance of CSCs and is linked with the development of cancer. Therefore, it is crucial for understanding CSC metabolism in order to develop effective therapies for cancer.

While CSCs mainly depend on glycolysis, several studies indicate that CSCs depend on mitochondrial oxidative metabolism (Lee K. M.-Z.-F., 2017). Slow cycling tumor initiating CSCs have been seen to be less glycolytic, with lower glucose uptake and lactate secretion. However, they contain higher ATP levels than their differentiated progeny this suggests an OXPHOS phenotype (Young Chan Chae, 2018). In addition, CSCs have been reported to contain increased mitochondrial mass and increased oxygen consumption rates. Furthermore, NANOG induces tumorigenesis through metabolic reprogramming to OXPHOS (Chen CL, 2016). The OXPHOS phenotype is negatively associated with tumor necrosis factor receptor-associated protein 1 (TRAP1), a mitochondria chaperone from the HSp90 family. Low expression of TRAP1 follows a high reliance on OXPHOS and is associated with chemoresistance (Nayak, 2018). Increased chemo-resistant ovarian cancer cells display increased OXPHOS activity and even survive in mice when fed 2-deoxyglucose (Dar S., 2017). It is obvious that CSC metabolism has crucial implications for cancer treatment and therefore a potential target in cancer therapy.

Hypoxia-induced factor 1 alpha (HIF1α) is a transcription factor that constantly is synthesized but quickly degraded under normoxic conditions. Hypoxic conditions activate HIF1α (Denko, 2008). Overexpression of HIF1A has been reported in a variety in cancers including, renal, colorectal, breast, cervical, and head and neck carcinomas (Pin-Yi, 2007). The expression of HIF1α has been associated with lymph node metastasis and survival rate and prognosis of cancer (Pin-Yi, 2007). In addition, HIF1α is a crucial regulator of a large number of genes including, metabolism, angiogenesis, and stemness (Tanimoto, 2017). As a critical mediator of the Warburg effect, HIF1α stimulates the expression and activation of glycolytic enzymes such as GLUT1. HK2, and LDHA (Parks, 2017). Furthermore, HIF1α promotes the transcription of proangiogenic cytokines such as vascular endothelial growth factor (VEGF), which plays a crucial role in breast tumorigenesis (Xiao G. , 2019). Targeting HIF1α has been reported to eliminate cancer stem cells in hematological malignancies (Wang, Liu, Malek, & Zheng, 2012). Therefore, targeting HIF1α pathway to inhibit glycolysis and stemness has been considered a potential therapeutic strategy in cancer therapy.

Nuclear factor of activated T-cell (NFATc) is a family of five isoforms (NFATc1-5) which act as transcription factors (Rao, Luo, & Hogan, 1997). Although first identified in immune cells, NFAT signaling is found to be expressed in all cells and tissues, including epithelial cells. Moreover, studies have now shown that all NFAT isoforms play a role in human cancer (Mancini & Toker, 2009). However, studies have shown that NFAT signaling may play a tumor suppressive role demonstrating a conflicting role in different types of cancers (Glud, Sorensen, Wang, Kondo E, & Jessen , 2005) (Robbs & Cruz, 2008). Studies have also shown that NFAT plays a role in the maintenance of cancer stemness in various human cancers (Gerlach, Daniel, &

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Lehr, 2012). NFAT signaling has been demonstrated to play an oncogenic role in cancer by promoting cancer stemness (Lee, et al., 2019). NFAT plays a role in glycolysis as well as contributes to tumor progression and malignancy by promoting cell growth, survival, invasion, and angiogenesis (Muller & Rao, 2010) (Yongsheng Jiang, 2019). Comprehension of the role NFAT signaling plays within tumor development can bring insight in effective therapies against tumor progression.

NFATc2 is linked to many different types of cancer (Perotti, et al., 2012) (Malsy, Graf, & Almstedt, 2019) (Xiao, et al., 2017). Studies show that NFATc2 is upregulated in cancer (Jiang-JiangQin, 2017). Constitutively active expression of NFATc2 has been associated with metastasis and invasion in vitro. In addition, NFATc2 has been reported to play a role in self-renewal capacity in colorectal cancer (Lang, Ding, Kong, & Zhou, 2018). Knockdown of NFATc2 demonstrated a reduction in cell proliferation in pancreatic cancer (Malsy, Graf, & Almstedt, 2019). In addition, chemical inhibition of NFATc2 has been demonstrated to suppress MDM2 oncogene as well as cMYC, a gene known to be involved in cancer stemness (Jiang-JiangQin, 2017). Furthermore, NFATc2 has been reported to enhance CSC phenotype through the NFATc2/Sox2ALDH axis in lung adenocarcinoma (Xiao, et al., 2017). It is obvious NFATc2 specifically is heavily involved in both cancer cells and cancer stem cells. Understanding of the role of NFATc2 within tumor development can have important implications for cancer treatment as NFATc2 is highly involved in CSCs

NFAT signaling is known to be associated with cell metabolism. For example, analyses of common transcription factor binding motifs revealed the presence of consensus NFAT binding

motifs in the regulatory regions of HK2, ENO1, and cMYC (Vaeth M. M.-H.-S., 2017). In addition, NFAT directly controls the expression of GLUT3 and can activate HIF1α expression, a transcription factor involved in glycolysis (Vaeth & Feske, 2018) (Walczak-Drzewiecka , Ratajewski, Wagner, & Dastych, 2008). This shows a direct association of NFAT and glycolysis. However, studies have also demonstrated that NFAT and glycolysis may have an inverse relationship. For example, lactic acid is reported to prevent the upregulation of NFAT signaling (Brand, et al., 2016). Obviously, NFAT signaling is associated with glycolysis. Understanding of the role of NFAT in cell metabolism can help lead to therapeutic solutions in cancer.

Studies demonstrate there is a serious increase in cancer risk with alcohol consumption (Menzes, Bergmann, & Santos Thuler, 2013). With an increasing rate of alcohol consumption, therapeutic treatments must be supported in alcohol progressed cancers. We hypothesize targeting glycolysis within alcohol progressed cancer will suppress the tumor initiating populations within cancer. Therefore, we investigated the local effect of alcohol on cancer cell metabolism and oral cancer stemness as well as its underlying mechanism. Our study found that chronic alcohol treatment increases glycolysis in OSCC as well as cancer stemness. We found that EtOH increased glycolysis is required for cancer stemness properties. Interestingly, HIF1α is not required for EtOH-Induced glycolysis but is necessary for maintenance of CSC population. Furthermore, our study demonstrates that NFATc2 is upregulated in chronic EtOH treatment in OSCC. In addition, we found that EtOH-induced NFATc2 is essential for EtOH mediated glycolysis and maintenance of CSC population. Our study suggests that NFATc2 may be a potential therapeutic target for eradicating CSCs in OSCC by suppressing glycolysis in alcohol related cancer patients.

MATERIALS AND METHODS

Cell Culture - Two human OSCC cell lines, SCC9 and UM6 were cultured in DMEM/F12 (LifeTechnologies) supplemented with 10% SuperCalf Serum (Gemini Bioproducts), 0.4 µg/ml hydrocortisone (Sigma-Aldrich), and 5 µg/ml Gentamycin aminoglycoside antibiotic (Invitrogen). All cell lines were grown in a humidified incubator with 5% CO_2 at 37℃. Cells were treated with Absolute ethanol (200 Proof) obtained from Fisher Bioreagents (BP2818100) by pipetting the ethanol into the cell culture medium. 2-Deoxy-D-glucose was obtained from Sigma Chemical Co. (D8375-1G) (St.Louis, MO, USA) and dissolved in PBS.

Total RNA isolation - Total RNA was isolated from cells using Trizol reagent RNA isolation method. Cell pellet was combined with 1ml of Trizol (Ambion) and 200ul of chloroform and centrifuged at 12,000 RPM for 15 minutes at 4℃. After centrifugation, the aqueous layer containing the RNA was removed while the layers containing the DNA and protein (organic layers) were discarded. Isopropanol (2-propynol) was added to the aqueous layer to form RNA precipitate and wash the RNA. Lastly, the RNA was washed with 70% 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 was around 1.85- 1.95 (260/280) and concentration between 500-800 ng/µL.

Quantitative reverse transcriptase real-time polymerase chain reaction (qPCR) - cDNA was synthesized from 5 µg of total RNA using SuperScript first-strand synthesis system (Invitrogen)

in 30 μl of a reaction mixture. We used 1 μl cDNA per well (in a 10 μl reaction volume) for qPCR

amplification using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). qPCR was

performed using a QuantStudio 3 (Thermo Fisher Scientific). The reactions were conducted by

heat denaturing at 95℃ for 10 minutes, and then 48 cycles of 95℃ for 15 seconds followed by

58℃ for 30 seconds and lastly 72℃ for 45 seconds. mRNA expression 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 manufacturer's

instructions. *B-Actin* was used as a control. The primer sequences were obtained from the

Universal Probe Library.

Primer name	Sequence
B-Actin	Forward: 5' CCAACCGCGAGAAGATGA 3' Reverse: 5' CCAGAGGCGTACAGGGATAG 3'
HIF1α	Forward: 5' TTCACCTGAGCCTAATAGTCC 3' Reverse: 5' CAAGTCTAAATCTGTGTCCTG 3'
NFATc2	Forward: 5' GATAGTGGGCAACACCAAAGTCC 3' Reverse: 5' TCTCGCCTTTCCGCAGCTCAAT 3'

Western blotting - Whole cell extracts were isolated using the lysis buffer (50 mM Tris-HCl, pH

8.8, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid sodium salt). The extracts

were then fractionated by SDS-PAGE and transferred to Protran nitrocellulose membrane

(Genesee) by a semi-dry transfer apparatus (Bio-Rad). The membranes were blocked with 5% non-fat milk in TBST for 30 minutes at room temperature, and then incubated with the primary antibodies diluted in TBST overnight at 4℃. The next day, membranes were washed in TBST for 3 rounds of five minutes and were incubated in their respective secondary antibodies (1:3000) diluted in TBST for one hour at room temperature and then exposed to the chemiluminescence reagent (Amersham) for signal detection. We used the following primary antibodies: anti-NFATc2 (1:500; sc- 4G6-G5; Santa Cruz) (4389; Cell Signaling), anti-HIF1α (1:1000; sc-13515; Santa Cruz), and mouse anti-GAPDH (1:1000; sc-47724; Santa Cruz). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz.

Cell proliferation assay - To determine the growth of cells over time, the cells were seeded with twenty thousand cells per well in a 6-well tissue culture plate in triplicate for each cell type. SCC9 EtOH, UM6 EtOH cells and their controls were then collected at day 2, 4, 6, and 8 after the initial seeding using 250 µl of 0.25% Trypsin-EDTA (ThermoFisher Scientific) and then neutralized with 250 µl of DMEM/F12. The triplicate wells were suspended into a single centrifuge tube. A 10 µl sample of that cell suspension was used to count the total number of cells using a hemocytometer. The average number of cells per well were then calculated. In addition, a tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell proliferation assay kit (ATCC) was used to determine the chemosensitivity of cells. The cells were plated at 2×10^3 cells per well into a 96-well tissue culture plate. They were then incubated in culture medium containing 10, 30, and 50 µM of Cisplatin (Sigma-Aldrich) for 2 days. Absorbance at 570 nm was determined using a microplate reader.

Anchorage-independent growth - To determine anchorage-independent growth ability, 1×10^4 cells were plated in culture medium containing 0.4% agarose over a base layer of serum-free medium containing 0.8% agarose. Three weeks after incubation, colonies were counted. The experiment was performed in triplicates with a 12-well tissue culture plate.

In vivo **xenograft tumor assay** - Cells were collected and washed with 1 X PBS. Prior to injections, five to ten million cells were mixed with 100 µl of Matrigel Basement Membrane Matrix (Corning) and subcutaneously injected into the flank of immunocompromised mice using a 27-gauge needle (strain *nu/nu*, Charles River Laboratories). Six to eight-week-old male mice were used in this study. The assay was performed according to guidelines approved by the Chancellor's Animal Research Committee (ARC#2006-017-01). A total of seven immunocompromised mice were used in this study per experimental group. The kinetics of tumor growth was determined by measuring the volume in three perpendicular axes of the nodules using micro-scaled calipers. Tumor growth measurements were obtained biweekly for a total of 7 weeks. The tumors were harvested at 7 weeks post-implantation.

ALDH1 assay - ALDH enzymatic activity was determined using Aldehyde Dehydrogenase-Based Cell Detection Kit (STEMCELL). One million cells were resuspended in 1 ml of the ALDEFLUOR Assay Buffer. Fluorescent nontoxic ALDEFLUOR Reagent BODIPY (2.5 µl) was added as a substrate to measure ALDH enzymatic activity in cells. Immediately after adding the substrate reagent, 0.5 ml of the cell suspension was transferred into the control tube which contains 2.5

µl of diethylaminobenzaldehyde (DEAB), an ALDH specific inhibitor. Then, cells were incubated

at 37℃ for 40 minutes and fluorescence data acquisition was made by using a BD LSRFortessa

X-20 SORP (BD Biosciences).

Tumor sphere formation assay - Ten thousand cells were grown in 3 ml of serum-free

DMEM/F12 media supplemented with 100X N-2 supplement (Invitrogen), 5 µg/ml Gentamicin

(Fisher Scientific), 5 ng/ml human recombinant epidermal growth factor (Peprotech), and 5

ng/ml human recombinant basic fibroblast growth factor (Peprotech) in Ultra-Low Attachment

6-well plates (Corning) for 6-10 days. The number of tumor spheres formed were observed and

counted under a microscope.

Migration Assay - Cell migration was measured using transwell chambers (Corning) in a 24-well

tissue culture plate. Thirty thousand cells in 100 µl of serum-free DMEM/F12 medium were

placed inside the upper compartment of the transwell chamber (6.5 mm diameter, 8.0 µm pore

size). 600 µl of DMEM/F12 supplemented with 1% serum medium was added to the lower

chamber, which serves as an angiogenic chemoattractant to encourage cells in the upper

compartment to migrate through the transwell membrane. Cells were incubated at 37℃ for 48

hours. The transwell insert was gently washed with 600 µl of 1 X PBS and then fixed with 600 µl

of 3% Formalin for 10 minutes. The insert was stained with 5% Crystal Violet for 90 minutes.

Non-migrated cells in the inner chamber were wiped out and the migrated cells, those that

penetrated to the bottom of the membrane, were counted under the microscope in three

randomly selected viewing fields.

Glucose uptake – 1 x10^4 cells were seeded into 96 well plate 24 hours prior to assay. Cells within the plate were washed with 200ul 1X PBS and harvested. 25ul of PBS and 12.5ul of inactivation solution (0.6N HCl) was added to the well and left on a shaker for 5 mins. 12.5ul of neutralization solution (1M Tris Base) was added to well for 3 mins on shaker. Afterwards 50ul of lactate detection reagent was added on shaker for 1 hour. Glucose uptake activity was measured with a microplate. Measurements were normalized to cell counting.

Lactate secretion – 1 x10^4 Cells were seeded into 96 well plate 24 hours prior to assay. Medium was collected from the plate and diluted 100-fold. The Lactate -Glo assay (Promega) was performed according to the manufacturer's instruction. Lactate secretion activity was measured with a microplate reader. Measurements were normalized to cell counting.

Luciferase reporter assay – 1x10^5 Cells were seeded into 6 well plate; after 24hr, cells were transfected with the pGL3-NFAT-luc vector (500ng) and pSV40RL vector (10ng) for 36 hours. Cells were then harvested and washed with 1X PBS. Protein was isolated with 125ul of Passive Lysis Buffer (Promega), underwent a freeze-thaw cycle, and centrifuged at 12,000rpm for 5 mins. Dual Luciferase Reporter Assay System (Promega) was performed according to the manufacturer's instructions and measured using microplate reader with integration time of 10 secs. Firefly luciferase values were normalized to Renilla luciferase activity using vector pSV40RL (5ng) and protein concentration.

Small Interfering RNA (siRNA) transfection –HIF1α siRNA (sc-35561; Santa Cruz Biotechnology), NFATc2 siRNA (sc-36055; Santa Cruz Biotechnology) and control siRNA (sc-37007; Santa Cruz Biotechnology) were purchased and introduced into cells using Lipofectamine RNAiMAX (Invitrogen #13778100). Cells ($2x10^5$) were plated in 60-mm dishes and transfected with 10ug siRNA. The cultures were harvested after one day post-transfection for expression and functional analyses.

RESULTS

EtOH concentration at 100-150 mM contains no cytotoxic effect in OSCC.

In order to investigate the local effect of EtOH on OSCC, we needed to determine whether EtOH had any cytotoxic effects on the cells (Figure 1). Oral cancer is two to three times more prevalent in men than women in most ethnic groups (Rivera, 2015). Cell lines SCC9 and UM6 are male tongue cancer cell lines known for their low tumorigenic potential. We found that EtOH relatively had no cytotoxic effects except at a concentration above 200 mM for both cell types. This finding allowed us to safely treat OSCC with a concentration of 150 mM and below in order to investigate the effect of EtOH on OSCC.

Acute EtOH exposure has no significant impact on glycolysis in OSCC (UM6).

We also investigated the effect of EtOH exposure on glycolysis in OSCC. In order to characterize glycolytic phenotypes we measured the glucose uptake and lactate secretions of the cells. We treated UM6 with EtOH concentrations of 0, 10 mM, 50 mM, 100 mM, and 200 mM for 24 and 48hrs and measured the glucose uptake rate (Figure 2A). We found that EtOH at various concentrations had a minimal effect on the glucose uptake rate. In addition, we measured the lactate secretion of the cells in similar conditions (Figure 2B) and found similar results, that acute EtOH treatment had minimal effect on the lactate secretion. These findings indicate that acute EtOH exposure has no significant impact on glycolysis in OSCC.

Chronic EtOH exposure increases OSCC growth *in vitro* and *in vivo*.

Having seen minimal effects of acute EtOH on OSCC, we further investigated the effect

of chronic EtOH exposure in OSCC. Chronic EtOH conditions were established by culturing OSCC

in media containing gradually increasing concentrations starting with 50 mM to 150 mM EtOH

for 15 weeks (Figure 3). Although 20 mM of EtOH represents 0.08% blood alcohol

concentration, a concentration of 150 mM EtOH will test the direct effect of alcohol on oral

cancer. After 15 weeks, we recognized the growth rate of the chronically treated cells had

increased. In order to confirm this, we compared the cell proliferation by cell counting with

untreated control SCC9, EtOH-treated SCC9 (SCC9/EtOH), untreated UM6, and EtOH-treated

UM6 (UM6/EtOH). SCC9 and UM6 were chosen as they are known to be low tumorigenic

squamous cell carcinoma and contain low self-renewal capacity. For both cell lines we found

that chronic EtOH treatment significantly increased their proliferation rates (Figure 4A). Eight

days after the initial seeding of twenty thousand cells, SCC9/EtOH had grown up to 2,500,000

cells which was significantly more than SCC9 which had grown to 700,000. Similarly, eight days

after initial seeding, UM6/EtOH had grown up to 1,500,000 cells which was significantly more

compared to UM6 which had grown to 500,000 cells. In addition, we investigated the effect of

chronic EtOH treatment on tumorigenicity of OSCC *in vitro* by performing soft agar assay. An

important feature of malignant cancers is their ability to sustain anchorage-independent

growth (Mori, et al., 2009). Chronic EtOH treatment significantly increased the number of

colonies form in soft agar for both UM6/EtOH and SCC9/EtOH (Figure 4B). Furthermore,

anchorage-independent growth is associated with tumorigenic potential in vivo, we

investigated the effect of chronic EtOH treatment on tumorigenicity of OSCC *in vivo* by

performing xenograft tumor assay in nude mice (Figure 4C). We subcutaneously injected

SCC9/EtOH and SCC9 into 8 nude mice and monitored their growth of the xenograft assay for over 7 weeks. Comparatively, SCC9/EtOH formed a larger tumor mass compared to its control. Interestingly, after four weeks the tumor mass began to regress over time. Similarly, UM6/EtOH and UM6 were injected into 7 nude mice each. UM6/EtOH formed a larger tumor mass over time compared to UM6 and after 2 weeks both UM6/EtOH and UM6 cells tumor mass began to regress with UM6/EtOH with the larger mass. These findings collectively indicate that chronic EtOH treatment increases OSCC growth *in vitro* and *in vivo.*

Chronic EtOH exposure increases glycolytic phenotype in OSCC.

Rapid cell division is a hallmark trait of cancer and in order to compensate for the energy required by cell division it is possible increased cell metabolism is developed. In order to investigate whether the alcohol-induced OSCC growth was caused by increased cell metabolism we investigated the effect of chronic EtOH treatment on glycolysis in OSCC. First, we measured the glucose uptake of the chronically EtOH exposed OSCC (Figure 5A). We found that chronic EtOH exposure of OSCC increased glucose uptake. In addition, we measured the lactate secretion of the chronic EtOH treated OSCC (Figure 5B) and discovered an increase in lactate secretion. To further confirm the effect of chronic EtOH exposure on glycolysis in OSCC we measured the relative mRNA levels for the glycolytic genes within the OSCC (Figure 5C). Although, a few glycolytic enzymes were decreased HK1, HK2, and LDHA, overall glycolytic genes in OSCC were increased with chronic EtOH treatment. Taken together, our findings indicate that chronic EtOH exposure to OSCC elevated glycolytic phenotype.

Chronic EtOH exposure increases CSC properties in OSCC.

Discovering increased glycolytic characteristics of our chronic EtOH treated OSCC, metabolism could play an important role in CSC and thus we investigated the effect of chronic EtOH exposure on CSC properties. *ALDH1* has been widely used as a marker for isolating CSCs. Therefore, we measured the gene expression of CSC biomarker, *ALDH1* (Figure 6A). We discovered that long term EtOH treated cells had upregulated *ALDH1* gene expression. In addition, using flow cytometry we measured the CSC-like population by measuring the ALDH1^{High} population within the OSCC (Figure 6B). SCC9 EtOH had a 45% increase in ALDH1^{High} population compared to SCC9 control. In addition, UM6 EtOH had 300% increase in ALDH1^{High} compared to its control. Chronic EtOH treatment OSCC had a higher *ALDH1^{high}* population compared to their control counterparts. To further validate our results, we did functional studies to confirm the effect of chronic EtOH treatment on CSC properties in OSCC. The key feature of CSCs is their ability to self-renew. CSC self-renewal capacity is known to be a driving force for the initiation and maintenance of tumorigenicity (O'Brien, Kreso, & Jamieson, 2010). We conducted tumor sphere formation assay with the EtOH treated OSCC and their control cells (Figure 6C). The EtOH treated OSCC demonstrated increased tumor sphere formation compared to their control. In addition to sphere forming ability, isolated CSCs have displayed increased migration ability. To test the effect of EtOH treatment on metastatic capability *in vitro*, we performed the transwell migration assay (Figure 6D). EtOH treatment significantly increased the number of migrated cells in both OSCC cell lines. Therefore, we concluded that chronic EtOH increases CSC population and phenotype in OSCC.

Glycolytic inhibition suppresses CSC properties in EtOH-treated OSCC

In order to investigate the association between EtOH-induced glycolysis and CSC phenotype we used 2-Deoxy-D-glucose (2-DG), a glycolytic inhibitor. 2-DG acts as a glucose mimic and has the 2-Hydoxyl group replaced by hydrogen. This replacement causes competitive inhibition of hexokinase which results in the inhibition of glucose metabolism. We measured the lactate secretion of the OSCC to confirm the properties of 2-DG. Lactate secretion in both chronic EtOH-treated cells was suppressed in a dose-dependent manner (Figure 7). Therefore, we concluded that 2-DG inhibited the EtOH-induced glycolysis in OSCC.

We continued to investigate the association between glycolysis and cancer stemness. To test the effect of glycolytic inhibition on self-renewal ability in EtOH treated OSCC, sphere forming assay was performed with 2-DG treatment (Figure 8A). The assayed displayed a significant reduction in self-renewal capacity in a dose-dependent manner. We also examined the effect of 2-DG on metastatic capability in EtOH treated OSCC (Figure 8B). The assay displayed a significant reduction in migration capability in a dose-dependent manner. Therefore, the inhibition of glycolysis inhibits EtOH increased CSC phenotypes in OSCC.

Chronic EtOH treatment upregulates HIF1α expression in OSCC

In order to explore the underlying mechanism by which chronic EtOH treatment increases glycolysis and cancer stemness, hypoxia-inducible factor 1-alpha (HIF1-α) is a transcription factor known to be involved in both glycolysis and stemness. We explored the effect of chronic EtOH treatment in HIF1α expression (Figure 9A and Figure 9B). We found that

chronic EtOH treatment increased both mRNA and protein expression of HIF1α. This data

indicates that HIF1α is be a possible candidate for the EtOH induced glycolysis and stemness.

HIF1α knockdown does not affect glycolysis of EtOH-treated OSCC

To investigate the role of HIF1α in glycolysis of EtOH-treated OSCC, we suppressed gene

expression of HIF1α using siRNA (Figure 10A). We transfected SCC9/EtOH and UM6/EtOH with

siRNA plasmid that precisely prevents the translation of HIF1α my degrading the mRNA after

transcription. We confirmed the knockdown of HIF1α of SCC9/EtOH and UM6/EtOH by

measuring the mRNA levels of HIF1α. After confirmation, we measured glycolysis by measuring

glucose uptake (Figure 10B) and lactate secretion (Figure 10C) as glycolytic phenotypes.

Interestingly, our results displayed that HIF1α knockdown had minimal effect on glycolysis. This

data indicates that HIF1α, a master transcription factor for glycolysis played an inconsistent role

in the EtOH induced glycolysis in OSCC.

HIF1α is required in CSC-phenotype of EtOH-treated OSCC.

Although it seemed HIF1α failed to suppress glycolysis of EtOH-treated OSCC, we

continued to explore whether it played a role in cancer stemness. We measured HIF1α

knockdown cell's ability to form spheres (Figure 11A). Interestingly, HIF1α knockdown

suppressed the sphere forming ability by more than 50%. In order to further validate HIF1α's

role in cancer stemness we measured the migration ability in the knock down cells (Figure 11B).

Our results displayed a 70% decrease in migration ability in HIF1α knockdown cells. These data

indicate that HIF1α plays a role stemness but not glycolysis.

NFAT signaling is upregulated in EtOH-treated OSCC

In order to continue exploring the underlying mechanism by which chronic EtOH

treatment increases glycolysis and cancer stemness, we investigated the role of NFAT signaling

in the EtOH-induced phenotypic changes in OSCC. Since, activation of NFAT is known to be

involved in both cancer cell metabolism and migration, it could be a potential candidate.

Therefore, we investigated the effect of chronic EtOH exposure on NFAT signaling using NFAT

reporter vector pGL3-NFAT (Figure 12A and 12B). The vector contained 3x NFAT binding

sequence upstream of the luciferase reporter. Therefore, activation of NFAT signaling drives

expression of the luciferase gene which can be detected (Panomics, 2008). Our results

demonstrated that NFAT signaling in both chronic EtOH treated OSCC increased by at least 7-

fold. Furthermore, we investigated the effect of chronic EtOH in NFAT signaling in order to

narrow down the isoform (Figure 12C). Our results demonstrated a significant upregulation of

NFATc2 and downregulation of NFATc1 in UM6/EtOH. Interestingly, we found no significant

change in NFATc3 expression as well. In addition, NFATc1, NFATc2, and NFATc3 were increased

in SCC9/EtOH. Therefore, NFATc2 was the likely candidate to explore the molecular mechanism

of EtOH induced glycolysis and stemness in OSCC.

NFATc2 is required on glycolysis of EtOH-treated OSCC

To investigate the role of NFATc2 in EtOH-induced glycolysis we performed knockdown

of NFATc2 (Figure 13A) and measured the glycolytic phenotype of the EtOH treated OSCC. We

measured the glucose uptake of the EtOH OSCC (Figures 13B). We found the inhibition of

NFATc2 suppressed the glucose uptake ability of the EtOH OSCC. In addition, we measured the lactate secretion of chronic EtOH OSCC (Figure 13C). We found that NFATc2 knockdown had suppressed lactate secretion as well. Therefore, this data indicates that EtOH induced NFATc2 is required for increased glycolysis activity.

NFATc2 is required for EtOH induced cancer stemness

To further explore NFATc2's role in EtOH induced cancer stemness, we measured the sphere forming ability in the OSCC (Figure 14A). We discovered when NFATc2 is knockdown the sphere forming ability is also suppressed. In addition, we measured the migration ability (Figure 14B). We report that when NFATc2 is knocked down so is the migratory ability of the cells. This data indicates that NFATc2 is required for EtOH induced stemness.

NFATc2/PKM2 axis is activated by chronic EtOH exposure in OSCC and enriched in CSC populations.

In order to determine the molecular mechanism by which NFATc2 regulates glycolysis and cancer stemness in chronic EtOH treated, we knocked down NFATc2 (Figure 13A) and measured the glycolytic genes that were overly expressed in the chronic EtOH treated OSCC (Figure 15A) such as GPI, TPI, ENO1, and PKM2. Our results demonstrated that knockdown of NFATc2 suppressed GPI, TPI, ENO1, and PKM2, all glycolytic genes that were once enhanced by chronic EtOH treatment. PKM2 is a known glycolytic gene associated with self-renewal capacity and metastasis (Xiao, et al., 2017). Therefore we hypothesize that chronic alcohol increased NFATc2 may regulate glycolysis and stemness through PKM2. In order to explore the

relationship between NFATc2 and PKM2, we investigated the correlation of their expression in 14 different squamous cell carcinoma. Our results collected a value of R^2= 0.596, meaning 59.6% of the time NFATc2 may cause expression of PKM2. This demonstrates that NFATc2 may be associated with PKM2. To further validate the NFATc2/PKM2 axis, we investigated the expression of NFATc2 and PKM2 in CSC populations vs non-CSC populations (Figure 15C). NFATc2 and PKM2 had increased expression in tumorspheres compared to monolayer, except for Fadu which had a decrease in NFATc2 expression in tumorsphere population. NFATc2 was reported to promote cancer resistance (Xiao, et al., 2017). To further validate the expression of NFATc2 and PKM2 in CSC populations, we explored the expression levels of NFATc2 and PKM2 in cisplatin-resistant SCC4 (CR-SCC4) (Figure 15D). Our results exhibit CR-SCC4 cells have increased self-renewal capacity, demonstrating a higher CSC-like population. In addition, increased expression of both NFATc2 and PKM2 further validate that NFATc2/PKM2 axis is enriched in CSC population. Therefore, this data indicates that NFATc2/PKM2 is activated by chronic EtOH exposure in OSCC and enriched in CSC populations.

DISCUSSION

The objective of this study is to investigate the effect of alcohol on cancer cell metabolism and oral cancer stemness. Our study revealed chronic EtOH increased cell proliferation. Next, we demonstrated that chronic EtOH exposure increased glycolysis in OSCC. In addition, chronic EtOH exposure increased cancer stemness in OSCC. Using 2-DG, we demonstrated that glycolysis is necessary for the maintenance of cancer stemness in EtOH induced OSCC. Importantly, we demonstrated that NFATc2 is the dominate isoform involved in chronic in OSCC. Using siRNA, we demonstrated that NFATc2 is required to maintain glycolysis of EtOH induced OSCC. The suppression of NFATc2 inhibited glucose uptake and lactate secretion. Finally, we demonstrated that NFATc2 is required to maintain cancer stemness. The transcriptional suppression of NFATc2 led to abolishment of self-renewal capacity. In addition, knockdown of NFATc2 reduced migration ability. Our study has important significance for developing advanced treatments of OSCC by targeting glycolysis and cancer stemness by means of NFATc2 activity.

An EtOH concentration of 20 mM represents a blood alcohol concentration of 0.08%. Although the use of 150 mM of EtOH can be deemed high, in order to investigate the direct effect of EtOH a higher concentration was used in order to represent the alcohol in the oral cavity as opposed to alcohol in the blood. Obviously, alcohol consumption is not as long as a duration of 3 months. However, in order to ensure the effects of chronic alcohol were not fleeting we selected cells that were tolerant to the concentration and duration of the ETOH.

Alcohol has been reported to increase the cell proliferation of cancer (Singletary, Frey, & Yan, 2001). Our study demonstrated that chronic alcohol exposure increased the growth rate of OSCC *in vitro* and *in vivo*. Interestingly, the tumor regressed overtime. Although, the chronic EtOH treated cells created a larger volume overall, the tumorigenic potential of the cell lines may have been too weak *in vivo*. Reprogrammed cell metabolism is a hallmark of cancer (Hanahan & Weinberg, 2011). In the presence of oxygen, most differential cells primarily metabolize glucose through oxidative phosphorylation for a total of 36 ATP as opposed to aerobic glycolysis which produces 4 ATP (Jones & Bianchi, 2015). Cancer cells with a high proliferation rate tend to produce glucose through aerobic glycolysis rather than energy efficient oxidative phosphorylation, even in the presence of oxygen and fully functioning mitochondria. The rate of glucose uptake and lactate production is dramatically increased. This phenomenon is known as the Warburg effect (Liberti & Localsale, 2016). In concurrence with the Warburg Effect, our study demonstrated that the EtOH treated OSCC with higher growth rate indeed had an increased glycolytic phenotype compared to untreated OSCC. Thus, we hypothesized chronic EtOH treated OSCC enhanced glycolytic phenotypes. In order to further validate that chronic EtOH treated OSCC enhances glycolytic phenotypes, we must ensure that oxidative phosphorylation activity is inverse to glycolytic activity.

CSCs have been identified in a wide range of cancers, especially OSCC (Shin & Kim, 2018). Enhanced ALDH activity is a common marker for CSC population (Abdullah & Chow, 2013). Through flow cytometry, we revealed that chronic EtOH treatment increased ALDH[high] population. In addition, CSCs contain characteristics similar to normal stem cells such as their self-renewal ability. Therefore, they are considered to play a major role metastasis,

tumorgenicity, and recurrence and thus are regarded as the source of the cancer. Through our study, we demonstrated that chronic EtOH treatment increased the self-renewal capacity of the OSCC as well as increased the migratory ability. Thus, we hypothesized that chronic EtOH treatment enhanced cancer stemness in OSCC. A suggested method in which CSC evade chemotherapy is through ALDH activity (Abdullah & Chow, 2013). To further validate that chronic EtOH treatment enhances cancer stemness in OSCC, we must perform chemoresistance assays.

The Warburg effect is a unique feature of cancer cells (Schwartz, Seyfried, Alfarouk, Moreira, & Fais, 2017). However, cancer tumors are not uniform but heterogeneous and contain a subset population with stemness abilities. The differentiated counterpart may rely primarily on glycolysis; however, CSCs may rely on either glycolysis or OXPHOS depending on the cancer type (Sancho, Barneda, & Heeschen, 2016). Although chronic alcohol treatment led to acquisition of increased glycolysis and CSC-like properties, the underlying mechanism was not understood. Studies have demonstrated the relationship between aerobic glycolysis and CSC (Xu, et al., 2016) (Ciavardelli, 2014). Glycolytic inhibition, using 2-DG, is known to target glycolysis and cancer stem cell phenotype (O'Neill, Porter, McNamee, Marrtinez, & O'Driscoll, 2019). In agreement, our study demonstrated that 2-DG inhibited glycolysis and multiple cancer stem cell phenotypes such as self-renewal capacity and migration ability in chronic EtOH treated cells. Previous reports have demonstrated that CSCs have an increased dependence on glycolysis compared to their differentiated counterparts (Zhou, et al., 2011)

HIF1α, a transcription factor, is known to be upregulated in hypoxic conditions (Peng, 2018). Hypoxia has been linked to the Warburg effect and therefore HIF1α modulates glycolysis

by increasing glucose uptake and lactate secretion (Vaupel, 2019). Our study demonstrated that HIF1α expression is upregulated in chronic EtOH exposed OSCC. However, our study demonstrated that HIF1α is not involved in glycolysis of chronic EtOH treated OSCC. Knockdown of HIF1α did not suppress glucose uptake and lactate secretion. Our results conflicted with (Denko, 2008) and may be due to genetic differences in our cell types; however it is reported that loss of HIF1α does not impact glycolysis under hypoxic conditions (Wierenga, et al., 2019). This may be a possible explanation as chronic ethanol treatment has been reported to cause hypoxia (Arteel, Limuro, Yin, Raleigh, & Thurman, 1997). Therefore, we hypothesized that HIF1α is not involved in glycolysis in chronic EtOH treated OSCC.

In addition, hypoxia occurs in the majority of solid tumors, and therefore, HIF1α is highly expressed in a variety of human cancers. Increased expression of HIF1α is linked to high metastasis as well as poor prognosis (Semenza, 2010). Our study demonstrated that chronic EtOH treatment in OSCC upregulated HIF1α expression. In addition, HIF1α knockdown suppressed self-renewal capacity of chronic EtOH OSCC. This suggested that suppression of HIF1α is associated with diminished CSC-like population. Furthermore, HIF1α knockdown demonstrated lowered migration ability in chronic EtOH OSCC. HIF1α knockdown inhibited CSC phenotype in chronic EtOH OSCC. This indicated that HIF1α is required for cancer stemness maintenance in chronic EtOH OSCC. In breast cancer stem cells, CD44+/CD24- populations demonstrate stem-like characteristics (Brooks, Burness, & Wicha, 2015). In addition, HIF1α is associated with CD44+/CD24- populations (Oliveira-Costa, et al., 2011). In order to further validate, HIF1α is linked to cancer stemness, we must distinguish the CD44+/CD24- population with HIF1α.

NFAT signaling contributes an oncogenic role in tumorigenesis (Mancini & Toker, 2009). NFATc3 was highly expressed in gastric cancer and promoted tumor progression (Zhang, Kang, & Zhang, 2017). Interestingly, other studies have displayed suppressive activity of NFATc2 and NFATc3 (Glud, Sorensen, Wang, Kondo E, & Jessen , 2005) (Robbs & Cruz, 2008). This suggests that NFAT signaling contributes to different types of cancer depending on the context. Our study demonstrated that NFAT signaling is activated by chronic EtOH-treated OSCC cells. Therefore, it must be determined which NFAT isoform is prominent in chronic EtOH treated OSCC and its role in glycolysis and oral cancer stemness. Previous reports indicate that deletion of NFATc2 strongly impaired glycolytic gene expression (Vaeth M. M.-H.-S., 2017). Our results displayed that NFATc2 is upregulated in chronic EtOH treated OSCC. Transcriptional knockdown of NFATc2 in OSCC significantly diminished glycolytic properties such as glucose uptake and lactate secretion. According to our results, we hypothesized NFATc2 may directly regulate PKM2, a key enzyme in glycolysis. Studies have demonstrated that knockdown of PKM2 decreases lactate production and therefore glycolysis (Yang, et al., 2014). Thus, further investigation is required to fully understand the role of PKM2 in NFATc2 mediated glycolysis in chronic EtOH treated OSCC.

Furthermore, functional studies have indicated NFATc2 silencing significantly suppressed sphere forming ability along with migration ability (Xiao, et al., 2017). Furthermore, NFATc2 is known to be involved with oncogenes. For example, NFATc2 activates MDM2 oncogene, promoting breast cancer cell metastasis (Zhang, Zhang, & Cheng, 2012) along with overexpression of NFATc2 activates c-Myc (Muller & Rao, 2010). Knockdown of NFATc2 in chronic EtOH OSCC cells suppressed stemness properties such as self-renewal capacity and

migration ability. These observations led us to hypothesize that NFATc2 is critical for maintenance of oral CSCs in EtOH induced OSCC; however, further investigation will be necessary to determine the functional role of NFAT signaling in oral CSCs. For example, NFATc2 was reported to be involved in self-renewal, chemoresistance, and tumorigenesis in lung adenocarcinoma (Xiao, et al., 2017). Interestingly, we reported HIF1α inhibition failed to suppress glycolysis but continued to suppress cancer stemness. It is possible that HIF1α is downstream of the signaling pathway of NFATc2. Activated mast cells have been reported to upregulate HIF1α mediated by NFAT (Walczak-Drzeiecka, Ratajewski, Wagner, & Dastych, 2008). In addition, *in silico* analyses of common transcription factor binding motifs revealed the presence of consensus NFAT binding motifs in the regulatory regions of HIF1α (Vaeth M. M.-H.-S., 2017). Therefore, we hypothesize that NFATc2 may be a possible mediator of HIF1α. Furthermore, reports have demonstrated that PKM2 is associated with CSC phenotypes such as metastasis and chemoresistance (Bian, et al., 2018) (Shi, et al., 2018). Therefore, further investigation is necessary to understand the role of PKM2 in NFATc2 enhanced cancer stemness in chronic EtOH treated OSCC.

In order to further explore the molecular mechanism by which chronic ETOH exposure regulates glycolysis and stemness in OSCC, we believe PKM2 may be another potential candidate to investigate as NFATc2 knockdown suppressed PKM2 expression. In breast cancer, PKM2 has been reported to be involved in both CD44^{HIGH}/CD24^{LOW} populations along with self-renewal capacity (Zhao, et al., 2015). Our data indicated that NFATc2/PKM2 axis is enriched in CSC populations. In addition, silencing of PKM2 has been demonstrated to enhance apoptosis and cell differentiation (Morfouace, et al., 2014). Preliminary investigation of PKM2 to be a

potential candidate demonstrated that NFATc2 consensus binding site (5'- GGAAA -3') is found

four times when looking -756 bp, -1092 bp, -1514 bp, and -1756 bp upstream from the start

codon using the complete coding sequence provided by GenBank (Ref code: AY352517.1)

(Badran, Wolinsky, Burny, & Willard-Gallo, 2002) (Macian, Lopez-Rodriguez, & Rao, 2001)

(Rieder, et al., 2003). In addition, our results demonstrated a high correlation between NFATc2

and PKM2 expression in various human SCC cell lines. This led us to believe that PKM2 is a

strong candidate that may be regulated by chronic EtOH-induced NFATc2. Further investigation

is necessary to fully understand the role of PKM2 as potential candidates in chronic EtOH

regulation of glycolysis and stemness through NFATc2.

Future studies must be conducted in order to further investigate the effect of chronic

alcohol consumption on oral cancer progression. Many unexplored avenues arise as the study

progresses such as the effect of chronic alcohol consumption *in vivo.* We plan on investigating

the effect of chronic EtOH in a chemical-induced tongue cancer mouse model. Studies have

demonstrated that 4-nitroquinoline-1-oxide (4NQO) carcinogenesis is an appropriate mouse

model of OSCC (Hawkins, et al., 1994). In addition, a study has demonstrated that ethanol

promotes oral carcinogenesis of the 4NQO mouse model (Guo, Wang, Zhang, Sun, & Chen,

2011). In addition, in order to further validate the role of alcohol on cancer progression, we

must investigate the effects of alcohol on oral precancerous cell lines. For example,

immortalized non-tumorigenic oral epithelial cell lines NOKSI and HOK-16B can be an

appropriate model to explore the carcinogenic potential of alcohol (Lee, et al., 2019). We plan

on investigating the effects that alcohol exposure can have on cell metabolism and whether

malignant properties can be induced.

In conclusion, our findings confirmed that chronic EtOH has a significant impact on glycolysis and cancer stemness in OSCC cells. Our results revealed a molecular mechanism wherein NFATc2, a major transcription factor, is upregulated and required for glycolysis and stemness in chronic EtOH-treated OSCC cells. Therefore, we speculate that the challenge of OSCC cells with chronic alcohol consumption may further increase the glycolytic and cancer stem-like phenotype which would promote the malignancy of OSCC (Figure 16). These findings will be helpful in further identifying molecular mechanisms associated with alcohol-induced progression of oral cancer.

FIGURE LEGENDS AND FIGURES

Figure 1. EtOH treatment at 100-150 mM had no cytotoxic effect on OSCC.

The cytotoxic effect of alcohol was determined by MTT assay. SCC9 and UM6 were treated with the indicated EtOH concentrations for 3 and 7 days, and subject to MTT assay. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's t test.

SCC9

UM6
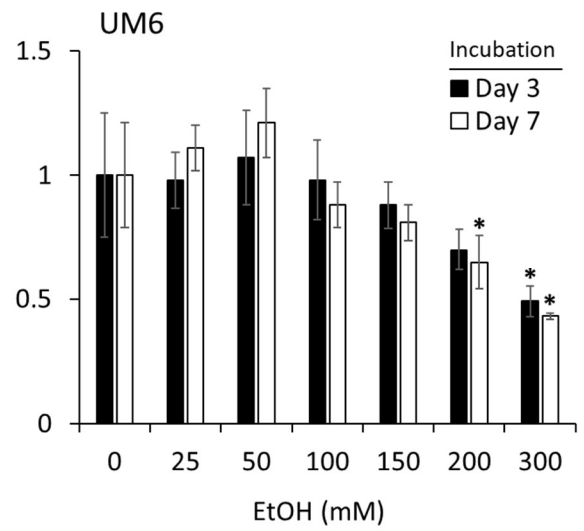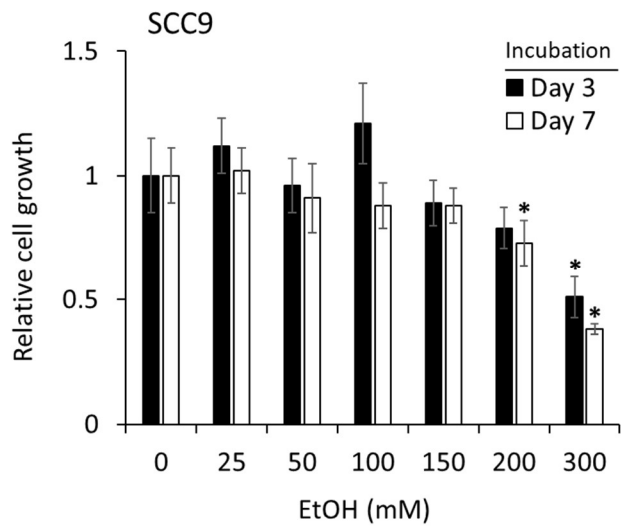
Figure 2. Acute EtOH exposure had no significant impact on glycolysis in OSCC.

(**A**) Effect of acute EtOH exposure on glucose uptake in OSCC was determined by glucose-glo assay. 10,000 cells were incubated in various concentrations of EtOH (0-200 mM) for 24 and 48hrs, and glucose update was measured. Data are means ±SD of triplicate experiment. (**B**) Effect of acute EtOH exposure on lactate secretion in OSCC was determined by lactate-glo assay. 10,000 cells were incubated in various concentrations of EtOH (0-200 mM) for 24 and 48hrs, and glucose update was measured. Data are means ±SD of triplicate experiment.

A
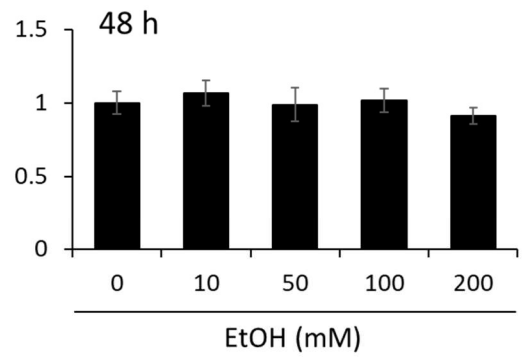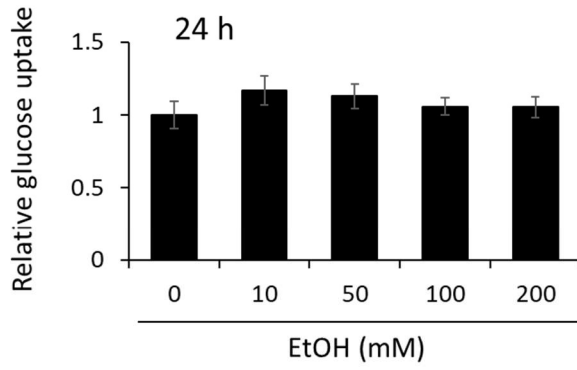
B
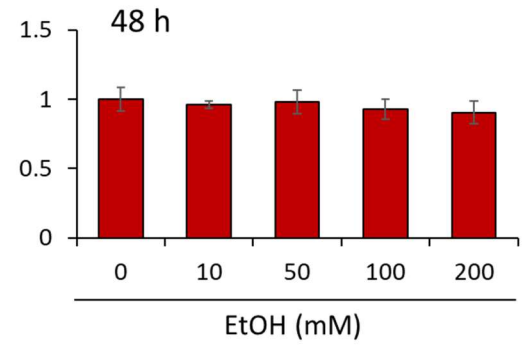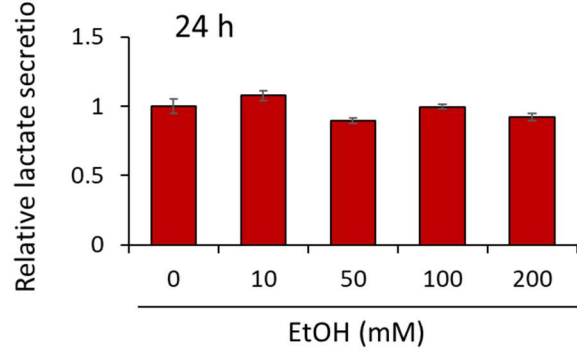
Figure 3. Experimental design for chronic alcohol exposure in vitro.

In order to mimic chronic alcohol exposure, SCC9 and UM6 were cultured in media containing gradually increasing EtOH concentrations (50 mM to 150 mM) for 15 weeks. The medium was changed every 3-4 day. After 15 weeks, we observed an increased cell proliferation. When increased cell proliferation was confirmed, we named chronic EtOH-treated SCC9 as SCC9/EtOH and chronic EtOH-treated UM6 as UM6/EtOH

SCC9 and UM6
Untreated cells

SCC9/EtOH and UM6/EtOH
Chronic EtOH treated OSCC

EtOH treatment (50 ~ 150 mM)
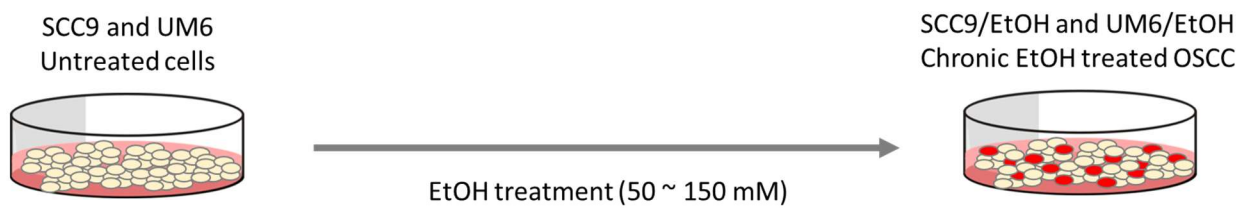
SCC9/EtOH and UM6/EtOH cells were established by culturing
SCC9 and UM6, respectively, in media containing gradually
increasing concentrations starting from 50 mM to 150 mM
EtOH for 15 weeks

Figure 4. Chronic EtOH exposure increased OSCC growth *in vitro* and *in vivo*.

(A) Effect of chronic EtOH treatment on cell proliferation was determined by cell counting. Data are means ± SD of triplicate experiments. *$P < 0.001$ by two-tailed Student's t test. **(B)** Effect of chronic EtOH on anchorage-independent growth ability of OSCC was determined by soft agar assay. Representative images of anchorage-independence assay were shown on the right of each bar graph. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's t test. **(C)** Effect of chronic EtOH on in vivo tumorigenicity was determined by xenograft tumor assay. 20,000 cells were injected subcutaneously into 5 nude mice. Tumor sizes were measured twice a week for 7 weeks. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's t test.
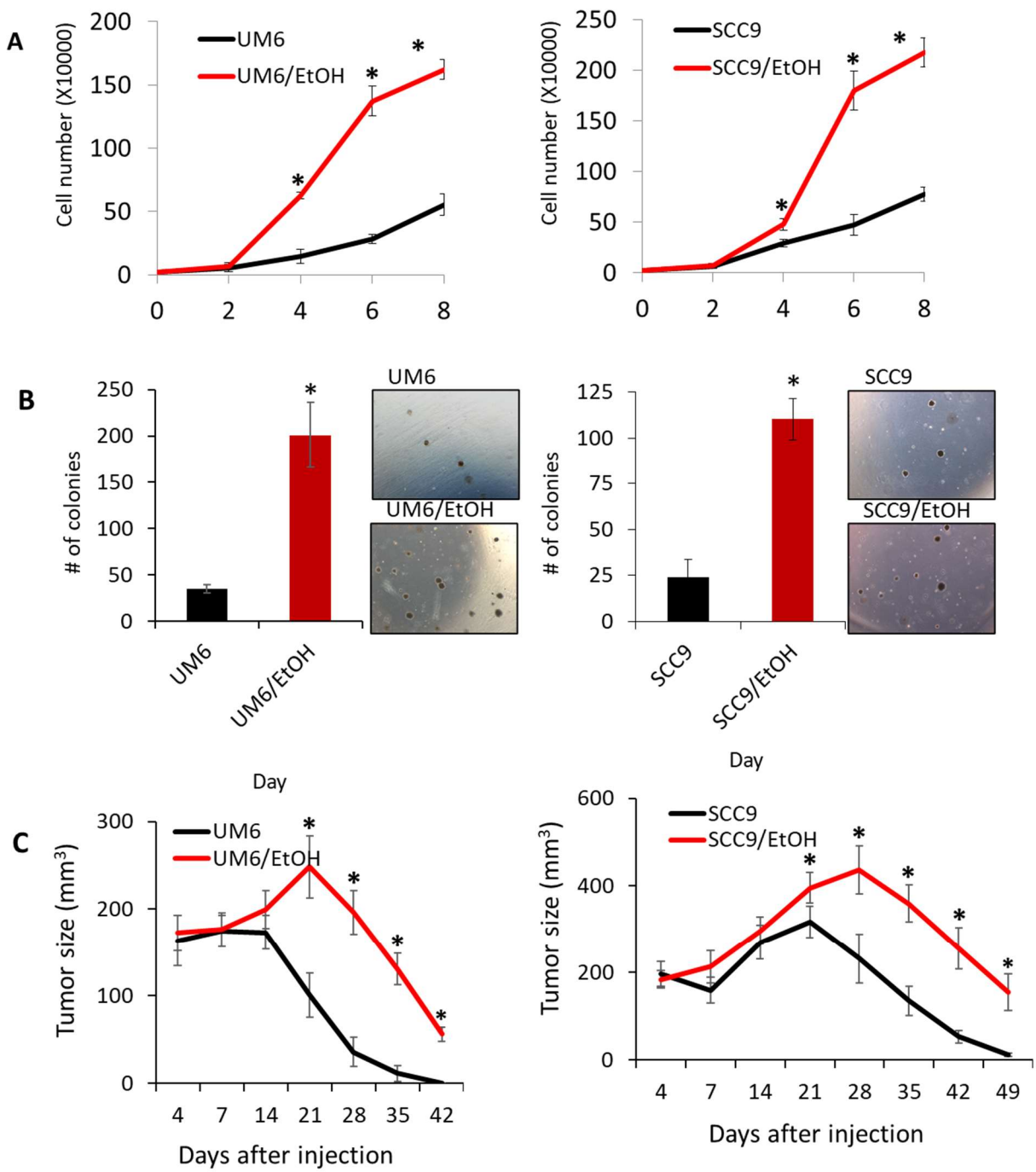
Figure 5. Chronic EtOH exposure increased glycolytic phenotype in OSCC.

(A) Effect of chronic EtOH exposure on glucose uptake was determined by glucose-glo assay. Data are means ±SD of triplicate experiment. * P < 0.05 was determined by two-tailed Student's t test. **(B)** Effect of chronic EtOH exposure on lactate secretion was determined by lactate-glo assay. Data are means ±SD of triplicate experiment. * P < 0.05 was determined by two-tailed Student's t test. **(C)** Effect of chronic EtOH exposure on expression of glycolytic genes was determined by qPCR. Data are means ±SD of triplicate experiment. * P < 0.05 was determined by two-tailed Student's t test.
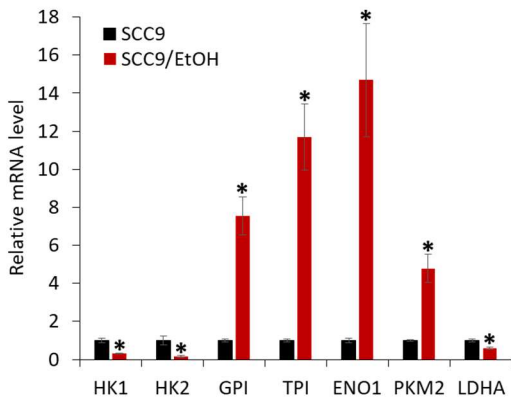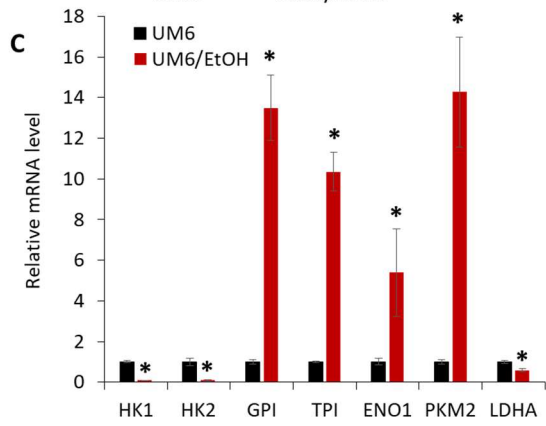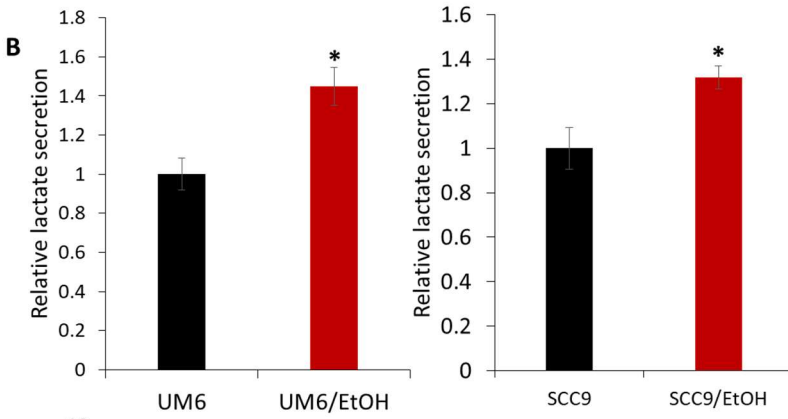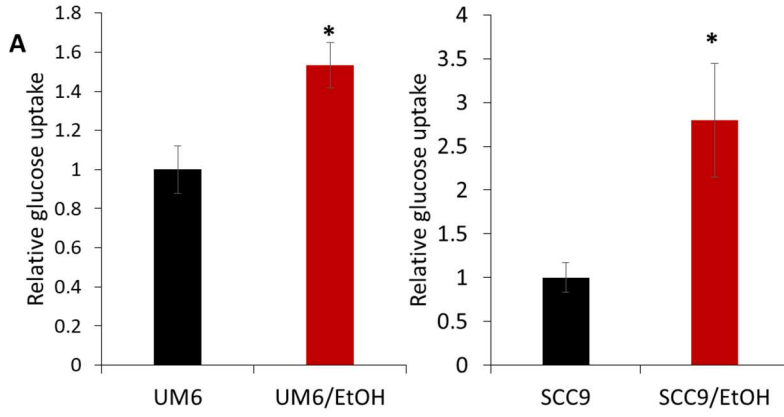
Figure 6. Chronic EtOH exposure increased CSC properties in OSCC.

(A) Effect of chronic EtOH exposure on ALDH1 expression by qPCR. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's t test. **(B)** Effect of chronic EtOH exposure on ALDH1HIGH cell population (CSC population) in OSCC was determined by flow cytometry. The number shown in each panel reflects the percentage of ALDH1HIGH cells in each cell type. * $P < 0.05$ was determined by two-tailed Student's t test. **(C)** Effect of chronic EtOH on renewal capacity in OSCC was determined by tumor sphere formation assay. Single cells were plated in Ultra-Low attachment plates at a density of 10,000 cells/ml in serum-free tumor sphere medium. Tumor spheres were counted on day 7. Representative images of tumor spheres were shown on the right. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's t test. **(D)** Effect of chronic EtOH on migration ability of OSCC was determined by transwell migration assay. Migration ability was described as number of migrated cells per field with data as mean ±SD for three randomly selected fields. Representative images of transwell migration assay shown on the right. * $P < 0.05$ was determined by two-tailed Student's t test.
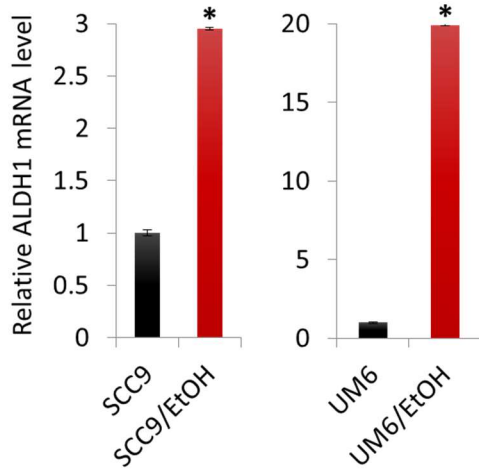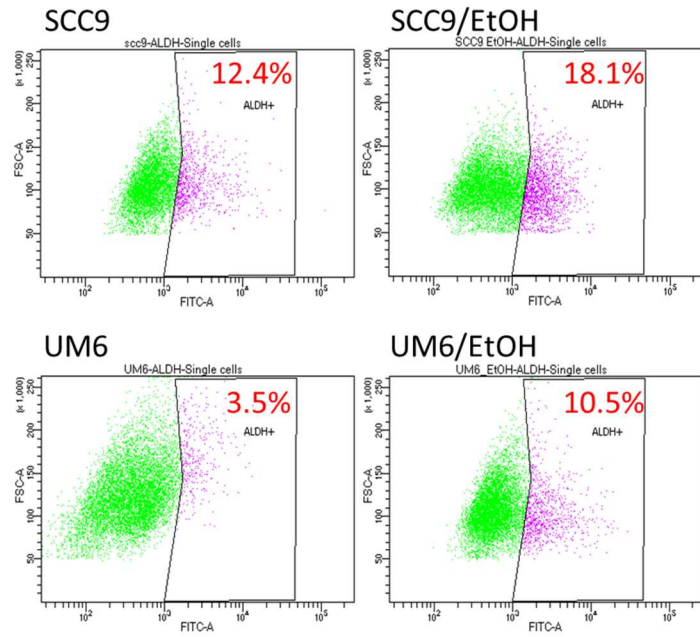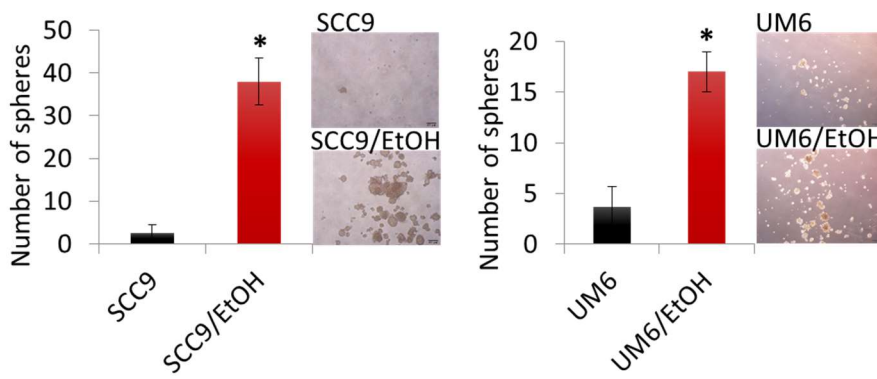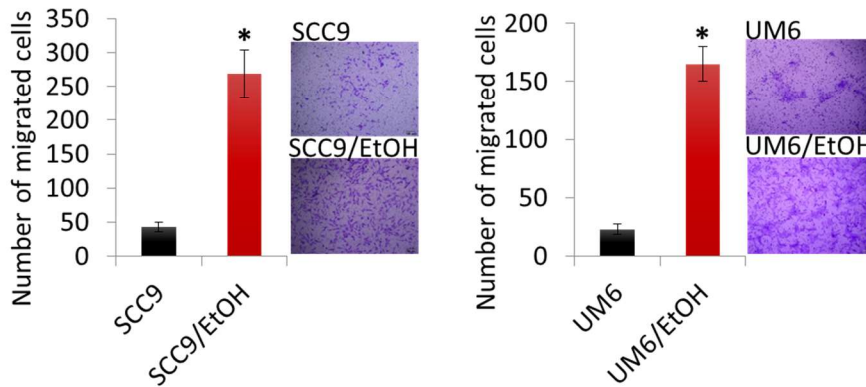
Figure 7. 2-Deoxy-D-glucose (2-DG) suppressed glycolysis in EtOH-treated OSCC.

Effect of 2-DG on lactate secretion was determined by lactate-glo assay. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's t test.
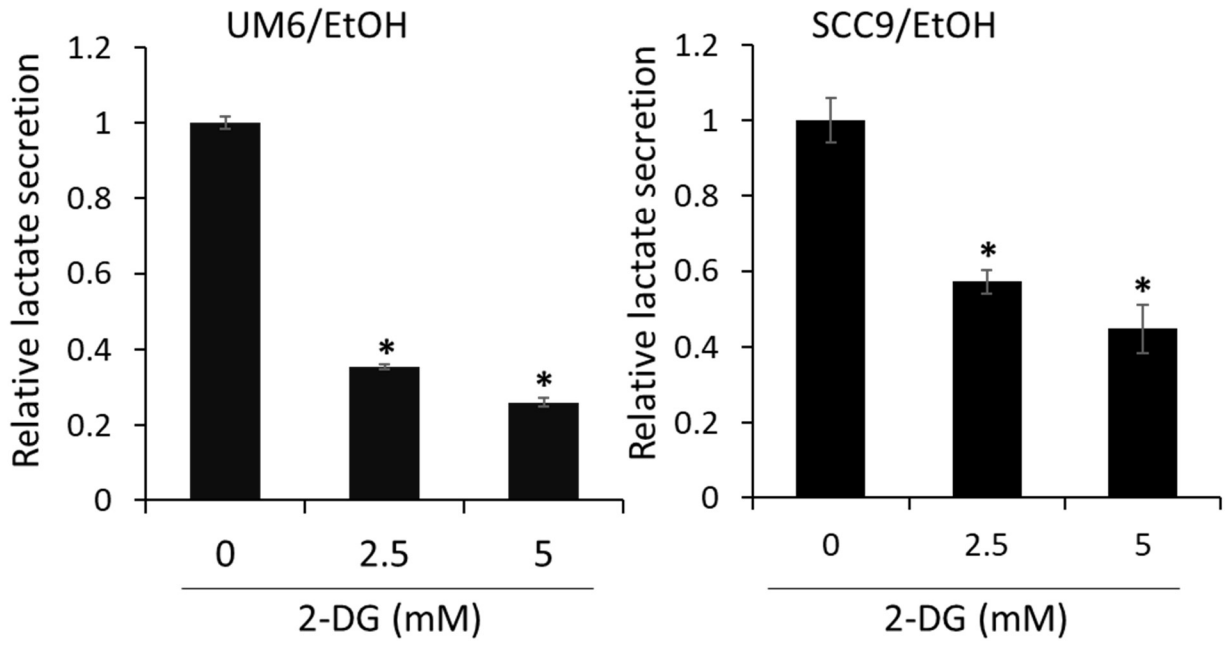
Figure 8. 2-DG inhibited CSC properties in OSCC.

(A) Effect of 2-DG on self-renewal capacity in OSCC determined by tumor sphere formation assay. Representative images of tumor spheres shown on the right. Data are means ±SD of triplicate experiment. * P < 0.05 was determined by two-tailed Student's t test. **(B)** Effect of 2-DG on migration ability of OSCC was determined by transwell migration assay. Migration ability was described as number of migrated cells per field with data as mean ±SD for three randomly selected fields. Representative images of transwell migration assay shown on the right. * P < 0.05 was determined by two-tailed Student's t test

A

B
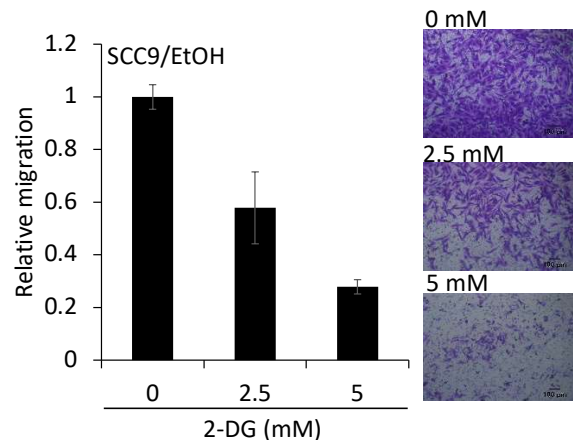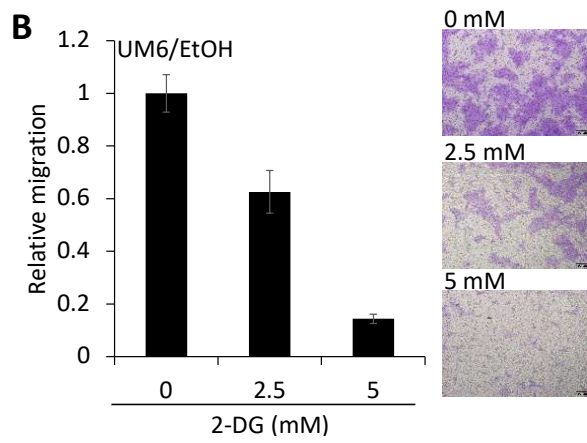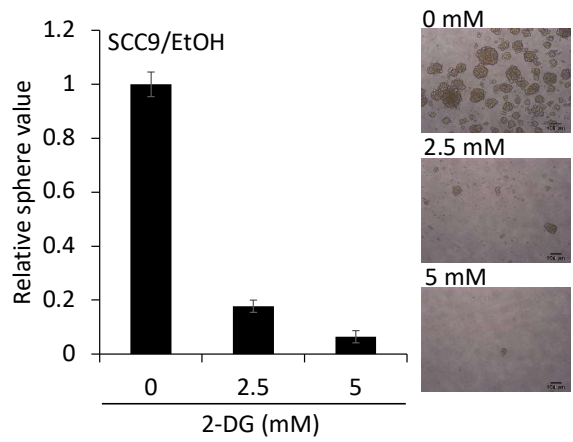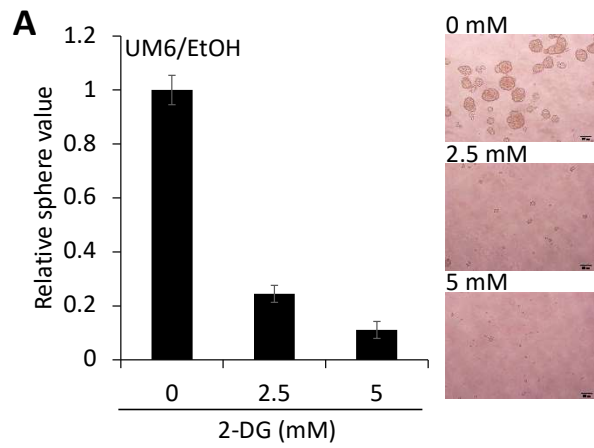
Figure 9. EtOH exposure increased HIF1α expression in OSCC.

Effect of chronic EtOH exposure on HIF1α gene expression was determined by **(A)** qPCR and **(B)** Western Blot. GAPDH was used as a loading control.
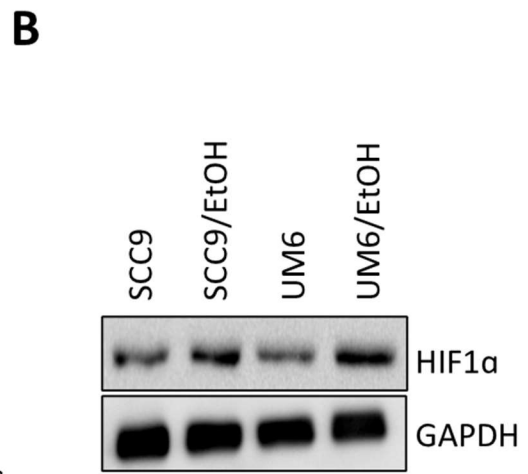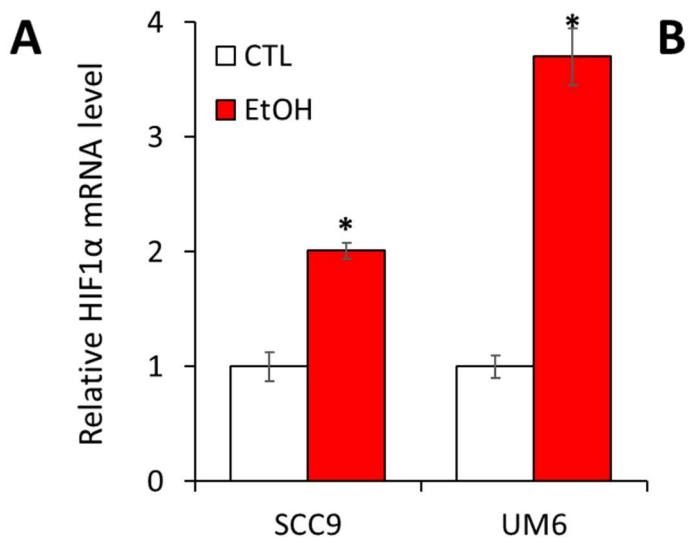
Figure 10. Suppression of HIF1α failed to suppress glycolysis of EtOH-treated OSCC.

(A) Endogenous HIF1α in EtOH-treated OSCC (SCC9/EtOH and UM6/EtOH) was knocked by siRNA against HIF1α (HIF1αi). Control siRNA (CTLi) was also transfected as a control. The knockdown of HIF1α was validated by qPCR. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's t test. **(B)** Effect of HIF1α knockdown on glucose uptake was determined by glucose-glo assay. Data are means ±SD of triplicate experiment. **(C)** Effect of HIF1α knockdown on lactate secretion was determined by lactate-glo assay. Data are means ±SD of triplicate experiment.
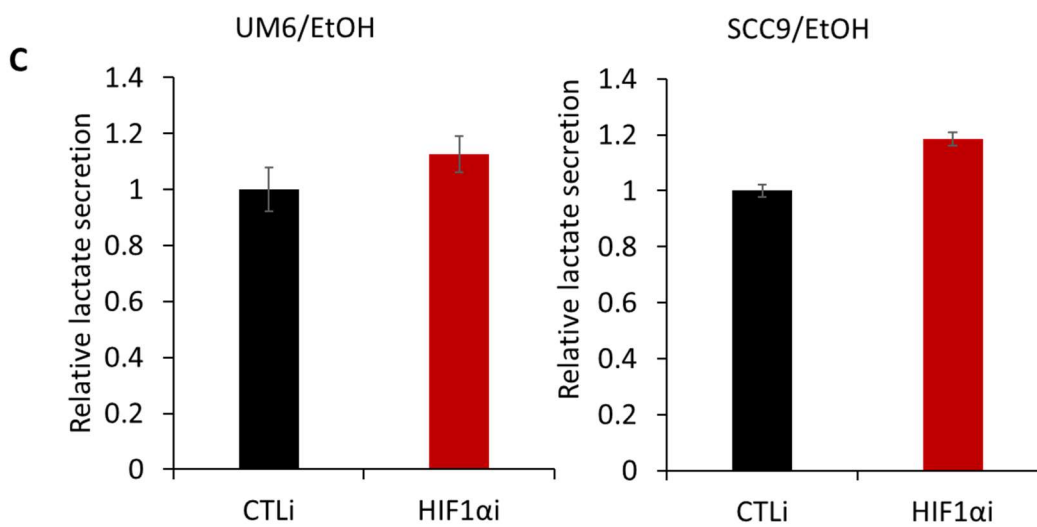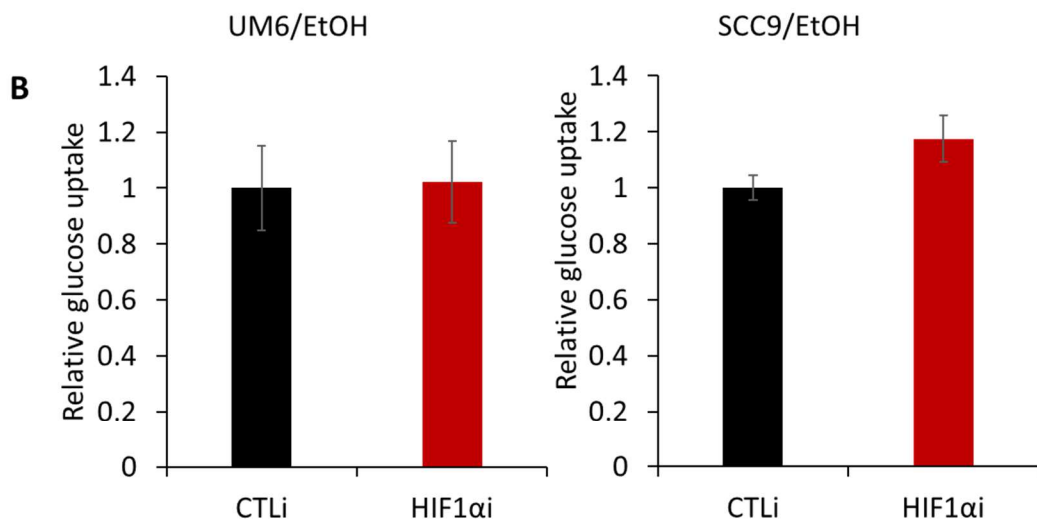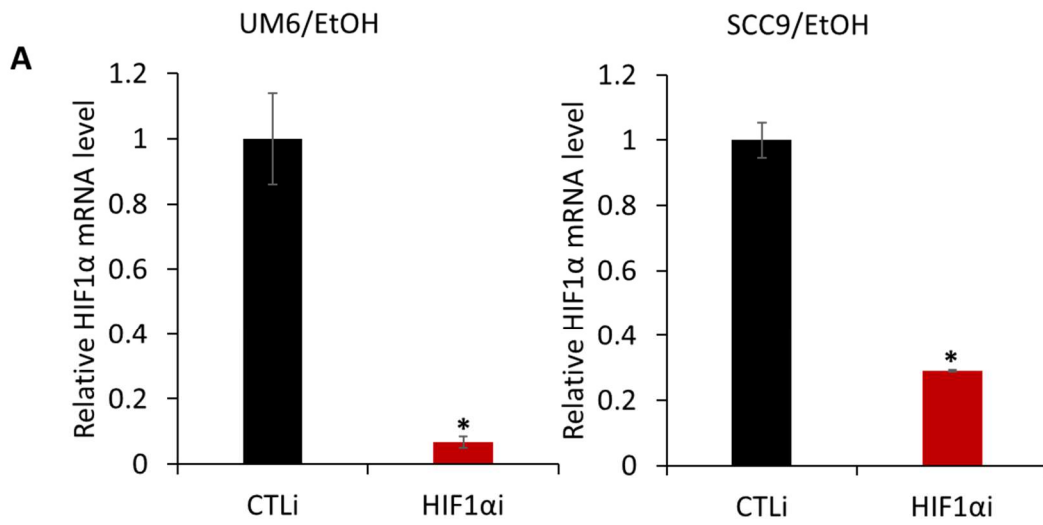
Figure 11. Suppression of HIF1α inhibited CSC properties of EtOH-treated OSCC.

(A) Effect of HIF1α knockdown on self-renewal capacity in the EtOH-treated OSCC was determined by tumor sphere formation assay. * $P < 0.05$ was determined by two-tailed Student's t test. **(B)** Effect of HIF1α knockdown on migration ability of the EtOH-treated OSCC was determined by transwell migration assay. Migration ability was described as number of migrated cells per field with data as mean ±SD for three randomly selected fields. * $P < 0.05$ was determined by two-tailed Student's t test.
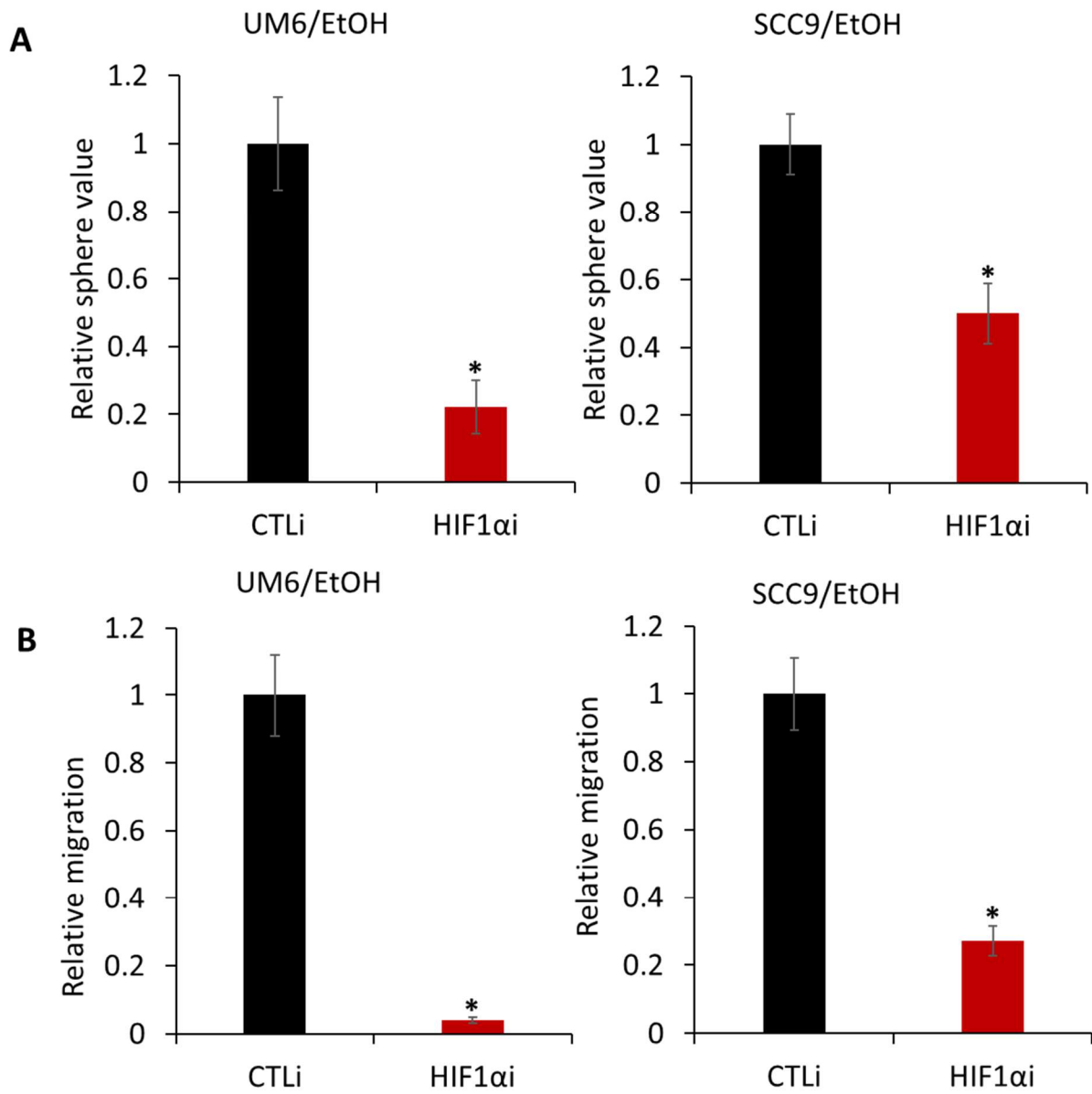
A

UM6/EtOH

SCC9/EtOH

B

UM6/EtOH

SCC9/EtOH

Figure 12. EtOH exposure increased NFATc2 isoform signaling in OSCC.

(A) Representation of vector map for pGL3-NFAT luciferase. NFAT binding sequence upstream of luciferase reporter. **(B)** Effect of chronic EtOH exposure on NFAT activation in OSCC was determined by luciferase assay. Renilla Luciferase was used as transfection control. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's t test. **(C)** Effect of chronic EtOH exposure on NFATc1-3 gene expression in OSCC was determined qPCR. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's t test.
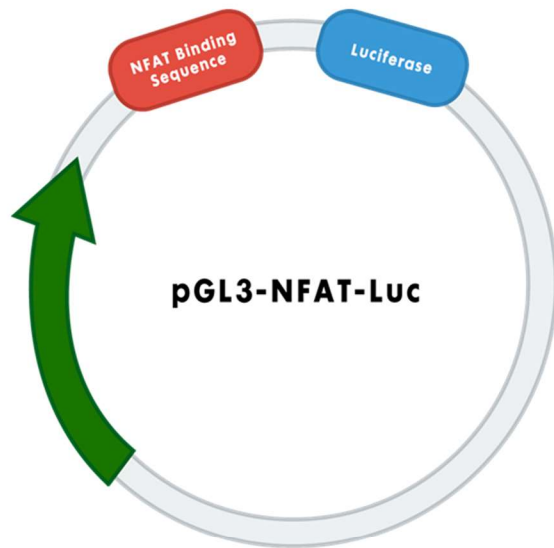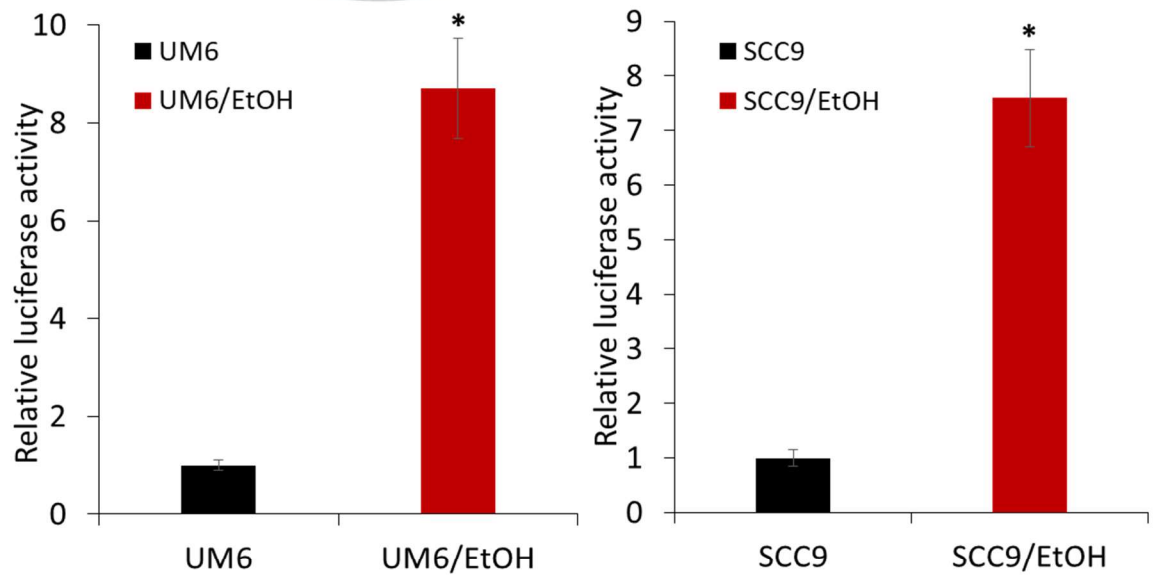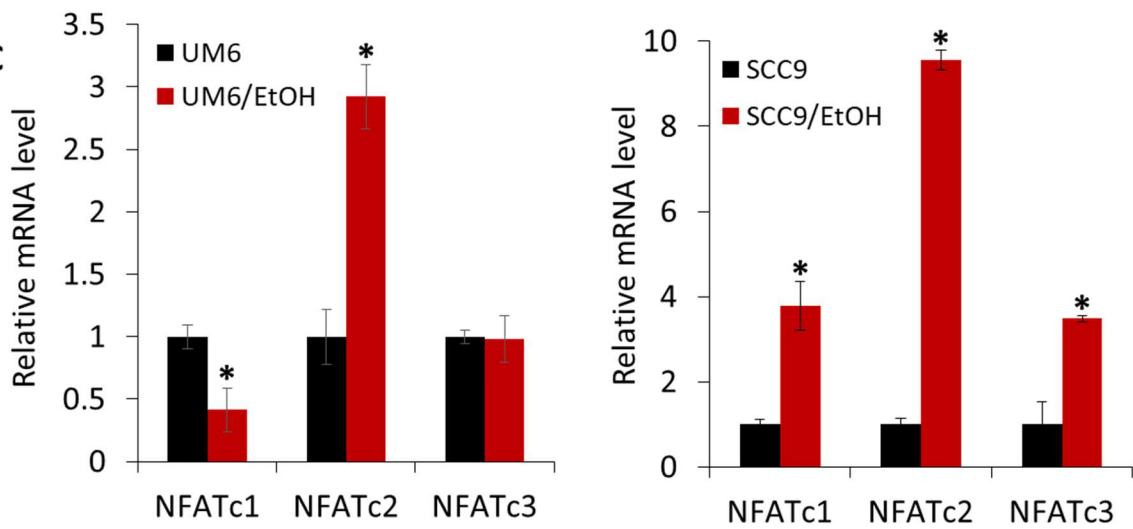
Figure 13. NFATc2 was required for EtOH induced glycolysis

(A) Endogenous NFATc2 in EtOH-treated OSCC (SCC9/EtOH and UM/6/EtOH) was knocked down by siRNA against NFATc2 (NFATc2i). Control siRNA (CTLi) was also transfected as a control. The knockdown of NFATc2 was validated by qPCR. **(B)** Effect of NFATc2 knockdown on glucose uptake in EtOH-treated OSCC (SCC9/EtOH and UM6/EtOH) was determined by glucose-glo assay. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's t test. **(C)** Effect of NFATc2 knockdown on lactate secretion in the EtOH-treated OSCC was determined by lactate-glo assay. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's t test.
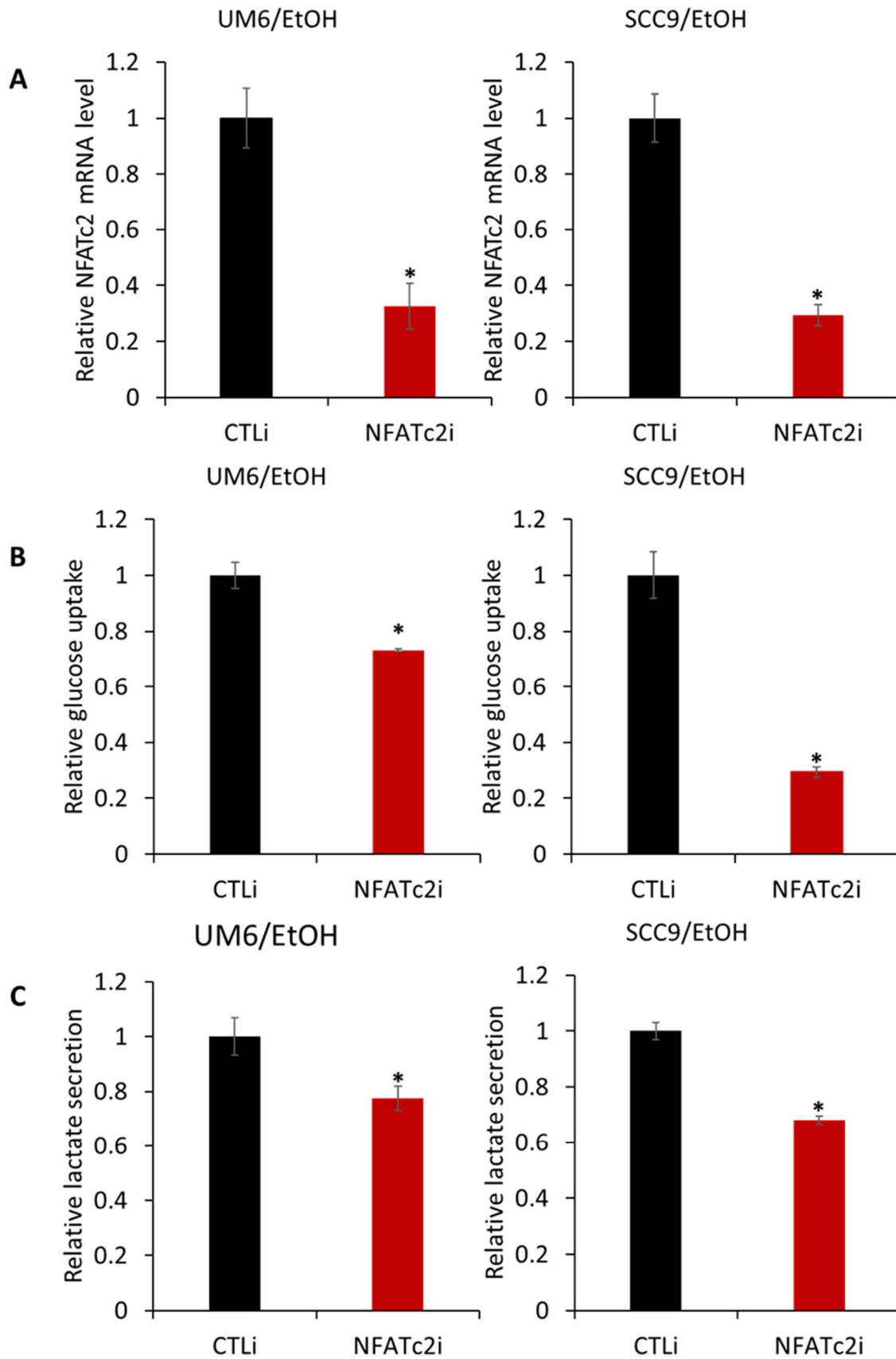
Figure 14. NFATc2 was required for EtOH-induced cancer stemness in OSCC.

(A) Effect of NFATc2 knockdown on self-renewal capacity in EtOH-treated OSCC (SCC9/EtOH and UM6/EtOH) was determined by tumor sphere formation assay. Single cells were plated in Ultra-Low attachment plates at a density of 10,000 cells/ml in serum-free tumor sphere medium. Tumor spheres were counted on day 7. Representative images of tumor spheres were shown on the right. Data are means ±SD of triplicate experiment. * P < 0.05 was determined by two-tailed Student's t test. **(B)** Effect of NFATc2 knockdown on migration ability of EtOH-treated OSCC was determined by transwell migration assay. Migration ability was described as number of migrated cells per field with data as mean ±SD for three randomly selected fields. Representative images of transwell migration assay shown on the right. * P < 0.05 was determined by two-tailed Student's t test.
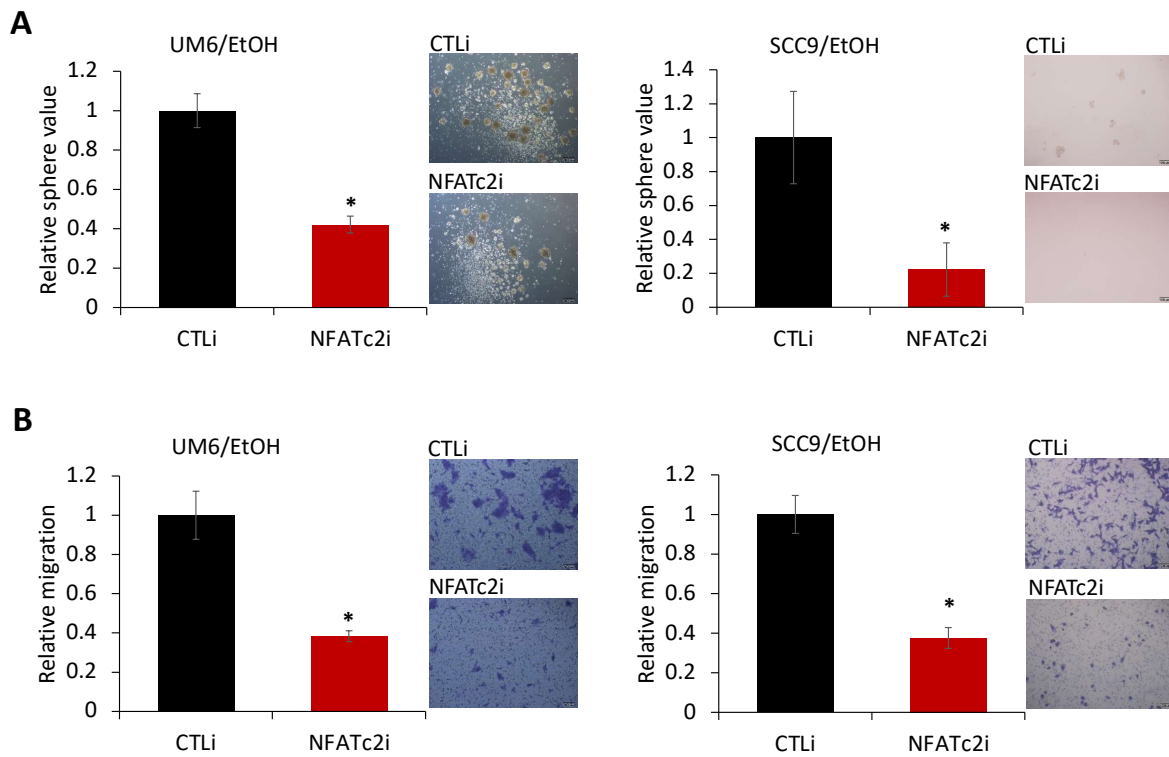
A

UM6/EtOH

SCC9/EtOH

B

UM6/EtOH

SCC9/EtOH

Figure 15. NFATc2/PKM2 axis was activated by chronic EtOH exposure in OSCC and enriched in CSC populations.

(A) Effect of NFATc2 knockdown on EtOH-increased glycolysis gene expression in EtOH-treated OSCC (SCC9/EtOH) was determined by qPCR. Data are means ±SD of triplicate experiment. * P < 0.05 was determined by two-tailed Student's t test. **(B)** Correlation analysis of NFATc2 and PKM2 expression was determined based on their mRNA levels in 14 human SCC cell lines by qPCR. **(C)** Expression of NFATc2 and PKM2 was assessed in tumor spheres (sphere) and adherent monolayer cells (Mono) by qPCR. Data are means ±SD of triplicate experiment. * P < 0.05 was determined by two-tailed Student's t test. **(D)** Expression of NFATc2 and PKM2 of cisplatin-resistant OSCC cells. Data are means ±SD of triplicate experiment. * P < 0.05 was determined by two-tailed Student's t test. Cisplatin-resistant OSCC cell line was established by treating SCC4 with 10 μM of cisplatin for 24 hours.

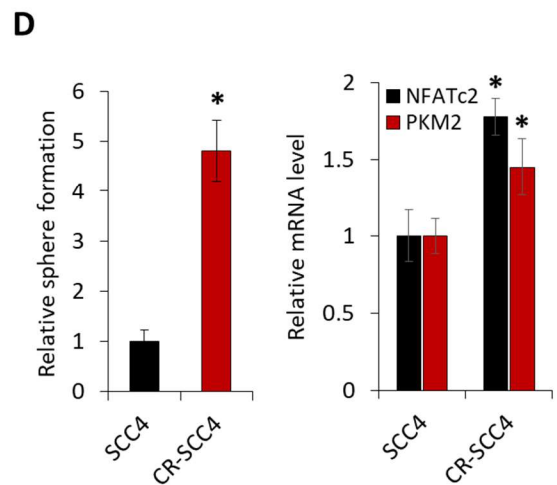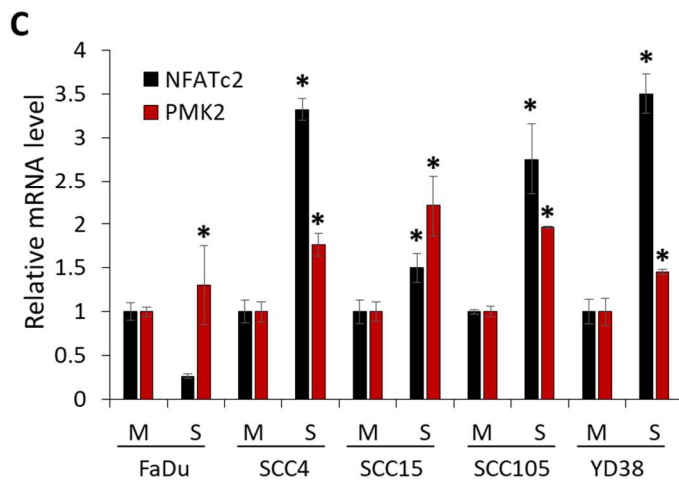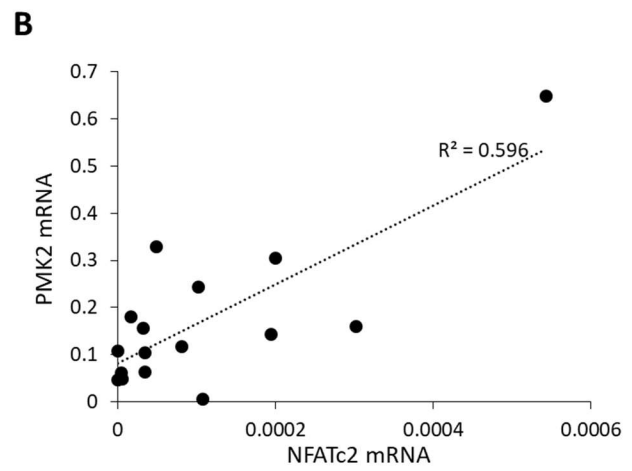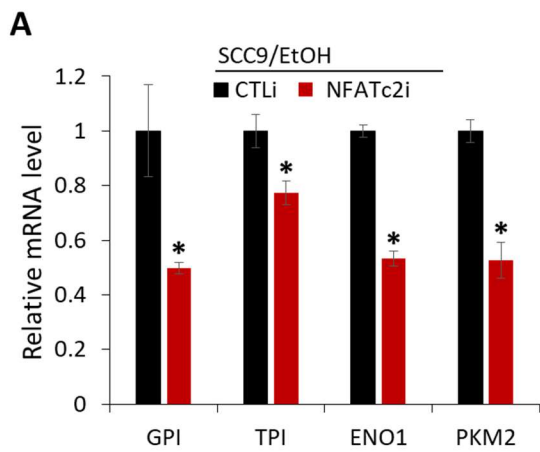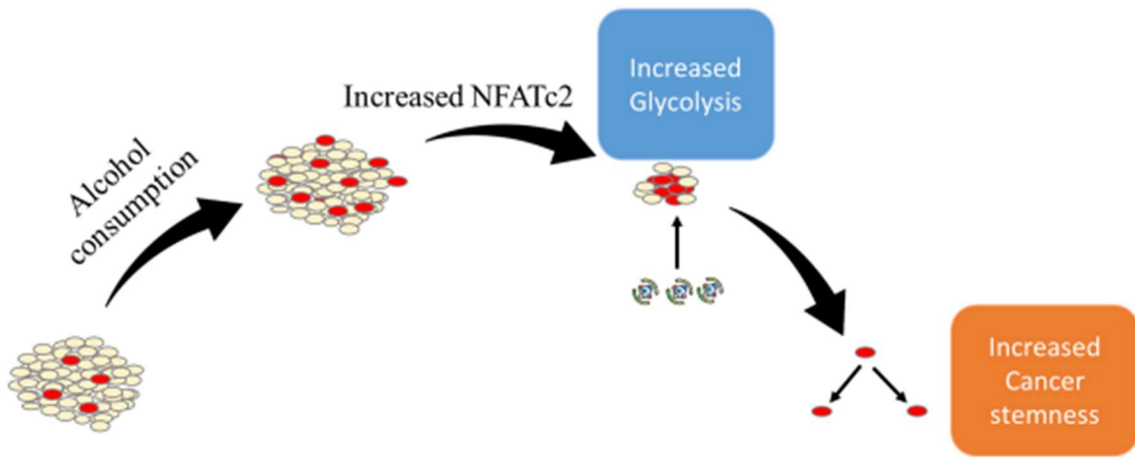**Figure 16. Proposed model for NFAT activation in chronic alcohol-induced cancer stemness in OSCC**

Chronic alcohol consumption increases glycolysis and cancer stemness of OSCC through NFAT activation.

Proposed Model

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