Title
DYRK1A is a Novel Molecular Determinant for Oral Cancer Stemness

Author
Martin, Charlotte Ellen

Publication Date
2018

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

DYRK1A is a Novel Molecular Determinant for Oral Cancer Stemness

A thesis submitted in partial satisfaction

of the requirements for the degree of Master of Science

in Oral Biology

by

Charlotte Ellen Martin

2018

ABSTRACT OF THE THESIS

DYRK1A is a Novel Molecular Determinant

for Oral Cancer Stemness

by

Charlotte Ellen Martin

Master of Science in Oral Biology

University of California, Los Angeles, 2018

Professor Ki-Hyuk Shin, Co-Chair

Professor Mo K Kang, Co-Chair

Cancer stem cells (CSCs) are a small subpopulation of the overall cancer cell population and display enhanced tumorigenicity, metastasis, and drug resistance, which make them plausible targets for cancer therapies. Dual-specificity tyrosine kinases (DYRK1-4) are a family of protein kinases involved in cancer development; however, their role in oral cancer and stemness remains unknown. In this study, we investigated the role of DYRK in oral carcinogenesis by screening the expression of the DYRK isoforms in normal human oral keratinocytes and oral squamous cell carcinomas (OSCC). Among DYRK members, DYRK1A was the dominant DYRK isoform in oral epithelial cells and was upregulated in OSCC. Genetic deletion and chemical inhibition of DYRK1A suppressed aggressive tumor phenotypes, such as tumor growth *in vitro* and *in vivo,* migration, and chemoresistance. DYRK1A was also essential for self-renewal of CSCs and was highly expressed in CSC-enriched populations. The DYRK1A

deletion inhibits FGF2 production in OSCC and concomitantly suppresses ERK signaling pathway, a downstream signaling activated by FGF2. Furthermore, FGF2 treatment in DYRK1A-deleted OSCC cells rescued CSC phenotype. Our study first identifies DYRK1A as a novel molecular determinant for oral cancer stemness and aggressiveness through FGF2/ERK signaling. Since DYRK1A is chemically modifiable by harmine, our study suggests that DYRK1A may be a potential therapeutic target for eradicating oral CSCs.

The thesis of Charlotte Ellen Martin is approved.

Yong Kim

Reuben Han-Kyu Kim

Mo K Kang, Co-Committee Chair

Ki-Hyuk Shin, Co-Committee Chair

University of California, Los Angeles

2018

ACKNOWLEDGEMENTS

I would first like to express my gratitude to my mentor Dr. Ki-Hyuk Shin for his encouragement, dedication, and guidance. His mentorship over the past two years has been invaluable and I'm extremely grateful for the opportunity to conduct research with him. His enthusiasm, curiosity, and passion for scientific research have inspired and motivated me to complete this thesis work.

I would also like to thank Dr. Mo Kang, Dr. Reuben Kim, and Dr. Yong Kim for their insightful comments and assistance throughout the completion of this work as members of my thesis committee.

I am very appreciative of Mr. Matthew Dingman at the Oral Biology department for all his administrative help and advice during my time in the MS program.

Finally, I want to thank all past and current members of The Shapiro Family Laboratory for their support, kindness, and encouragement.

TABLE OF CONTENTS

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a serious and growing worldwide epidemic. It is the sixth most common cancer globally with approximately over 50,000 new cases and 10,000 deaths in the United States alone (Siegel, Miller, & Jemal, 2018; Warnakulasuriya, 2009). Oral cancer, similar to other cancers, often progress due to a series of genetic and epigenetic changes (Kang & Park, 2001). The major risk factors for OSCC development are smoking, alcohol use, and human papillomavirus (HPV) infections (Kumar, Nanavati, Modi, & Dobariya, 2016). Surgery, often followed by radiotherapy and chemotherapy, is the main therapy treatment for oral cancer (Chi, Day, & Neville, 2015). Unfortunately, recurrence and distant metastasis following surgery is still common in OSCC. (Huang et al., 2010; Sano & Myers, 2007). Furthermore, metastasis can lead to a poorer prognosis (Larsen, Johansen, Sorensen, & Krogdahl, 2009). Larsen et al. found that five-year survival rates drastically reduced from 65% to 35% with nodal involvement. Therefore, identifying various mechanisms which drive OSCC progression and metastasis are imperative.

Cancer stem cells (CSCs) are one well-studied theory to explain OSCC development. CSCs are a very small population within the overall heterogeneous tumor cell population that share similar stemness properties to embryonic and adult stem cells (Clevers, 2011). One of those shared properties is self-renewal. Self-renewal is the ability of cancer stem cells to divide and increase the overall cell population without differentiation or loss of proliferation capacity. Through this mechanism, it is proposed that CSCs are able to initiate and sustain tumor development (Soltysova, Altanerova, & Altaner, 2005). CSCs also have multi-lineage differentiation capability (Vermeulen, Sprick, Kemper, Stassi, & Medema, 2008). CSCs

are able to differentiate into several types of cell, which contribute to tumor heterogeneity. Tumors taken from different patients have shown to exhibit significant heterogeneity in terms of morphology, cell surface markers, cell proliferation rates, and response to therapy (Dick, 2008). Phenotypic and functional variety in cancer cells complicate the treatment of these cancers.

CSCs have been identified using cell surface protein markers, such as CD133, CD44, EpCAM, and ALDH activity, in multiple solid tumors, including lung, colon, prostrate, and breast (Visvader & Lindeman, 2012). These biomarkers are important for identifying the CSC population in solid tumors and directing treatment strategies. However, these markers are not entirely universal for all CSC populations even within the same cancer subtype. For example, CD44 high/CD24 low fractions have been extensively utilized as a marker of CSCs in breast cancer. Compared to CD44 high/CD24 high populations, the CD44 high/CD24 fraction displayed enhanced in vivo tumorigenicity, invasion, and metastasis (Abraham et al., 2005; Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Sheridan et al., 2006). However, it has been found that in estrogen receptor (ER) alpha-negative breast cancers, CSCs have been isolated in both CD44 high/CD24 low and CD44 high/CD24 high cell populations (Meyer et al., 2010). Cell populations from both CD44 high/CD24 low and CD44 high/CD24 high fractions were capable of forming tumors in the fat pad of NOD-SCID mice. A similarly contradictory story of markers has been found in colorectal cancer (Dalerba et al., 2007). Dalerba et al. found the colorectal CSC population, which was defined by the cell's ability to engraft *in vivo* in immunodeficient mice, was identified by EpCAM high/CD44+ markers. However, the tumors formed by those EpCAM high/CD44+ cells did not consistently express CD133, a known co-CSC marker. Therefore, CSC markers cannot unfailingly isolate all CSC populations within all solid tumors. More research is required to identify protein markers for cancer-specific CSCs.

CSCs have been shown to be highly tumorigenic (Schatton, Frank, & Frank, 2009). The self-renewal properties of cancer stem cells are the true driving force behind tumor initiation as these cells are immortal and can continually proliferate. The most widespread *in vivo* assay to validate a cell's tumorigenic capacity is transplantation in immunocompromised mice (Ailles & Weissman, 2007). An *in vitro* model to study tumor-forming ability of potential cancer stem cells is a tumorsphere formation assay (Johnson, Chen, & Lo, 2013). A tumorsphere is a solid, spheroid that develops in culture due to the proliferative capacity of one cancer stem cell. Cancer stem cells are unique in that they can survive and proliferate in extremely harsh conditions. Therefore, the cells are grown in serum-free, non-adherent conditions in order to enrich the cancer stem cell population. The number and size of the spheroids in culture can then be used to characterize the population of cancer stem cells in the overall cell population (Dontu et al., 2003; J. C. Liu, Deng, Lehal, Kim, & Zacksenhaus, 2007).

CSCs are also thought to generate metastasis and may be metastatic precursors (Shiozawa, Nie, Pienta, Morgan, & Taichman, 2013). Expression of several CSC markers, such as CD133, CD44, and CD166, have been shown to correlate with high rates of recurrence and metastasis in glioblastoma, lung adenoma, and colorectal carcinoma (Horst, Kriegl, Engel, Kirchner, & Jung, 2009; Pallini et al., 2008; Woo et al., 2010). The combination of CSC marker expression and metastasis is also shown to associate with poorer prognosis and survival. Another mechanism by which CSCs may promote metastasis is through the epithelial to mesenchymal transition (EMT) (Shiozawa et al., 2013). Most adult cells have the ability to undergo this cellular transformation during the process of wound-healing, tissue regeneration, and organ fibrosis. However, CSCs may capitalize on this process by overexpressing epithelial and mesenchymal markers in addition to stemness markers, which results in cell metastasis

(Aktas et al., 2009; Armstrong et al., 2011; Peter, 2010). Further experiments have demonstrated *in vivo* metastatic tumors using mouse models. CD44 high/CD24 low breast CSC populations have developed lung metastases after the generation of primary tumors at an orthotopic site in mice (H. Liu et al., 2010). Similarly, orthotopic injection of CSC-rich (CD133+/CXCR4+) human pancreatic cancer cells in mice developed liver metastasis (Hermann et al., 2007). Currently, anti-cancer drugs and treatments are not specific to these cancer stem cells, which means the CSCs are able to survive and cause metastasis and relapse. Therefore, CSCs have important implications for cancer treatment because metastasis and relapse are involved in more than 90% of cancer deaths (Chaffer & Weinberg, 2011).

Most importantly to therapeutic interventions, CSCs are highly resistant to chemotherapy drugs. Chemoresistance has been attributed to 90% of treatment failure for patients with metastatic cancer (Longley & Johnston, 2005). One proposed mechanism by which cancer stem cells evade death by chemotherapy is through aldehyde dehydrogenase (ALDH) activity (Abdullah & Chow, 2013). Enhanced ALDH activity is a common marker for CSC populations, but it may also endow the cells with chemoresistance. Even before ALDH activity was used to characterize cancer stem cell populations, John Hilton found that inhibiting ALDH activity with disulfrim could reverse the leukemic cell line's cyclophosphamide-resistant (Hilton, 1984). Further studies of ALDH inhibition have shown that ALDH activity is crucial for chemoresistance of human pancreatic adenocarcinoma, colorectal, prostrate, and lung cancers (Duong et al., 2012; Dylla et al., 2008; Hellsten, Johansson, Dahlman, Sterner, & Bjartell, 2011; Sullivan et al., 2010). Other CSC-related signaling pathways such as the WNT/β-Catenin and Notch have also been shown to maintain chemoresistance in hepatocellular carcinoma and colon cancer (Meng et al., 2009; Noda et al., 2009).

Dual-Specificity Tyrosine Regulated Kinases (1-4) are a family of kinases, which have phosphorylation activity on tyrosine, serine, and threonine residues. However, DYRK tyrosine phosphorylation is restricted to auto-phosphorylation. The auto-phosphorylation event is required in order to achieve full kinase activity (Walte et al., 2013). DYRK has five family members including DYRK1A, DYRK1B, DYRK2, DYRK3, and DYRK4 (Aranda, Laguna, & de la Luna, 2011). DYRK1A and DYRK1B are located to the nucleus, whereas DYRK2, DYRK3, and DYRK4 are located to the cytoplasm.

DYRK1A is the most ubiquitously expressed and well-studied isoform. DYRK1A is involved in various biological processes such as apoptosis and cell cycle regulation (Aranda et al., 2011; Yoshida, 2008). For example, DYRK1A plays a protective role against apoptosis by phosphorylating caspase 9 at T125 (Seifert, Allan, & Clarke, 2008). This specific DYRK1A regulation is especially important in caspase 9-mediated developmental cell death during mouse retina development (Laguna et al., 2008). DYRK1A also regulates the G_0/G_1 phase of the cell cycle by phosphorylating the DREAM component LIN52 at S28 (Litovchick, Florens, Swanson, Washburn, & DeCaprio, 2011). This regulatory event controls senescence and quiescence (Litovchick et al., 2011). DYRK1A is also an important regulator of neuronal development (Tejedor & Hammerle, 2011). Many studies have focused on the effect of DYRK1A gene dosage and implicated irregular DYRK1A expression in several neural diseases, such as Down syndrome and Alzheimer's disease (Ferrer et al., 2005; Park, Song, & Chung, 2009).

DYRK has also been shown to be important in carcinogenesis. In glioblastoma DYRK1A prevents the degradation of EGFR which is responsible for tumor growth (Pozo et al., 2013). The inhibition of DYRK1A in GBM cell lines and neural progenitor cells caused a sharp reduction in the self-renewal capacity of the cancer cells and decreased tumor burden. Similarly,

an increased expression of DYRK1A phosphorylates STAT3 at Y705 and enhances STAT3 activity and astrogliogenesis (Kurabayashi, Nguyen, & Sanada, 2015; Matsuo, Ochiai, Nakashima, & Taga, 2001). Enhanced STAT3 has been linked to cancer progression by inhibiting apoptosis or inducing angiogenesis, proliferation, and metastasis (Kamran, Patil, & Gude, 2013). Therefore, DYRK1A could potentially promote cancer development by enhancing STAT3 activity. Meanwhile, certain studies have found that DYRK1A can also play a tumor suppressive role. In acute myeloid leukemia, overexpression of DYRK1A inhibits tumor cell proliferation by downregulating c-Myc degradation (Q. Liu et al., 2014). Furthermore, Down syndrome patients show a significant reduced incidence of most solid tumors (Hasle, Clemmensen, & Mikkelsen, 2000). Therefore, the 1.5-fold increase in DYRK1A gene dosage in DS patients may correlate to their reduction in tumor formation.

Some studies have shown that DYRKs play a role in tumor aggressiveness, thereby suggesting that they may directly interact with the CSC population. Lee et al. found a novel mechanism by which DYRK1A regulates the glioblastoma CSC population (S. B. Lee et al., 2016). DYRK1A-mediated phosphorylation of ID2 leads to HIF2α destabilization, which then leads to loss of glioma stem cell traits and tumor reduction. In endothelial cells, DYRK1A was found to positively regulate VEGF-dependent nuclear factor of activated T cells (NFAT) transcription (Rozen et al., 2018). The NFAT signaling pathway regulates the transcription of several genes which control angiogenesis, which is a critical process for tumor growth and metastasis (Folkman, 2002; Muller & Rao, 2010). DYRK2 regulates breast cancer stem cells (Mimoto et al., 2013). Downregulation of DYRK2 activity caused an increase in androgen receptor (AR) transcription of KLF4. The increase in KLF4 expression promoted cancer stem-like traits in vitro, tumorigenesis in vivo and the proportion of the cancer stem cell population in

human breast cancer tissues. Although DYRK has been explored in several cancers, the role of DYRK in oral cancer and their effect on oral CSCs is unknown and underexplored.

In this study, we investigated the role of DYRK in oral cancer and their effect on oral CSCs. Our study demonstrates that DYRK1A is the dominant isoform of the DYRK family members and is upregulated in OSCC. We also found that DYRK1A is not only essential for OSCC tumorigenicity but required for the maintenance of their CSC population and its properties. Moreover, our mechanistic and functional investigation suggests that DYRK1A regulates cancer stemness via fibroblast growth factor 2 (FGF2)/extracellular signal-regulated kinase (ERK) signaling pathway. Since DYRK1A is chemically modifiable, our study suggests that DYRK1A may be a potential therapeutic target for eradicating CSCs in OSCC.

MATERIALS AND METHODS

Cell Culture - Primary normal human oral keratinocytes (NHOK) were prepared from oral mucosa and cultured in Keratinocyte Growth Medium (KGM, Lonza). Eleven human OSCC cell lines, BapT, FaDu, SCC4, SCC9, SCC15, SCC105, UM6, YD38, and SNU1066, were used. BapT, SCC4, SCC9, SCC15, SCC105, UM6, YD38, and SNU1066 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). FaDu was cultured in RPMI (LifeTechnologies) supplemented with 10% SCS (Gemini Bioproducts) All cell lines were grown in a humidified incubator with 5% CO_2 at 37℃.

Total RNA isolation - Total RNA was isolated from cells using Trizol reagent RNA isolation method. 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. The primer sequences for *DYRK1A*, *DYRK1B*, *DYRK2*, *DYRK3*, *DYRK4* and *GAPDH* were obtained from the Universal Probe Library.

Primer name	Sequence
GAPDH	Forward: 5' AGCCACATCGCTCAGACAC 3' Reverse: 5' GCCCAATACGACCAAATCC 3'
DYRK1A	Forward: 5' AGTTCTGGGTATTCCACCTGCTCA 3' Reverse: 5' TGAAGTTTACGGGTTCCTGGTGGT 3'
DYRK1B	Forward: 5' GCCAGCTCCATCTCCAGTTCT 3' Reverse: 5' CACAATATCGGTTGCTGTAGCGGT 3'
DYRK2	Forward: 5' TCCACCTTCTAGCTCAGCTTCCAA 3' Reverse: 5' TGGCAACACTGTCCTCTGCTGAAT 3'
DYRK3	Forward: 5' TGCAATCCTTCTGAACCACCTCCA 3' Reverse: 5' GCTGTTCTACCTTCATCTCACCTCCA 3'
DYRK4	Forward: 5' AGGCTGTCATCACTCGAGCAGAAA 3' Reverse: 5' AGTCCTGCTGATCACCTGAATGCT 3'

CRISPR/Cas9 Knockout of DYRK1A - DYRK1A protein production was knocked out using a DYRK1A CRISPR/Cas9 KO plasmid or Control Scrambled gRNA CRISPR/Cas9 Plasmid with a DYRK1A HDR Plasmid (Santa Cruz Biotech), which was introduced using Lipofectamine 2000 (Life Technologies). Cells (2 x 10^5) were plated in a single well from a 6-well tissue culture plate and transfected with 1 µg of each plasmid. The cells were cultured in the transfection media for 48 hours and then changed to normal culture media. Successfully transfected cells were selected 48 hours post-transfection using 1 µg/ml of puromycin antibiotic for 2 weeks and then used for experiments.

Western blotting - Whole cell extracts were isolated using the lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 8.8, 150 mM NaCl, 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 for 30 minutes at room temperature, and then incubated with the primary antibodies overnight at 4℃. The next day, membranes were incubated in their respective secondary antibodies for one hour at room temperature and then exposed to the chemiluminescence reagent (Amersham) for signal detection. We used the following primary antibodies: anti-DYRK1A (1:500; sc-100376 Santa Cruz), anti-NFATc3 (1:500; sc-8405; Santa Cruz), anti-Lamin B1 (1:1000; sc-20682; Santa Cruz), anti-α-tubulin (1:1000; T9026; Sigma), anti-β-catenin (1:1000; sc-7199; Santa Cruz), anti-Bmi1 (1:1000; 05-637; Millipore), anti-c-Myc (1:1000; sc-764; Santa Cruz), anti-Oct4 (1:1000; SAB1306163; Sigma Aldrich), anti-FGF2 (1:500; sc-74412; Santa Cruz), anti-phospho ERK1/2 (1:1000; D13.14.4E; Cell Signaling), anti-ERK1/2 (1:1000; 137F5; Cell Signaling), mouse anti-ß-actin (1:1000; Santa Cruz) and mouse

anti-GAPDH (1:1000; Santa Cruz). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz. Cytoplasmic and nuclear proteins were isolated using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, Rockford) following the manufacturer's instructions.

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. SCC4 cells were then collected at day 2, 4, and 7 after the initial seeding and days 2, 4, 6, and 8 for FaDu cells 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.

DNA staining for cell cycle analysis – One million cells were collected and stained with a hypotonic staining buffer for DNA (0.25 g sodium citrate, 0.75 ml Triton X-100, 0.025 g Propidium iodide, 0.005 g Ribonuclease A, 250 ml Distilled Water). Stained cells were incubated away for light at 4°C for 15 minutes and then underwent acquisition using a BD LSRFortessa X-20 SORP (BD Biosciences). Data analysis was done using ModFit software.

FITC Annexin V Apoptosis Staining – Apoptosis was determined by staining cells with annexin V fluorescein isothiocyanate (FITC) and PI labeling as per manufacturer's instructions (BD Biosciences). Cells were washed twice with cold PBS, resuspended in 1 ml of binding

buffer at a concentration of 1 x 10^6 cells/ml. 100 µl of the resuspended culture was transferred to a 15 ml centrifuge tube and then stained with 5 µl Annexin V and 5 µl PI. The cells were analyzed on the BD LSRFortessa X-20 SORP (BD Biosciences).

Anchorage-independent growth - To determine colony-forming efficiency in semi-solid medium, 1 x 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 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). A total of five immunocompromised mice were used in this study. The animal study was performed according to the protocol approved by UCLA Animal Research Committee. 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 3 weeks. The tumors were harvested at 3 weeks post-implantation.

ALDH1 assay - ALDH enzymatic activity was determined using Aldehyde Dehydrogenase-Based Cell Detection Kit (STEMCELL). One million cells were resuspended in 1ml 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 - Three 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.

Chemoresistance Assay - 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 x 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.

Migration Assay - Cell migration was measured using transwell chambers (Corning) in a 24-well tissue culture plate. Twenty 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.

Confocal laser scanning microscopy - Five thousand cells were seeded on four-well Lab-Tek II chamber slides (Thermo Fisher Scientific) one day prior to the immunofluorescence staining. After 24 hours, cells were fixed with 200 µl of 3% Formaldehyde, permeabilized with 200 µl of TNT Buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.05% Tween-20), and blocked with TNBT Blocking Buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.05% Tween-20, 0.3 g/ml of BSA). Cells were then probed with NFATc3 primary antibody overnight (1:250; sc-8405; Santa Cruz). Cells were washed twice with TNT Buffer and then probed with Alexa Fluor 594 dye-conjugated secondary antibody (red) and DAPI (blue-green) for confocal laser scanning. Confocal laser scanning microscopy was performed using Fluoview FV10i Confocal Microscope (Olympus), and images were captured with 120x oil objective under different gain settings. The laser diode 559 nm was used to capture NFATc3 staining, and the diode 405 nm laser was used to capture DAPI nuclear stain. Image acquisition and further adjustment of brightness was performed using Olympus

fluoview Ver. 4.2a. Fluorescent images of cells were taken as single channel images then converted to overlay images and all images were saved in TIFF format.

FGF2 treatment - Recombinant human FGF2 was purchased from PeproTech Inc. Tumor sphere formation and migration assays were all performed as previously described in the absence or presence of FGF2 (20, 50, 100, or 200 ng/ml).

Harmine treatment - DYRK1A chemical inhibitor, harmine, was purchased from Sigma-Aldrich. Harmine specifically inhibits DYRK1A activity by competitively binding to DYRK1A's ATP substrate binding site and preventing DYRK1A kinetic activity (Adayev, Wegiel, & Hwang, 2011). The anchorage independence growth, migration, and tumor sphere formation assays were all conducted as previously described in the absence or presence of harmine (1 μg/ml or 2 μg/ml).

RESULTS

Expression profile of DYRKs in normal human oral keratinocytes and oral squamous cell carcinoma cell lines.

The Human Protein Atlas has found that expression of DYRK1A is detected in 20 different cancer types including head and neck cancer (Uhlen et al., 2017). Seventy five percent of head and neck squamous cell carcinoma (HNSCC) specimens showed a high to medium expression of DYRK1A. However, the expression pattern of all 5 family members of DYRK (DYRK1A, DYRK1B, DYRK2, DYRK3, and DYRK4) remains inconclusive and not well studied in oral cancer. To investigate the role of DYRKs in oral cancer, we first measured the expression level of five DYRK isoforms in normal human oral keratinocytes (NHOK) and 9 human OSCC cell lines (BapT, FaDu, SCC4, SCC9, SCC15, SCC105, UM6, YD38, and SNU1066) by qPCR (Figure 1A). We found that DYRK1A was the most dominantly expressed isoform in NHOK and 6 OSCC cell lines, *i.e,* BapT, FaDu, SCC4, SCC15, SCC105, and YD38 compared to the other isoforms. Interestingly, there were some exceptions to DYRK's dominance in some OSCC cell lines. For example, DYRK1A expression was superseded by DYRK2 in SCC9. DYRK4 had the highest expression level, followed closely by DYRK1A in both UM6 and SNU1066. Furthermore, except SCC9, the tested OSCC cell lines express higher level of DYRK1A than NHOK. To extend our findings, we examined the protein expression of DYRK1A in NHOK, non-tumorigenic immortalized oral epithelial cell lines (OKF6, NOK-SI, and HOK-16B), and OSCC cell lines (SCC15, UM6, UM17B, and SCC105) by Western blot (Figure 1B). Similar to the mRNA expression pattern (Figure 1A), DYRK1A protein is expressed higher in OSCC cells compared to normal and non-tumorigenic immortalized oral

epithelial cells. Taken together, our findings indicate that elevated DYRK1A expression correlates with oral carcinogenesis, which lead us to hypothesize that DYRK1A may play an oncogenic role in oral cancer.

Effect of genetic deletion of DYRK1A on OSCC growth *in vitro* and *in vivo*

To determine the functional role of DYRK1A in OSCC, we generated DYRK1A knockout OSCC cells using CRISPR/Cas9 genome editing technique. We transfected SCC4 and FaDu with CRISPR/Cas9 plasmids that specifically caused a frameshift mutation in the DYRK1A gene. The frameshift mutation is caused by the insertion of a puromycin resistance gene within the DYRK1A gene site. This event prevents proper protein translation of DYRK1A and also allows for the selection of successfully transfected cells. We successfully selected transfected cells using puromycin as a selection marker and confirmed the deletion of DYRK1A in SCC4 and FaDu using Western blot (Figure 2A).

Abnormal and rapid cell division is a hallmark trait of cancer cells (Bresciani et al., 1974; Prescott, 1972). Therefore, we initially investigated the effect of DYRK1A deletion on cancer phenotype by measuring cell proliferation rates. We compared the cell proliferation by cell counting with SCC4 wild-type (WT), SCC4 DYRK1A Knockout (KO), FaDu WT, and FaDu DYRK1A KO. For the both cell lines, we found that DYRK1A deletion significantly suppressed their proliferation rate (Figure 2B). Seven days after the initial seeding of twenty thousand cells, SCC4 DYRK1A KO had grown to 660,000 cells, which was significantly fewer cells compared to SCC4 WT which had grown to 1,995,000 cells. Similarly, six and eight days after initial seeding, FaDu DYRK1A KO had significantly diminished cell numbers (221,250 at day 6 and 645,000 at day 8) compared to FaDu WT (506,250 at day 6 and 1,035,000 at day 8).

Further, we investigated the effect of DYRK1A deletion on cell cycle and apoptosis. Our earlier results demonstrated that DYRK1A deletion caused a significant decrease in cellular proliferation. The inhibited proliferation may be attributed to aberrant cell cycle progression. In mammalian cells, antiproliferative signals, such as Cyclin-Dependent Kinase (CDK) inhibitory genes Cip1/p21 and Kip1/p27, arrest cell cycle progression at the G1 phase (Wainwright, Lasorella, & Iavarone, 2001). Therefore, to explore a potential pathway affected by DYRK1A deletion, we performed cell cycle analysis using propidium iodide (PI) staining followed by flow cytometry. Cell cycle arrest was characterized by a significant increase of cells in G1 phase or decrease of cells in S and G2 phase. However, DYRK1A KO cells did not display these results (Figure 2C). Instead, we observed a slight decrease in the number of KO cells in G1 phase (66.05% in WT compared to 56.75% in KO) and a slight increase in the number of KO cells in S and G2 phase (30.35% and 3.61% in WT compared to 36.15% and 7.05% in KO). DYRK1A deletion didn't cause cell cycle arrest and therefore, was hindering cell proliferation through a different cellular mechanism. DYRK1A deletion could potentially inhibit proliferation by inducing apoptosis. To test this, the WT and KO cells were co-stained with Annexin V-FITC and PI and subjected to flow cytometry to evaluate apoptosis. The assay revealed that there was no difference in the number of apoptotic cells (Annexin V positive/PI negative) and dead cells (Annexin V negative/PI positive) in both cell types (Figure 2D). These findings together with Figure 2C indicate that the OSCC growth inhibition by DYRK1A deletion is not associated with cell cycle arrest and cell death. Instead, DYRK1A deletion may suppress OSCC malignancy, which manifested in stunted proliferation rates.

We continued our exploration of DYRK1A's role in OSCC malignancy by investigating the effect of DYRK1A deletion on anchorage-independent growth ability by performing a soft

agar assay. An important feature of malignant cancers is their ability to sustain anchorage-independent growth (Mori et al., 2009) DYRK1A deletion significantly reduced the number of colonies formed in soft agar for both SCC4 and FaDu (Figure 3A), indicating that DYRK1A inhibition decreases the anchorage-independent growth ability of OSCC. Since anchorage-independent growth is strongly correlated with tumorigenic potential in vivo, we explored the effect of DYRK1A deletion on tumorigenicity of OSCC by performing xenograft tumor assay in nude mice. We subcutaneously injected FaDu WT and FaDu DYRK1A KO cells into the flank of five nude mice and monitored the growth of the xenograft tumors over three weeks. Three out of five nude mice developed tumors with an average weight of 240 mg and average size of 645 mm^3 (Figure 3B). However, the FaDu DYRK1A KO cells failed to form tumors *in vivo*. These findings collectively indicate that DYRK1A is required to maintain tumorigenic growth of OSCC *in vitro* and *in vivo*.

Effect of genetic deletion of DYRK1A on CSC population and phenotype

Tumorigenicity is known to be driven by CSCs (Beck & Blanpain, 2013). Having concluded that DYRK1A is essential for tumorigenicity in OSCC, we wanted to determine whether DYRK1A plays a role in maintaining oral CSC population. We first investigated aldehyde dehydrogenase 1 (ALDH1) activity in WT and DYRK1A KO cells. ALDH1 has been widely used as a marker for isolating CSCs. Its expression and activity are enriched in CSCs isolated from various solid malignancies including head and neck cancer (Monroe, Anderson, Clayburgh, & Wong, 2011; Zou, Sun, Qi, & Ji, 2012). Moreover, cancer cells with high ALDH1 activity ($ALDH1^{HIGH}$ cells) displayed higher CSC properties compared to those with low ALDH1 activity ($ALDH1^{low}$ cells). Thus, to test whether DYRK1A deletion decreases

ALDH1^{HIGH} CSC population, we sorted ALDH1^{HIGH} and ALDH1^{low} cells from the DYRK1A KO cells and their corresponding WT controls by performing flow cytometry analysis. The assay revealed that DYRK1A KO cells had a significantly lowered number of ALDH1^{HIGH} cells compared to the control WT cells (Figure 4A). Our findings indicate that DYRK1A is important for the maintenance of the ALDH1^{HIGH} CSC population for both SCC4 and FaDu cell lines.

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). Because DYRK1A deletion markedly reduced *in vitro* and *in vivo* tumorigenicity of OSCC, we further investigate the role of DYRK1A in self-renewal capacity. Self-renewing CSCs can be enriched in non-adherent tumor spheres cultured in ultra-low attachment plates that support the undifferentiated growth of stem cells. Therefore, abundance and the growth kinetics of non-adherent tumor spheres are indicative of self-renewing CSC content in a given culture of heterogeneous cancer cells. Tumor spheres derived from OSCC cells are CSC-enriched cell population as stemness transcription factors, NANOG, OCT4, KLF4, LIN28, and SOX2 were enriched in tumor spheres (S. H. Lee et al., 2016). We conducted tumor sphere formation assay using the DYRK1A KO cells and their control cells. The DYRK1A KO cells demonstrated significantly decreased tumor sphere formation compared to their control cells (Figure 4B), indicating that DYRK1A is critical for maintaining self-renewing CSC population. Taken together, our data indicate that DYRK1A is essential to maintain oral CSC population.

We continued our investigation by determining whether DYRK1A could affect other CSC properties such as chemoresistance and metastasis (Shiozawa et al., 2013; Zhao, 2016). To determine the effect of DYRK1A on chemoresistance, we used cisplatin which is a common

treatment for many solid malignancies including OSCC (Galanski, 2006; Kohno et al., 2000). We treated the DYRK1A KO and WT control cells with 0 - 50 μM cisplatin for 2 days and determined cell survival by MTT assay (Figure 5A). The assay revealed that DYRK1A KO cells were more sensitive to cisplatin than control WT cells (Figure 5A), indicating that DYRK1A deletion increases cellular sensitive to cytotoxic effect of cisplatin. Moreover, to test the effect of DYRK1A on metastatic capability *in vitro,* we performed the transwell migration assay. DYRK1A deletion significantly reduced the number of migrated cells both in FaDu and SCC4 (Figure 5B). Our result indicates that DYRK1A is an important player in the migration ability of OSCC cells. Therefore, we concluded that DYRK1A is vital for the maintenance of CSC population and property in OSCC.

DYRK1A expression in CSC populations

Next, we wanted to further confirm the importance of DYRK1A in CSCs by comparing its expression in non-CSC and CSC-enriched populations derived from OSCC. In order to isolate a CSC-enriched population, we utilized the tumor sphere formation assay. CSCs proliferate and self-renew as non-adherent spheres in a low-serum environment. We then collected those spheres and extracted protein and mRNA to use in our subsequent assays. Our non-CSC population is designated as the heterogenous, adherent monolayer cell culture of OSCC. Similar to the result from Figure 1, DYRK1A is also the dominant isoform in both tumor spheres and their corresponding adherent monolayer cells derived from FaDu cells (Figure 6A). Moreover, DYRK1A expression is enriched in tumor spheres compared to their adherent monolayer cells both at the mRNA and protein levels (Figure 6B). Enrichment of DYRK1A in tumor spheres was consistently observed from several OSCC cell lines including FaDu, BapT,

and SCC9/TNF (Figure 6C). These results indicate that DYRK1 highly expresses in self-renewing CSCs.

To further confirm the enrichment of DYRK1A in CSC population, we investigated DYRK1 expression in chemoresistant OSCC cells. CSCs have demonstrated the ability to evade cell death induced by chemotherapy drugs (Zhao, 2016). Thus, cancer cells that exhibit an intrinsic resistance to chemotherapeutic treatment can be enriched in CSC population. We generated the cisplatin-resistant OSCC cell line. SCC4 cells were exposed to 10 μM of cisplatin for 24 hours, and surviving cells were isolated and cultured. We validated induced-cisplatin resistance in the surviving cells by examining their half maximal inhibitory concentration (IC50) value in compared with that of unexposed control cells (Figure 7A). The surviving cells (SCC4/CispR) exhibits a 2.2-fold increased IC50 value compared to their control SCC4 cells, indicating the successful development of cisplatin-resistant OSCC cell line. Using SCC4 and SCC4/CispR cell lines, we measured their levels of five DYRK isoforms (DYRK1A, DYRK1B, DYRK2, DYRK3, and DYRK4) by qPCR analysis (Figure 7B). Consistent with the expression of DYRKs in the self-renewing CSCs (Figure 6A), DYRK1A in SCC4/CispR was the dominant isoform and increased on the mRNA (Figure 7B) and protein level (Figure 7C) compared with DYRK1A in control SCC4. DYRK1A mRNA levels were increased by 2.81-fold in SCC4/CispR cells and 1.86-fold in FaDu sphere cells compared to their controls, SCC4 and FaDu monolayer cells, respectively. Furthermore, in both sets of CSC-enriched populations, DYRK1A is the highest expressed isoform. DYRK1A is 5.75 times higher in FaDu sphere cells and 6.5 times higher in SCC4/CispR cells compared to the second highest isoform, DYRK3.

ALDH1 activity is a well-established marker for CSCs. OSCC cells expressing high levels of ALDH1 contain high quantities of CSCs compared to cells expressing low levels of

ALDH1 (Baillie et al., 2017). Therefore, we sorted for $ALDH^{HIGH}$ and $ALDH^{low}$ cell population from SCC4 cells by using ALDEFLUOR assay. The $ALDH^{HIGH}$ population has a higher mRNA level of DYRK1A compared to the $ALDH^{low}$ population (Figure 8). However, the difference of DYRK1A level between these two populations was much smaller than that between self-renewing CSCs and monolayer non-CSCs, suggesting CSC heterogeneity depending on the specificity of CSC isolation. Taken together, our data clearly indicate that DYRK1A expression is augmented in CSC-enriched populations of OSCC. We conclude that DYRK1A is a molecular regulator for oral cancer stemness.

Effect of genetic deletion of DYRK1A on FGF2/ERK signaling pathway

Having established that DYRK1A was an important oncogenic regulator of oral cancer by promoting oral cancer stemness, we decided to explore the underlying mechanism by which DYRK1A regulates oral cancer stemness. DYRK1A is a well-known negative regulator of nuclear factor of activated T cells (NFATc); DYRK1A directly phosphorylates NFATc, which subsequently exports NFATc out of the nucleus (Arron et al., 2006; Gwack et al., 2006). NFATc are a family of five isoforms (NFATc1-5) which act as transcription factors and were first identified in immune cells (Shaw et al., 1988). Moreover, studies have now shown that all NFATc isoforms are ubiquitously expressed and play a role in human cancer (Jauliac et al., 2002). NFATc contributes to tumor progression and malignancy by promoting cell growth, survival, invasion, and angiogenesis (Mancini & Toker, 2009). Furthermore, a recent study found that NFATc3, the major isoform, is upregulated in OSCC and a positive regulator of oral cancer stemness (S. H. Lee et al., 2016). In contrast to what was expected, we found the positive correlation between NFAT and DYRK1A expression in OSCC. Therefore, we further explored

the effect of DYRK1A deletion on NFAT activity. We compared NFATc3 activity in WT and DYRK1A KO cells by measuring nuclear and cytoplasmic NFATc3. Because DYRK1A-mediated phosphorylation of NFATc3 causes its exportation to the cytoplasm, we would expect to see more NFATc3 located in the nucleus in the DYRK1A KO cell lines. However, we failed to observe a significant change in NFATc3 location between the KO and WT cell lines for both SCC4 and FaDu (Figure 9A), indicating that NFATc3 activity is not affected by a loss of DYRK1A. Additionally, after performing immunofluorescence staining, SCC4 WT and SCC4 DYRK1A KO cells displayed similar NFATc3 dominant cytoplasmic localization (Figure 9B). Our results indicate that the DYRK1A-mediated phenotype change in OSCC is independent of the NFAT signaling pathway.

Thus, we continued to investigate the underlying mechanism by which DYRK1A regulates cancer stemness. We determined whether there was a link between DYRK1A and well-known stemness factors in regulating OSCC CSCs. Western blot analysis showed that DYRK1A deletion did not affect the expression of stemness factors, β-catenin, Bmi1, c-Myc, and Oct4 (Figure 10A). Interestingly, DYRK1A deletion notably decreased FGF2 protein expression (Figure 10A and B). It is well documented FGF2 activates the ERK signaling pathway in human cancer (Lau, So, & Leung, 2013; J. F. Liu, Crepin, Liu, Barritault, & Ledoux, 2002; Maehara et al., 2017). Thus, we hypothesize that DYRK1A deletion suppresses ERK signaling pathway by reducing FGF2. To test this, we examined a downstream protein kinase of the ERK1/2 signaling pathway, ERK1/2, which is known to be activated by FGF2 ligand binding. ERK1/2 is the final protein kinase activated in the signaling cascade and is responsible for activating transcription factors to induce gene expression (Wortzel & Seger, 2011). We found that phosphorylated ERK (p-ERK), the active form of ERK, was decreased by DYRK1A

deletion in both SCC4 and FaDu (Figure 10C). Our data indicate that DYRK1A deletion suppresses the FGF2/ERK signaling pathway in OSCC.

Effect of exogenous FGF2 treatment on CSC properties in DYRK1A-deleted OSCC cells

We sought to determine whether there was a functional link between FGF2 reduction in the DYRK1A deletion-mediated suppression of CSC phenotype. We first examined the effect of exogenous FGF2 treatment on self-renewal ability in SCC4 DYRK1A KO cells and found that FGF2 treatment increased the number of spheres formed by the DYRK1A KO cells in a dose dependent manner (Figure 11A). At the higher concentrations of 100 ng/ml and 200 ng/ml of FGF2, there was a significant rescue effect in self-renewal capability. We also examined the rescue effect of FGF2 on cell migration in SCC4 DYRK1A KO cells. The transwell migration assay showed that addition of exogenous FGF2 increased the number of migrated cells in the DYRK1A KO cells (Figure 11B). Therefore, we conclude that DYRK1A deletion inhibits oral CSC phenotype by reducing FGF2. However, FGF2-activated MEK/ERK1/2 signaling might be responsible for the rescue and maintenance of oral cancer phenotype and stemness traits in the absence of DYRK1A.

Effect of chemical inhibition of DYRK1A on malignant growth and CSC phenotype of OSCC

Our study suggests that targeting DYRK1A function may prove to be a promising therapeutic strategy for patients with OSCC. In order to test our hypothesis, we utilized harmine, a potent, selective DYRK1A inhibitor. Harmine functions as an ATP-competitive inhibitor of

DYRK1A kinase function (Adayev et al., 2011). Like many kinases, DYRK1A exhibits a two-substrate reaction mechanism by utilizing ATP (phosphate donor) and a phosphate acceptor as substrates (Adayev, Chen-Hwang, Murakami, Wegiel, & Hwang, 2006). Other chemicals, such as epigallocatechin-3-gallate (EGCG), have been shown to inhibit DYRK1A by functioning as a non-competitive inhibitor of both substrates (Adayev et al., 2006). Instead, harmine only competes with DYRK1A's ATP substrate binding by interacting with the ATP binding pocket of DYRK1A. EGCG is a less potent inhibitor of DYRK1A with an IC50 value of about 0.3 μM compared to harmine, which has an IC50 value of about 0.1 μM (Bain, McLauchlan, Elliott, & Cohen, 2003; Bain et al., 2007). Harmine has also been shown to inhibit the growth of several cancers *in vivo* and *in vitro* (H. Zhang et al., 2014; L. Zhang et al., 2015). For example, a recent study found that harmine suppressed growth and induced apoptosis in thyroid cancer both *in vivo* and *in vitro* (Ruan, Jia, & Li, 2017). Therefore, harmine's specificity for DYRK1A's ATP-binding pocket makes it the ideal chemical to inhibit DYRK1A kinase activity and to examine the effect of chemical inhibition of DYRK1A on CSC phenotype.

We first determined the effect of harmine on OSCC cell growth by performing MTT assay (Figure 12A). We found that 1 μg/ml and 2 μg/ml of harmine concentrations have a minimal effect on cell proliferation rate in SCC4 and FaDu (Figure 12A). Therefore, we continued our study using those two concentrations. Initially, we studied the effect of harmine on anchorage-independence growth ability in OSCC by preforming soft-agar assay. We found that harmine treatment significantly decreased the number of colonies formed by SCC4 and FaDu in a dose-dependent manner (Figure 12B), indicating that chemical inhibition of DYRK1A suppresses malignant growth of OSCC.

We further investigated the effect of harmine on CSC phenotype of OSCC. Using multiple OSCC cell lines (SCC4, FaDu, SCC15 and BapT), we determined the effect of harmine on self-renewal capacity by performing tumor sphere formation assay. Harmine treatment significantly diminished the number and size of spheres formed by all tested OSCC cells in a dose-dependent manner (Figure 13A and 13B). We also examined the effect of harmine on migratory ability of OSCC. Transwell migration assay revealed that a low concentration of harmine (1 μg/ml) markedly ablated the migration ability of FaDu and BapT cells (Figure 14A and 14B). Consistent with genetic deletion of DYRK1A, its chemical inhibition of DYRK1A displayed suppressive effect on CSC phenotype of OSCC. Overall, our results further confirm that blocking DYRK1A kinase activity by harmine diminishes OSCC growth and CSC properties, suggesting that harmine is a promising therapeutic strategy to treat OSCC by targeting CSCs.

DISCUSSION

The aim of this study is to understand the functional role of DYRK in OSCC. Our study reveals for the first time that among 5 DYRK isoforms (DYRK1A, 1B, 2, 3, and 4), DYRK1A is the dominant isoform in oral epithelial cells, and its expression is elevated in OSCC cells compared to normal oral epithelial cells. DYRK1A expression is further increased in various CSC-enriched OSCC populations compared to their corresponding non-CSC populations. Using CRISPR/Cas9 genome editing technique, we demonstrated that DYRK1A is essential to maintain malignant growth and cancer stemness of OSCC. Genetic deletion of DYRK1A abrogates tumorigenic potential of OSCC *in vivo*. The DYRK1A deletion also leads to suppression of self-renewal capacity that is considered as the driving force for tumorigenicity and key feature of CSC. We showed that the loss of DYRK1A diminishes important CSC properties, such as chemoresistance and migration ability. The DYRK1A deletion inhibits FGF2 production in OSCC and concomitantly suppresses ERK signaling pathway, a downstream signaling activated by FGF2. Importantly, we demonstrated that recombinant FGF2 promotes CSC phenotype suppressed by DYRK1A deletion, suggesting that DYRK1A regulates cancer stemness *via* FGF2/ERK signaling pathway. Lastly, we showed that chemical inhibition of DYRK1A by harmine successfully suppresses malignant growth properties and CSC phenotype in OSCC. Our findings have important implications for developing better approaches to treat OSCC by targeting CSCs *via* suppression of DYRK1A activity.

Kinases, such as DYRK, drive various cellular processes by enzymatically adding a phosphate group to their target protein (Hubbard & Till, 2000). The addition of a phosphate group to a protein can dramatically alter its function; either turning “on” or “off” protein activity

(Ardito, Giuliani, Perrone, Troiano, & Lo Muzio, 2017). These protein phosphorylation events are a mechanism of regulation for several cellular processes, such as cell division, protein synthesis, signal transduction, cell growth, and development. However, aberrant changes in these regulatory mechanisms can lead to disruption of signaling pathways and subsequent changes to normal cellular functions (Shchemelinin, Sefc, & Necas, 2006). Abnormal activation of these pathways caused by the hyperactivation or malfunction of kinases have been reported in several cancers, including oral cancer (Cohen, 2002; Gross, Rahal, Stransky, Lengauer, & Hoeflich, 2015; Hanahan & Weinberg, 2011). Gene mutations within the extracellular domain of EGFR caused its tyrosine kinase to remain constitutively active in glioblastomas, ovarian tumors, and non-small cell lung carcinoma (Nishikawa et al., 1994; Reguart & Remon, 2015; Wilken et al., 2012). Continuous activity and signal activation leads to uncontrolled cell proliferation, thereby eventually resulting in oncogenic cell transformation. Paradoxically, altered kinase activity can also elicit tumor suppressive activities. Expression of Syk tyrosine kinase is downregulated or absent in breast cancer, and its downregulation increases tumor growth and progression (Moroni et al., 2004). However, the upregulated expression of Syk supports tumor growth and migration in prostate cancer (Ghotra et al., 2015). Thus, the consequences of altered kinase activity and expression are not consistent across all cancer types. Indeed, contradictory effects of kinases and their downstream signaling on oral carcinogenesis have also been reported (Pramanik et al., 2016; Smolensky, Rathore, Bourn, & Cekanova, 2017).

DYRK family members (DYRK1A, 1B, 2, 3, and 4) play an important role in tumorigenesis by regulating downstream targets involved in cancer development; however, their role in cancer is controversial (Fernandez-Martinez, Zahonero, & Sanchez-Gomez, 2015). For instance, overexpression of DYRK1A, the most well-studied isoform, has been observed in

multiple human cancer types (Uhlen et al., 2015). Inhibition of DYRK1A suppressed tumor growth by promoting EGFR degradation in glioblastoma cells, suggesting an oncogenic role of DYRK1A (Pozo et al., 2013). However, tumor suppressive activity of DYRK1A was also demonstrated in human cancer. DYRK1A is reduced in acute myeloid leukemia (AML), and its ectopic overexpression inhibited cell proliferation by arresting cell cycle *via* c-Myc regulation (Q. Liu et al., 2014). These prior reports suggest DYRK members play unique roles in the context of cancer type. The role of DYRK members in OSCC has not been documented. Thus, the following questions remain to be answered; 1) which DYRK member is dominant isoform in OSCC and 2) its role in oral carcinongenesis. We showed that DYRK1A is most abundant isoform and highly expressed in OSCC compared to precancerous and normal oral epithelial cells. Our finding is the first report showing a stepwise elevation of DYRK1A during the progression of OSCC. Genetic deletion of DYRK1A in OSCC significantly suppressed malignant growth properties, such as anchorage-independent growth ability, self-renewal capacity, and *in vivo* tumor-forming ability. Moreover, pharmacological inhibition of DYRK1A caused reduced anchorage-independent growth ability and self-renewal capacity of OSCC. A recent study has found a similar effect of the DYRK1A inhibition on the malignant growth in head and neck squamous cell carcinoma (Radhakrishnan et al., 2016). Thus, we hypothesize that DYRK1A activity is required for malignant growth of OSCC, thus a potential molecular target for oral cancer therapy.

CSCs have been identified in a broad spectrum of solid tumors, including OSCC (Baillie, Tan, & Itinteang, 2017; Chiou et al., 2008; Kim, Pearson, & Nor, 2016). They retain characteristics similar to self-renewing normal stem cells, thus responsible for tumorigenicity and are considered as key contributing cancer cells for metastasis, drug resistance, and

recurrence. Therefore, targeting CSCs by suppressing unique molecular determinants of CSCs provides an effective therapeutic intervention. Using phenotypic and functional experimental approaches, we showed that DYRK1A is a novel molecular marker for oral CSCs, and its function is required for the maintenance of CSC population and property in OSCC. DYRK1A is highly expressed in various CSC-enriched populations, *i.e.,* self-renewing, $ALDH^{HIGH}$ and drug resistant OSCC cells. Inhibition of DYRK1A suppressed the number of self-renewing cell population in multiple OSCC cell lines, indicating that DYRK1A activity is essential to maintain self-renewal capacity of CSCs. Similar effects of DYRK1A inhibition on self-renewal have been demonstrated in glioblastomas (S. B. Lee et al., 2016). Genetic deletion of DYRK1A gene also diminished the number of $ALDH^{HIGH}$ cells in OSCC. ALDH1 has been found to be a marker for stem cells in different types of cancer, including OSCC (Clay et al., 2010b; M. E. Prince et al., 2007; M. E. P. Prince et al., 2016). $ALDH1^{HIGH}$ cancer cells displayed higher self-renewal potential than $ALDH1^{low}$ cells (Clay et al., 2010a; Ota, Ohno, Seno, Taniguchi, & Ozeki, 2014; Richard et al., 2013). Isolated CSCs have demonstrated enhanced metastatic potential and drug resistance, thereby these properties are considered as unique CSC phenotypes. In our study, both pharmacological and genetic inhibition of DYRK1A also suppressed migration ability of OSCC. Our finding is consistent with previous reports showing the importance of DYRK1A in cancer cell migration (Radhakrishnan et al., 2016; Rozen et al., 2018). Inasmuch as epithelial-to-mesenchymal transition (EMT) is the key cellular process in metastasis, the effect of DYRK1A on EMT and EMT-related gene expression should be warranted to investigate (Zheng et al., 2015). We also found that DYRK1A expression is increased in cisplatin-resistant OSCC cells, and its deletion sensitized OSCC response to cisplatin. Similarly, knockdown of DYRK1B sensitized ovarian cancer cells to cisplatin (J. Hu & Friedman, 2010). A contractor observation

was also reported that ectopic overexpression of DYRK1A increased drug sensitivity of blood cancer (Q. Liu et al., 2014). Since CSCs are play the key role in tumor growth and aggressiveness, our findings are of paramount important for the development of more effective oral cancer therapies by targeting DYRK1A. However, underlying mechanism by which DYRK1A regulates oral cancer stemness has not been understood.

Our study showed that DYRK1A deletion results in decreased FGF2 expression and its downstream signaling pathway, ERK1/2, in OSCC. FGF2 has been shown to play a significant role in malignant growth (Takahashi et al., 1992). FGF2 expression is upregulated in a variety of malignant tumors, such as melanomas, pancreas, head and neck, and non-small cell lung cancers (Berger et al., 1999; Dellacono, Spiro, Eisma, & Kreutzer, 1997; Halaban, 1996; Yamanaka et al., 1993). FGF2 overexpression is linked to poor prognosis, high invasiveness, and malignant transformation from potentially malignant oral lesions (PMOLs) to OSCC (Hase et al., 2006; Nayak et al., 2015). FGF2 also promoted CSC properties, including self-renewal capacity, migration and chemoresistance in human cancers (Chen et al., 2016). It is well known that FGF2 induces its biological effects by activating downstream signaling cascades after its binding to FGF receptors (X. Zhang et al., 2006). An important signaling cascade activated by FGF2 is extracellular signal-regulated kinases (ERK) pathway. This signaling pathway has four important components: Ras, Raf, MEK, and ERK. In response to the binding of an extracellular signal to its receptor, active Ras-GTP complex increases and recruits the binding of Raf kinases. Raf is then activated by autophosphorylation or phosphorylation by other kinases, resulting in phosphorylation of MEK. Phosphorylated MEK then subsequently phosphorylates and activates ERK, which in turn phosphorylates and activates several transcription factors, such as NF- κB, AP-1, and ETS-1 (Wortzel & Seger, 2011). FGF2-mediated induction of MEK/ERK signaling is

important for tumor cell proliferation, survival, and EMT (Grose & Dickson, 2005; Y. Hu, Mintz, Shah, Quinones-Hinojosa, & Hsu, 2014). Furthermore, the FGF2-mediated MEK/ERK signaling pathway was crucial for the maintenance of CSC population and properties in esophageal squamous cell carcinoma (Maehara et al., 2017).

Several studies have found interactions between FGF2 and DYRK1A activity. FGF2 promoted DYRK1A kinase activity and phosphorylation of the transcription factor, CREB, to promote neuronal differentiation (Yang, Ahn, & Chung, 2001). FGF2-induced DYRK1A-CREB phosphorylation was later found to promote oncogenesis of bladder carcinomas *via* NDY1/KDM2B transcriptional regulation (Kottakis et al., 2011). It was also demonstrated that DYRK1A promoted FGF2 signaling by phosphorylating and inactivating a receptor tyrosine kinase (RTK) signaling inhibitor, Sprouty2 (Aranda, Alvarez, Turro, Laguna, & de la Luna, 2008). All prior studies have found FGF2 to be an upstream regulator of DYRK1A activity. Interestingly, our study suggested that DYRK1A might work as an upstream regulator of FGF2. The DYRK1A deletion suppressed FGF2 production and its downstream ERK signaling pathway. When FGF2 was supplemented in the DYRK1-deleted OSCC cells, FGF2 successfully rescued their suppressed CSC properties. This observation leads us to hypothesize that DYRK1A regulates oral CSC phenotype *via* FGF2/ERK signaling pathway; however, further investigation will be necessary to examine the functional roles of ERK signaling in CSC regulation by DYRK1A/FGF2 axis.

Inhibition of DYRK1A activity in a clinical setting is possible with harmine, a chemical inhibitor of DYRK1A. Similar to DYRK1A genetic deletion, we found that harmine diminished oral cancer stemness. Harmine has been used to diminish tumor growth *in vitro* and *in vivo* in gastric, thyroid, and liver cancer (Ruan et al., 2017; H. Zhang et al., 2014; L. Zhang et al., 2015).

Therefore, it's possible to eradicate CSCs in oral cancer using harmine. Furthermore, in ovarian cancer, harmine suppressed both CREB and ERK1/2 phosphorylation, which suppressed proliferation and migration (Gao et al., 2017). Interestingly, they also observed a decrease in the expression of vascular endothelial growth factor (VEGF) as well as matrix metalloproteinase (MMP) family MMP-2 and MMP-9. This study mirrors our work and also suggests that inhibiting DYRK1A activity can lower production of growth factors such as FGF2. Diminished growth factors then cause a reduction of ERK1/2 phosphorylation levels and tumor growth. Further study is needed to evaluate other molecular pathways that are affected by DYRK1A inhibition.

Numerous research groups have reported successful isolation of oral CSC populations using various markers. In general, CSCs in OSCC can be isolated by either cell-surface markers or their unique functional properties (Baillie et al., 2017; Kim et al., 2016; W. Liu et al., 2013). Nevertheless, no single marker or CSC trait are capable of specifically isolating oral CSC populations from OSCC cells, suggesting the heterogeneity of CSC populations (Eun, Ham, & Kim, 2017). For example, in breast CSCs, both ALDH+ and CD44+/CD24- populations demonstrate stem-like characteristics (Brooks, Burness, & Wicha, 2015). However, they display different cellular phenotypes: ALDH+ CSCs are more epithelial-like and highly proliferative, whereas CD44+/CD24- CSCs are more mesenchymal-like, quiescent, and invasive. Therefore, this may be a plausible explanation for the differential effect of DYRK1 on CSC phenotype. For example, we observed a dramatic decrease in migration ability, but a subtler decrease in chemoresistance in the DYRK1A-deleted OSCC. Enrichment of DYRK1A is much greater in self-renewing and chemoresistant CSC population compared to $ALDH1^{HIGH}$ CSC population. These indicate that identifying additional oral CSC markers and understanding their biochemical

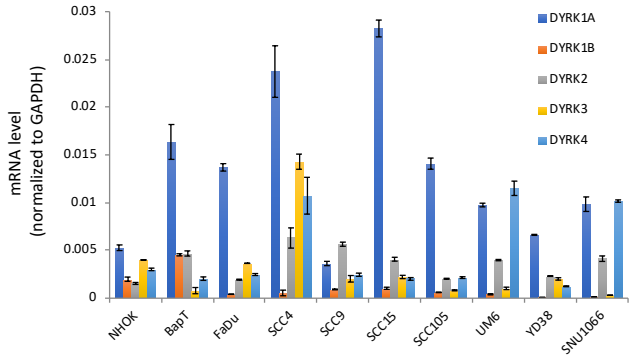
activity in the regulation of cancer stemness should be important research endeavors in oral cancer biology. Thus, our findings highlight a novel molecular stemness axis by which DYRK1A regulates oral cancer stemness *via* FGF2/ERK pathway.

FIGURE LEGENDS AND FIGURES

Figure 1. DYRK1A is the dominant isoform among DYRK family members and overexpressed in OSCC compared to normal human oral keratinocytes.

(A) Level of 5 DYRK isoforms (DYRK1A, 1B, 2, 3, and 4) was determined in normal human oral keratinocyte (NHOK) and 9 OSCC cell lines (BapT, FaDu, SCC4, SCC9, SCC15, SCC105, UM6, YD38, and SNU1066) by qPCR. The Ct values of 5 DYRK isoforms were normalized by the CT value of GAPDH. **(B)** Level of DYRK1A protein was determined in NHOK, 3 precancerous, non-tumorigenic immortalized oral epithelial cell lines (OKF6, NOK-SI, and HOK-16B), and 4 OSCC cell lines (SCC15, UM6, UM17B, and SCC105) by Western blot analysis. GAPDH was used as a loading control

A


mRNA level
(normalized to GAPDH)
0.03
0.025
0.02
0.015
0.01
0.005
0
DYRK1A
DYRK1B
DYRK2
DYRK3
DYRK4
NHOK
BapT
FaDu
SCC4
SCC9
SCC15
SCC105
UM6
YD38
SNU1066

B

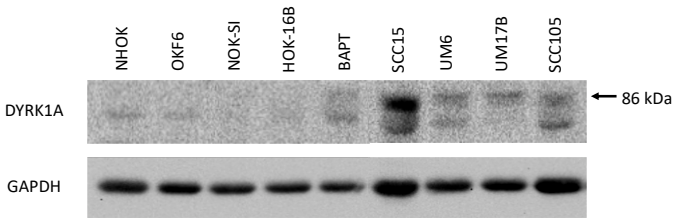

NHOK
OKF6
NOK-SI
HOK-16B
BAPT
SCC15
UM6
UM17B
SCC105
DYRK1A
86 kDa
GAPDH

Figure 2. DYRK1A deletion reduces OSCC proliferation. Genetic deletion of DYRK1A was performed in 2 OSCC cell lines (SCC4 and FaDu) by CRISPR/Cas9 genome editing technique. **(A)** Deletion of DYRK1A protein expression in SCC4 and FaDu was confirmed by Western blot. GAPDH was used as a loading control. WT; Wild-type, KO; DYRK1A Knockout. **(B)** Effect of DYRK1A deletion on cell proliferation was determined by cell counting. Data are means ± SD of triplicate experiments. *$P < 0.003$, and **$P < 0.001$ by two-tailed Student's *t* test. **(C)** Effect of DYRK1A deletion on cell cycle progression was determined by PI staining using SCC4 WT and SCC4 KO cells. **(D)** Effect of DYRK1A deletion on apoptosis was determined by Annexin V-FITC and PI staining followed by flow cytometry using SCC4 WT and SCC4 KO cells. Living cells were stained negative for both Annexin V-FITC and PI. Cells stained Annexin V positive/PI negative were classified as early-stage apoptotic cells, and double-positive cells were classified as late-stage apoptotic cells. Cells stained Annexin V negative/PI positive were classified as dead cells.

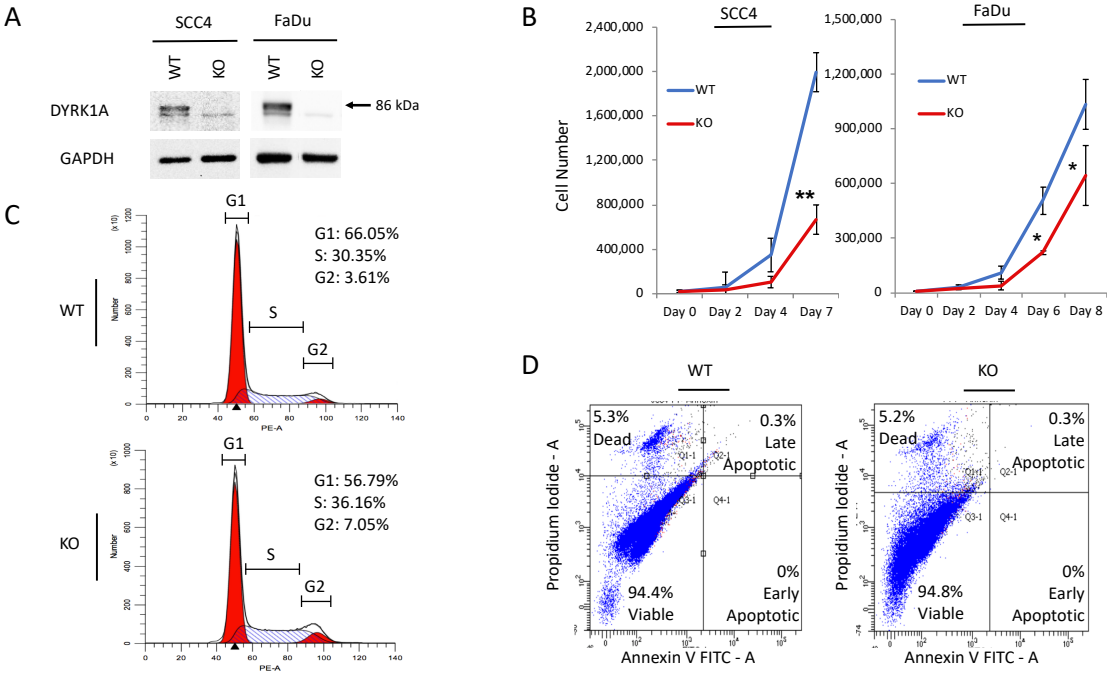

A
SCC4
FaDu
WT
KO
WT
KO
DYRK1A
86 kDa
GAPDH
B
SCC4
FaDu
Cell Number
WT
KO
Day 0 Day 2 Day 4 Day 7
Day 0 Day 2 Day 4 Day 6 Day 8
C
WT
G1
S
G2
G1: 66.05%
S: 30.35%
G2: 3.61%
KO
G1: 56.79%
S: 36.16%
G2: 7.05%
PE-A
D
WT
KO
Propidium Iodide - A
Annexin V FITC - A
5.3% Dead
0.3% Late Apoptotic
94.4% Viable
0% Early Apoptotic
5.2% Dead
0.3% Late Apoptotic
94.8% Viable
0% Early Apoptotic

Figure 3. DYRK1A deletion reduces OSCC tumorigenicity *in vitro* and *in vivo.*

(A) Effect of DYRK1A deletion on anchorage-independent growth ability was determined by soft agar assay. Data are means ±SD of triplicate experiment. * $P < 0.05$ was determined by two-tailed Student's *t* test. **(B)** Representative images of colonies formed in anchorage independent assay with SCC4 WT and SCC4 DYRK1A KO. Upper images were taken at 4X magnification and lower images were taken at 10X magnification. **(C)** Effect of DYRK1A deletion on *in vivo* tumorigenicity was determined by xenograft tumor assay. FaDu WT and FaDu DYRK1A KO were injected subcutaneously into 5 nude mice. Tumor sizes were measured twice a week for 3 weeks. After 3 weeks, tumors were excised and weighed.

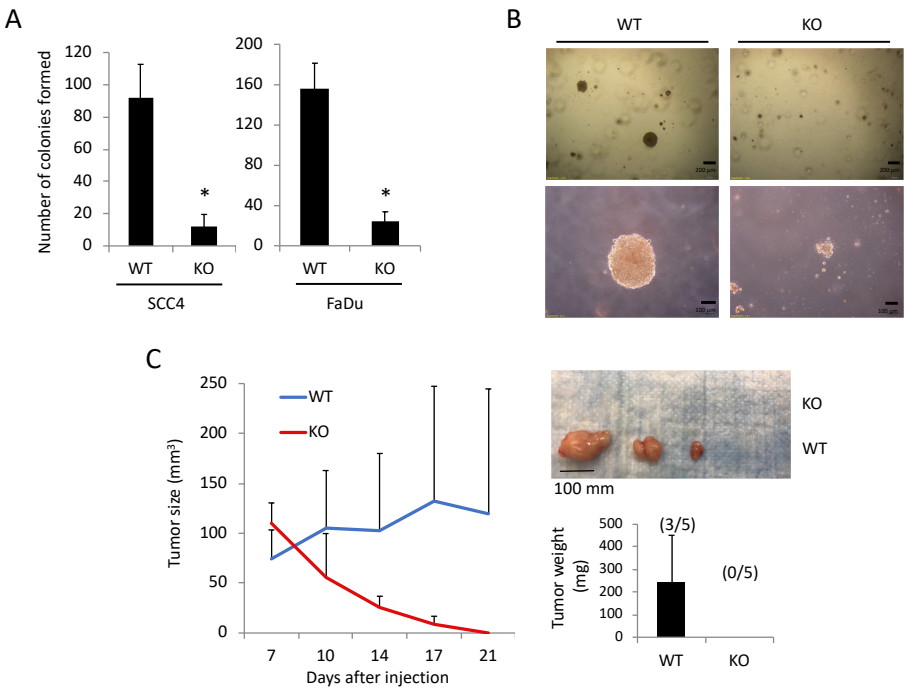

A
Number of colonies formed
120
100
80
60
40
20
0
WT
KO
SCC4
200
160
120
80
40
0
WT
KO
FaDu
B
WT
KO
C
Tumor size (mm³)
250
200
150
100
50
0
WT
KO
7
10
14
17
21
Days after injection
KO
WT
100 mm
Tumor weight (mg)
500
400
300
200
100
0
(3/5)
(0/5)
WT
KO

Figure 4: DYRK1A deletion diminishes the number of CSCs in OSCC.

(A) Effect of DYRK1A deletion on ALDH1^{HIGH} cell population (CSC population) in OSCC was determined by Aldefluor assay. Cells were labeled with Aldefluor with and without the ALDH inhibitor DEAB and analyzed by flow cytometry. The gate for ALDH + cells is determined in relation to the DEAB control (+DEAB) and shows the brightly fluorescent ALDH population versus the side scatter, a population that is absent/decreased in the presence of DEAB. The number shown in each panel reflects the percentage of ALDH1^{HIGH} cells in each cell type. **(B)** Effect of DYRK1A deletion on self-renewing CSC population in OSCC was determined by tumor sphere formation assay. Single cells were plated in ultralow attachment plates at a density of 3000 cells/ml in serum-free tumor sphere medium. Tumor spheres were counted on day 7. Representative images of tumor spheres formed by the WT and DYRK1A KO cells were shown on the right.

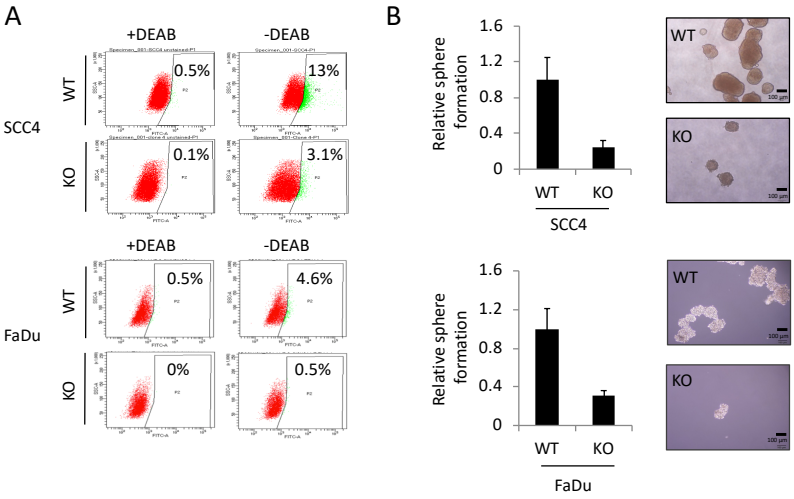

A
+DEAB
-DEAB
SCC4
WT
KO
0.5%
13%
0.1%
3.1%
FaDu
0.5%
4.6%
0%
0.5%
B
Relative sphere formation
1.6
1.2
0.8
0.4
0
WT
KO
SCC4
FaDu

Figure 5: DYRK1A deletion inhibits CSC properties in OSCC.

(A) Effect of DYRK1A deletion on chemoresistance of OSCC was determined by MTT assay. SCC4 WT and SCC4 DYRK1A KO cells were treated with 10, 30, and 50 μM of cisplatin for 2 days, and their viability was determined. *$P < 0.003$ and **$P < 0.0001$ **(B)** Effect of DYRK1A deletion 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. **(C)** Representative images of transwell migration assay.

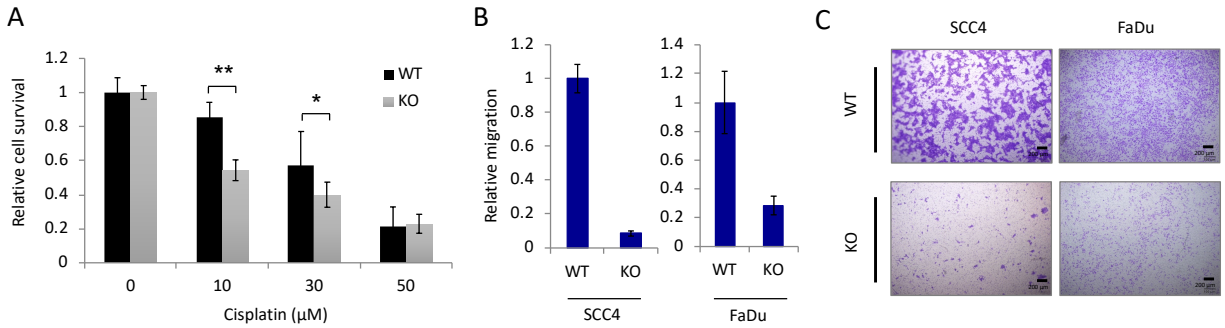

A
Relative cell survival
1.2
1
0.8
0.6
0.4
0.2
0
**
*
WT
KO
0
10
30
50
Cisplatin (μM)
B
Relative migration
1.2
1
0.8
0.6
0.4
0.2
0
WT
KO
SCC4
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
KO
FaDu
C
SCC4
FaDu
WT
KO

Figure 6: DYRK1A expression is enriched in self-renewing CSCs in OSCC.

(A) Expression of 5 DYRK isoforms was assessed in tumor spheres (Sphere) and adherent monolayer cells (Mono) derived from FaDu by qPCR. **(B)** Expression of DYRK1A was assessed in tumor spheres (Sphere) and adherent monolayer cells (Mono) derived from FaDu by Western blot analysis. **(C)** Expression of DYRK1A was assessed in tumor spheres (Sph.) and adherent monolayer cells (Mono.) derived from multiple OSCC cell lines (FaDu, BapT, and SCC9/TNF) by qPCR.

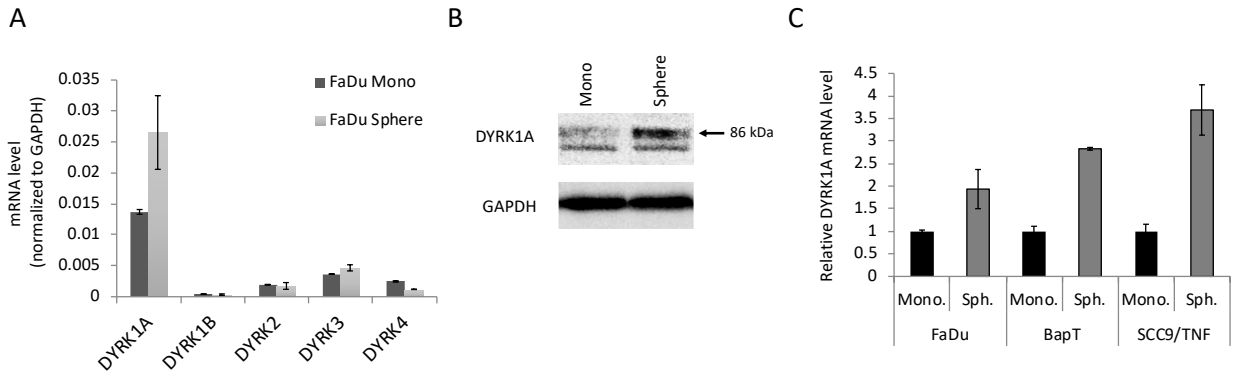

A
B
C
mRNA level (normalized to GAPDH)
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
FaDu Mono
FaDu Sphere
DYRK1A
DYRK1B
DYRK2
DYRK3
DYRK4
Mono
Sphere
DYRK1A
86 kDa
GAPDH
Relative DYRK1A mRNA level
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Mono.
Sph.
Mono.
Sph.
Mono.
Sph.
FaDu
BapT
SCC9/TNF

Figure 7: DYRK1A expression is enriched in chemoresistant OSCC cells.

(A) Schematic description of establishment of chemoresistant OSCC cells. Cisplatin-resistant OSCC cell line was established by treating SCC4 with 10 μM of cisplatin for 24 hours. After the treatment, surviving cells were isolated and named as SCC4/CispR. MTT assay revealed that the IC50 value of control SCC4 and SCC4/CispR cells are 31.5 μM and 70 μM, respectively, thus indicating a successful generation of cisplatin-resistant OSCC cell line. **(B)** Expression of DYRK isoforms was measured in SCC4 and SCC4/CispR by qPCR. **(C)** Expression of DYRK1A was measured in SCC4 and SCC4/CispR by Western blot analysis.

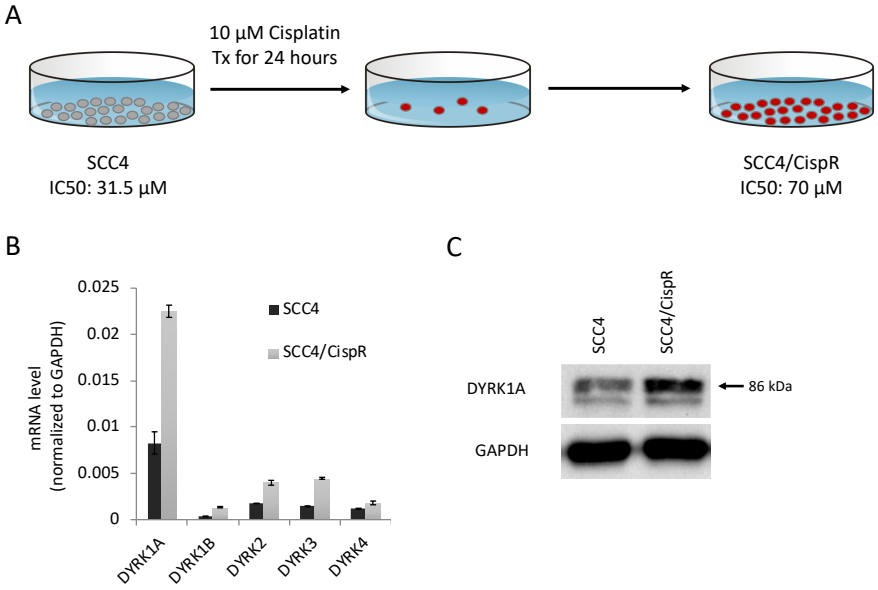

A
10 µM Cisplatin
Tx for 24 hours
SCC4
IC50: 31.5 µM
SCC4/CispR
IC50: 70 µM
B
mRNA level
(normalized to GAPDH)
0.025
0.02
0.015
0.01
0.005
0
SCC4
SCC4/CispR
DYRK1A
DYRK1B
DYRK2
DYRK3
DYRK4
C
SCC4
SCC4/CispR
DYRK1A
86 kDa
GAPDH

Figure 8: ALDH^{HIGH} OSCC cells express higher DYRK1A expression compared to ALDH^{low} OSCC cells.

ALDH1^{HIGH} (CSC-enriched population) and ALDH1^{low} (non-CSC population) cell populations were sorted from SCC4 cells by flow cytometry. Expression of DYRK1A was measured in the ALDH^{HIGH} and ALDH^{low} populations using qPCR. ** $P < 0.0002$ was determined by two-tailed Student's *t* test.

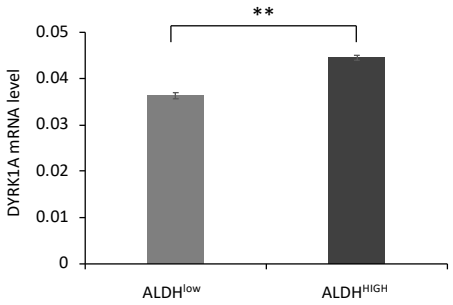

**
0.05
0.04
0.03
0.02
0.01
0
DYRK1A mRNA level
ALDHlow
ALDHHIGH

Figure 9: DYRK1A deletion does not affect NFAT activity

(A) Effect of DYRK1A deletion on the intracellular localization of NFATc3 was determined by Western blot analysis using the cytoplasmic (Cyto.) and nuclear (Nuc.) extracts isolated from the WT and DYRK1A KO cells. Lamin B is a nuclear protein and α-tubulin is a cytoplasmic protein, which were used as loading controls. **(B)** Intracellular localization of NFATc3 was determined in SCC4 WT and SCC4 DYRK1A KO by confocal laser scanning microscopy. After cell permeabilization and blocking, cells were probed with NFATc3 primary antibody overnight, then with Alexa Fluor 594 dye-conjugated secondary antibody and DAPI (blue-green) for confocal laser scanning. NFATc3 immunofluorescence staining (red) mainly in the cytoplasm of both cell types was observed. Representative images were taken at 120x magnification.

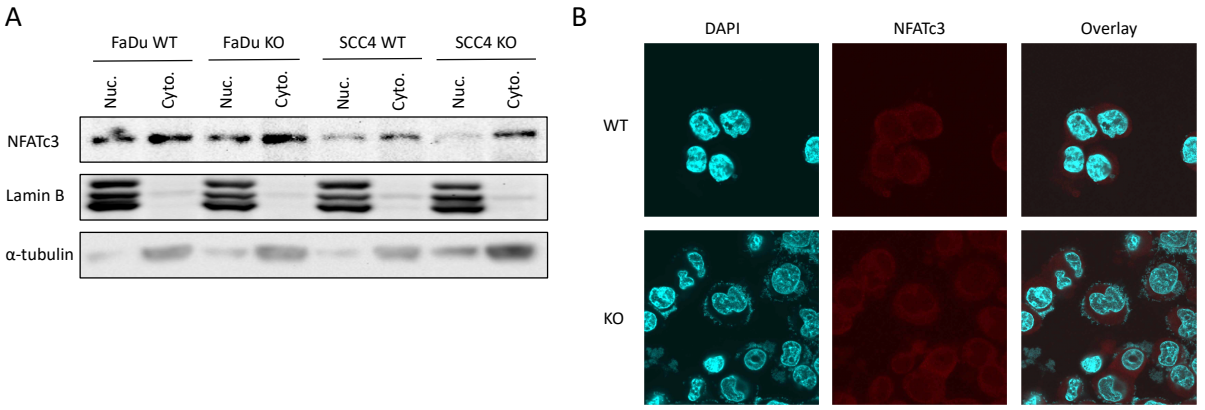

A
FaDu WT
FaDu KO
SCC4 WT
SCC4 KO
Nuc.
Cyto.
NFATc3
Lamin B
α-tubulin
B
DAPI
NFATc3
Overlay
WT
KO

Figure 10: DYRK1A deletion suppresses FGF2/ERK signaling pathway

(A) Expression levels of CSC-related factors (β-catenin, Bmi1, c-Myc, Oct4, and FGF2) were compared between WT and KO cell lines by Western blot analysis. **(B)** Effect of DYRK1A deletion on FGF2 expression was further confirmed by Western blot analysis using different protein amounts (8 μg, 24 μg, and 72 μg). **(C)** Effect of DYRK1A deletion on ERK1/2 signaling pathway was determined by Western blot analysis using a phospho-specific ERK1/2 antibody. ß-actin was used as a loading control.

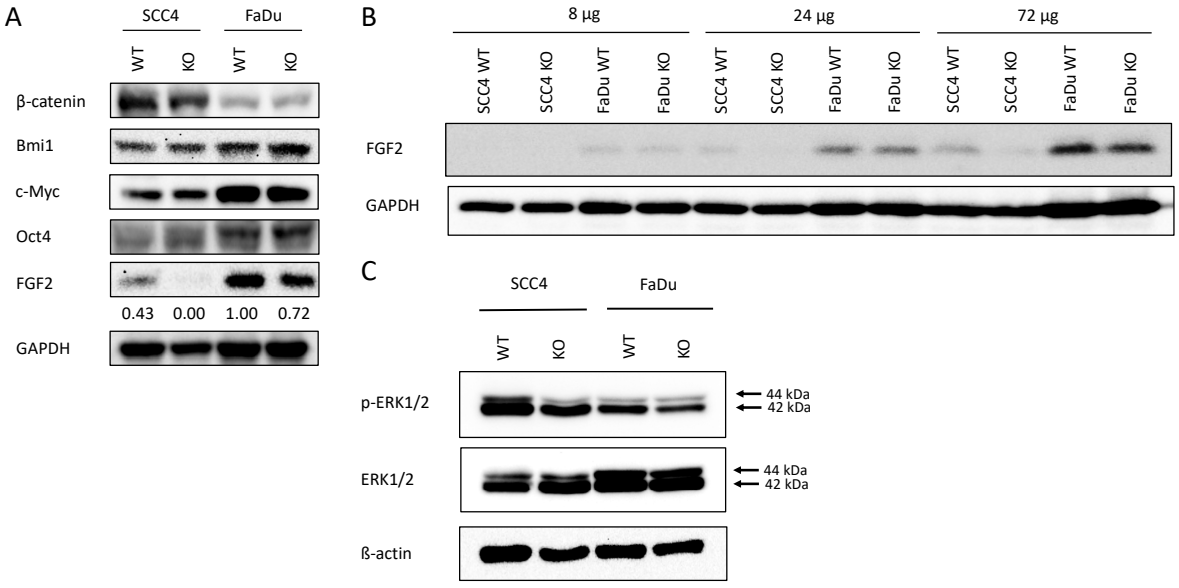

A
SCC4
FaDu
WT
KO
WT
KO
β-catenin
Bmi1
c-Myc
Oct4
FGF2
0.43
0.00
1.00
0.72
GAPDH
B
8 μg
24 μg
72 μg
SCC4 WT
SCC4 KO
FaDu WT
FaDu KO
FGF2
GAPDH
C
SCC4
FaDu
WT
KO
WT
KO
p-ERK1/2
44 kDa
42 kDa
ERK1/2
44 kDa
42 kDa
ß-actin

Figure 11: Exogenous FGF2 treatment rescues CSC properties in DYRK1A-deleted OSCC

(A) Effect of FGF2 treatment on self-renewal capacity of SCC4 DYRK1A KO was measured by tumor sphere formation assay. The assays were performed in the absence and presence of FGF2 (20, 50, 100, and 200 ng/ml). $*P < 0.0317$ and $**P < 0.0013$. **(B)** Effect of FGF2 treatment on migration ability of SCC4 DYRK1A KO was measured by transwell migration assay. The assays were performed in the absence and presence of FGF2 (50, 100, and 200 ng/ml). $*P < 0.0157$ and $**P < 0.0201$.

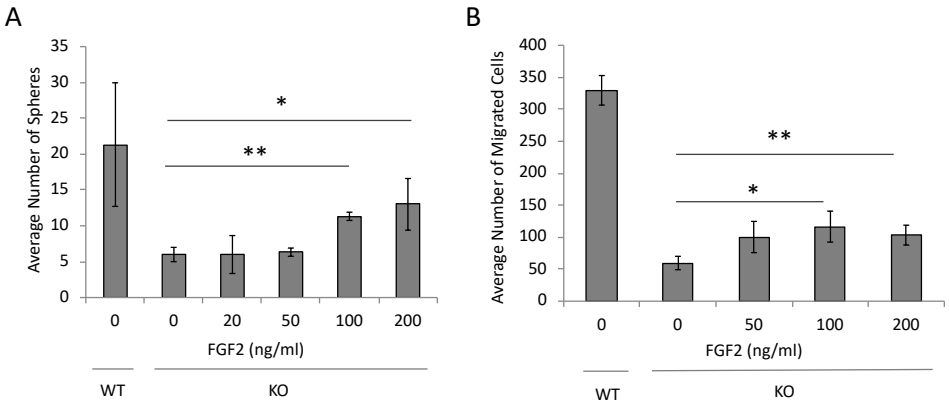

A
Average Number of Spheres
35
30
25
20
15
10
5
0
*
**
0
0
20
50
100
200
FGF2 (ng/ml)
WT
KO
B
Average Number of Migrated Cells
400
350
300
250
200
150
100
50
0
**
*
0
0
50
100
200
FGF2 (ng/ml)
WT
KO

Figure 12: Harmine, a DYRK1A chemical inhibitor suppresses malignant growth property of OSCC.

(A) Effect of harmine on OSCC growth was determined by MTT assay. Cell proliferation was measured 3 and 6 days after treatment with the indicated concentrations of harmine. **(B)** Effect of harmine on anchorage-independent growth ability of OSCC was determined by soft agar assay. The assays were performed in the absence and presence of harmine (1 and 2 μg/ml). Representative images of anchorage-independence assay were shown on the right of each bar graph.

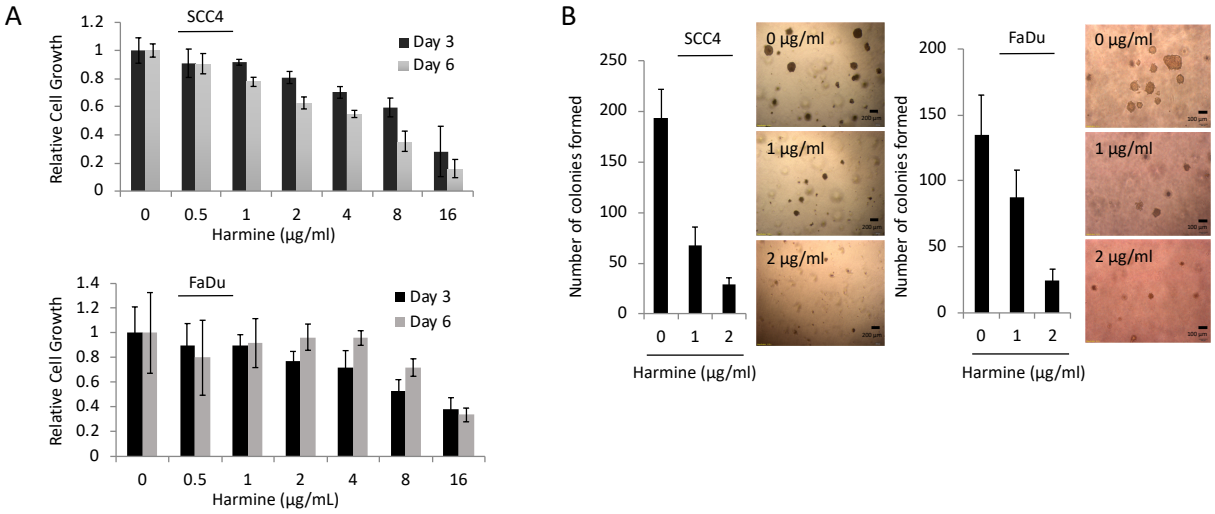

A
SCC4
Relative Cell Growth
Day 3
Day 6
Harmine (μg/ml)
FaDu
Relative Cell Growth
Day 3
Day 6
Harmine (μg/mL)
B
SCC4
Number of colonies formed
Harmine (μg/ml)
0 μg/ml
1 μg/ml
2 μg/ml
FaDu
Number of colonies formed
Harmine (μg/ml)
0 μg/ml
1 μg/ml
2 μg/ml

Figure 13: Harmine, a DYRK1A chemical inhibitor suppresses self-renewal capacity of OSCC.

(A) Effect of harmine on self-renewal capacity was measured by tumor sphere formation assay using multiple OSCC cell lines (SCC4, FaDu, SCC15, and BapT). The assays were performed in the absence and presence of harmine (1 and 2 μg/ml). Bar graph quantifies the relative number of tumor spheres formed by OSCC cells exposed to the indicated concentrations of harmine. **(B)** Representative images of tumor spheres formed by each groups.

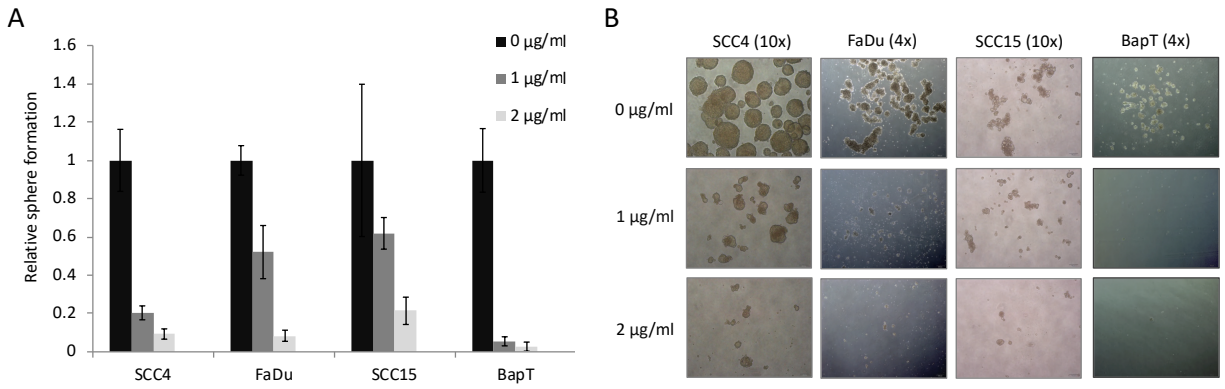
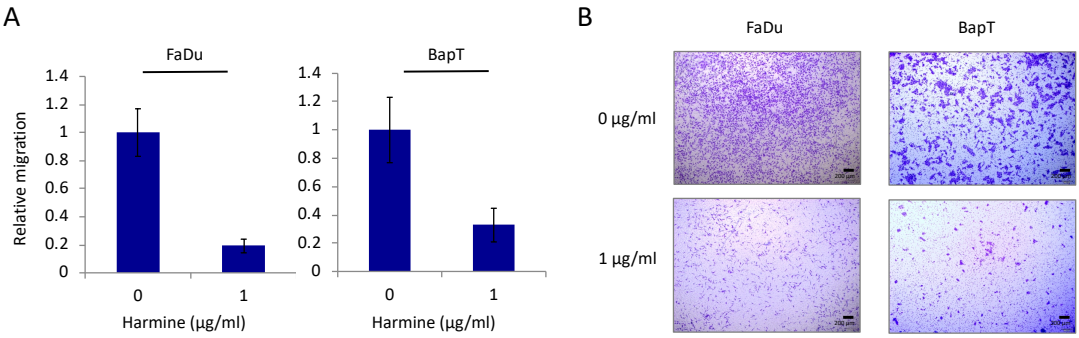

A
Relative sphere formation
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 µg/ml
1 µg/ml
2 µg/ml
SCC4
FaDu
SCC15
BapT
B
SCC4 (10x)
FaDu (4x)
SCC15 (10x)
BapT (4x)
0 µg/ml
1 µg/ml
2 µg/ml

Figure 14: Harmine, a DYRK1A chemical inhibitor suppresses migration ability of OSCC

(A) Effect of harmine on migration ability of OSCC was determined by transwell migration assay. The assays were performed in the absence and presence of harmine (1 μg/ml). Bar graph quantifies the relative number of migrated cells from control and harmine-treated OSCC cells . **(B)** Representative images of the migration assay with each groups.


A
FaDu
BapT
Relative migration
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
Harmine (µg/ml)
B
FaDu
BapT
0 µg/ml
1 µg/ml

REFERENCES

Abdullah, L. N., & Chow, E. K. (2013). Mechanisms of chemoresistance in cancer stem cells. *Clin Transl Med, 2*(1), 3. doi:10.1186/2001-1326-2-3

Abraham, B. K., Fritz, P., McClellan, M., Hauptvogel, P., Athelogou, M., & Brauch, H. (2005). Prevalence of CD44+/CD24-/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis. *Clin Cancer Res, 11*(3), 1154-1159.

Adayev, T., Chen-Hwang, M. C., Murakami, N., Wegiel, J., & Hwang, Y. W. (2006). Kinetic properties of a MNB/DYRK1A mutant suitable for the elucidation of biochemical pathways. *Biochemistry, 45*(39), 12011-12019. doi:10.1021/bi060632j

Adayev, T., Wegiel, J., & Hwang, Y. W. (2011). Harmine is an ATP-competitive inhibitor for dual-specificity tyrosine phosphorylation-regulated kinase 1A (Dyrk1A). *Arch Biochem Biophys, 507*(2), 212-218. doi:10.1016/j.abb.2010.12.024

Ailles, L. E., & Weissman, I. L. (2007). Cancer stem cells in solid tumors. *Curr Opin Biotechnol, 18*(5), 460-466. doi:10.1016/j.copbio.2007.10.007

Aktas, B., Tewes, M., Fehm, T., Hauch, S., Kimmig, R., & Kasimir-Bauer, S. (2009). Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. *Breast Cancer Res, 11*(4), R46. doi:10.1186/bcr2333

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., & Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A, 100*(7), 3983-3988. doi:10.1073/pnas.0530291100

Aranda, S., Alvarez, M., Turro, S., Laguna, A., & de la Luna, S. (2008). Sprouty2-mediated inhibition of fibroblast growth factor signaling is modulated by the protein kinase DYRK1A. *Mol Cell Biol, 28*(19), 5899-5911. doi:10.1128/MCB.00394-08

Aranda, S., Laguna, A., & de la Luna, S. (2011). DYRK family of protein kinases: evolutionary relationships, biochemical properties, and functional roles. *FASEB J, 25*(2), 449-462. doi:10.1096/fj.10-165837

Ardito, F., Giuliani, M., Perrone, D., Troiano, G., & Lo Muzio, L. (2017). The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review). *Int J Mol Med, 40*(2), 271-280. doi:10.3892/ijmm.2017.3036

Armstrong, A. J., Marengo, M. S., Oltean, S., Kemeny, G., Bitting, R. L., Turnbull, J. D., . . . Garcia-Blanco, M. A. (2011). Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. *Mol Cancer Res, 9*(8), 997-1007. doi:10.1158/1541-7786.MCR-10-0490

Arron, J. R., Winslow, M. M., Polleri, A., Chang, C. P., Wu, H., Gao, X., . . . Crabtree, G. R. (2006). NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. *Nature, 441*(7093), 595-600. doi:10.1038/nature04678

Baillie, R., Tan, S. T., & Itinteang, T. (2017). Cancer Stem Cells in Oral Cavity Squamous Cell Carcinoma: A Review. *Front Oncol, 7*, 112. doi:10.3389/fonc.2017.00112

Bain, J., McLauchlan, H., Elliott, M., & Cohen, P. (2003). The specificities of protein kinase inhibitors: an update. *Biochem J, 371*(Pt 1), 199-204. doi:10.1042/BJ20021535

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., McLauchlan, H., . . . Cohen, P. (2007). The selectivity of protein kinase inhibitors: a further update. *Biochem J, 408*(3), 297-315. doi:10.1042/BJ20070797

Beck, B., & Blanpain, C. (2013). Unravelling cancer stem cell potential. *Nat Rev Cancer, 13*(10), 727-738. doi:10.1038/nrc3597

Berger, W., Setinek, U., Mohr, T., Kindas-Mugge, I., Vetterlein, M., Dekan, G., . . . Micksche, M. (1999). Evidence for a role of FGF-2 and FGF receptors in the proliferation of non-small cell lung cancer cells. *Int J Cancer, 83*(3), 415-423.

Bresciani, F., Paoluzi, R., Benassi, M., Nervi, C., Casale, C., & Ziparo, E. (1974). Cell kinetics and growth of squamous cell carcinomas in man. *Cancer Res, 34*(9), 2405-2415.

Brooks, M. D., Burness, M. L., & Wicha, M. S. (2015). Therapeutic Implications of Cellular Heterogeneity and Plasticity in Breast Cancer. *Cell Stem Cell, 17*(3), 260-271. doi:10.1016/j.stem.2015.08.014

Chaffer, C. L., & Weinberg, R. A. (2011). A perspective on cancer cell metastasis. *Science, 331*(6024), 1559-1564. doi:10.1126/science.1203543

Chen, Y., Zhu, G., Wu, K., Gao, Y., Zeng, J., Shi, Q., . . . He, D. (2016). FGF2-mediated reciprocal tumor cell-endothelial cell interplay contributes to the growth of chemoresistant cells: a potential mechanism for superficial bladder cancer recurrence. *Tumour Biol, 37*(4), 4313-4321. doi:10.1007/s13277-015-4214-4

Chi, A. C., Day, T. A., & Neville, B. W. (2015). Oral cavity and oropharyngeal squamous cell carcinoma--an update. *CA Cancer J Clin, 65*(5), 401-421. doi:10.3322/caac.21293

Chiou, S. H., Yu, C. C., Huang, C. Y., Lin, S. C., Liu, C. J., Tsai, T. H., . . . Lo, J. F. (2008). Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. *Clin Cancer Res, 14*(13), 4085-4095. doi:10.1158/1078-0432.CCR-07-4404

Clay, M. R., Tabor, M., Owen, J. H., Carey, T. E., Bradford, C. R., Wolf, G. T., . . . Prince, M. E. (2010a). Single-Marker Identification of Head and Neck Squamous Cell Carcinoma Cancer Stem Cells with Aldehyde Dehydrogenase. *Head and Neck-Journal for the Sciences and Specialties of the Head and Neck, 32*(9), 1195-1201. doi:Doi 10.1002/Hed.21315

Clay, M. R., Tabor, M., Owen, J. H., Carey, T. E., Bradford, C. R., Wolf, G. T., . . . Prince, M. E. (2010b). Single-marker identification of head and neck squamous cell carcinoma cancer stem cells with aldehyde dehydrogenase. *Head Neck, 32*(9), 1195-1201. doi:10.1002/hed.21315

Clevers, H. (2011). The cancer stem cell: premises, promises and challenges. *Nat Med, 17*(3), 313-319. doi:10.1038/nm.2304

Cohen, P. (2002). Protein kinases--the major drug targets of the twenty-first century? *Nat Rev Drug Discov, 1*(4), 309-315. doi:10.1038/nrd773

Dalerba, P., Dylla, S. J., Park, I. K., Liu, R., Wang, X., Cho, R. W., . . . Clarke, M. F. (2007). Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci U S A, 104*(24), 10158-10163. doi:10.1073/pnas.0703478104

Dellacono, F. R., Spiro, J., Eisma, R., & Kreutzer, D. (1997). Expression of basic fibroblast growth factor and its receptors by head and neck squamous carcinoma tumor and vascular endothelial cells. *Am J Surg, 174*(5), 540-544.

Dick, J. E. (2008). Stem cell concepts renew cancer research. *Blood, 112*(13), 4793-4807. doi:10.1182/blood-2008-08-077941

Dontu, G., Abdallah, W. M., Foley, J. M., Jackson, K. W., Clarke, M. F., Kawamura, M. J., & Wicha, M. S. (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev, 17*(10), 1253-1270. doi:10.1101/gad.1061803

Duong, H. Q., Hwang, J. S., Kim, H. J., Kang, H. J., Seong, Y. S., & Bae, I. (2012). Aldehyde dehydrogenase 1A1 confers intrinsic and acquired resistance to gemcitabine in human pancreatic adenocarcinoma MIA PaCa-2 cells. *Int J Oncol, 41*(3), 855-861. doi:10.3892/ijo.2012.1516

Dylla, S. J., Beviglia, L., Park, I. K., Chartier, C., Raval, J., Ngan, L., . . . Gurney, A. L. (2008). Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS One, 3*(6), e2428. doi:10.1371/journal.pone.0002428

Eun, K., Ham, S. W., & Kim, H. (2017). Cancer stem cell heterogeneity: origin and new perspectives on CSC targeting. *BMB Rep, 50*(3), 117-125.

Fernandez-Martinez, P., Zahonero, C., & Sanchez-Gomez, P. (2015). DYRK1A: the double-edged kinase as a protagonist in cell growth and tumorigenesis. *Mol Cell Oncol, 2*(1), e970048. doi:10.4161/23723548.2014.970048

Ferrer, I., Barrachina, M., Puig, B., Martinez de Lagran, M., Marti, E., Avila, J., & Dierssen, M. (2005). Constitutive Dyrk1A is abnormally expressed in Alzheimer disease, Down syndrome, Pick disease, and related transgenic models. *Neurobiol Dis, 20*(2), 392-400. doi:10.1016/j.nbd.2005.03.020

Folkman, J. (2002). Role of angiogenesis in tumor growth and metastasis. *Semin Oncol, 29*(6 Suppl 16), 15-18. doi:10.1053/sonc.2002.37263

Galanski, M. (2006). Recent developments in the field of anticancer platinum complexes. *Recent Pat Anticancer Drug Discov, 1*(2), 285-295.

Gao, J., Zhu, H., Wan, H., Zou, X., Ma, X., & Gao, G. (2017). Harmine suppresses the proliferation and migration of human ovarian cancer cells through inhibiting ERK/CREB pathway. *Oncol Rep, 38*(5), 2927-2934. doi:10.3892/or.2017.5952

Ghotra, V. P., He, S., van der Horst, G., Nijhoff, S., de Bont, H., Lekkerkerker, A., . . . Danen, E. H. (2015). SYK is a candidate kinase target for the treatment of advanced prostate cancer. *Cancer Res, 75*(1), 230-240. doi:10.1158/0008-5472.CAN-14-0629

Grose, R., & Dickson, C. (2005). Fibroblast growth factor signaling in tumorigenesis. *Cytokine Growth Factor Rev, 16*(2), 179-186. doi:10.1016/j.cytogfr.2005.01.003

Gross, S., Rahal, R., Stransky, N., Lengauer, C., & Hoeflich, K. P. (2015). Targeting cancer with kinase inhibitors. *J Clin Invest, 125*(5), 1780-1789. doi:10.1172/JCI76094

Gwack, Y., Sharma, S., Nardone, J., Tanasa, B., Iuga, A., Srikanth, S., . . . Rao, A. (2006). A genome-wide Drosophila RNAi screen identifies DYRK-family kinases as regulators of NFAT. *Nature, 441*(7093), 646-650. doi:10.1038/nature04631

Halaban, R. (1996). Growth factors and melanomas. *Semin Oncol, 23*(6), 673-681.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. doi:10.1016/j.cell.2011.02.013

Hase, T., Kawashiri, S., Tanaka, A., Nozaki, S., Noguchi, N., Kato, K., . . . Nakagawa, K. (2006). Correlation of basic fibroblast growth factor expression with the invasion and the prognosis of oral squamous cell carcinoma. *J Oral Pathol Med, 35*(3), 136-139. doi:10.1111/j.1600-0714.2006.00397.x

Hasle, H., Clemmensen, I. H., & Mikkelsen, M. (2000). [Incidence of cancer in individuals with Down syndrome]. *Tidsskr Nor Laegeforen, 120*(24), 2878-2881.

Hellsten, R., Johansson, M., Dahlman, A., Sterner, O., & Bjartell, A. (2011). Galiellalactone inhibits stem cell-like ALDH-positive prostate cancer cells. *PLoS One, 6*(7), e22118. doi:10.1371/journal.pone.0022118

Hermann, P. C., Huber, S. L., Herrler, T., Aicher, A., Ellwart, J. W., Guba, M., . . . Heeschen, C. (2007). Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell, 1*(3), 313-323. doi:10.1016/j.stem.2007.06.002

Hilton, J. (1984). Role of aldehyde dehydrogenase in cyclophosphamide-resistant L1210 leukemia. *Cancer Res, 44*(11), 5156-5160.

Horst, D., Kriegl, L., Engel, J., Kirchner, T., & Jung, A. (2009). Prognostic significance of the cancer stem cell markers CD133, CD44, and CD166 in colorectal cancer. *Cancer Invest, 27*(8), 844-850. doi:10.1080/07357900902744502

Hu, J., & Friedman, E. (2010). Depleting Mirk Kinase Increases Cisplatin Toxicity in Ovarian Cancer Cells. *Genes Cancer, 1*(8), 803-811. doi:10.1177/1947601910377644

Hu, Y., Mintz, A., Shah, S. R., Quinones-Hinojosa, A., & Hsu, W. (2014). The FGFR/MEK/ERK/brachyury pathway is critical for chordoma cell growth and survival. *Carcinogenesis, 35*(7), 1491-1499. doi:10.1093/carcin/bgu014

Huang, T. Y., Hsu, L. P., Wen, Y. H., Huang, T. T., Chou, Y. F., Lee, C. F., . . . Chen, P. R. (2010). Predictors of locoregional recurrence in early stage oral cavity cancer with free surgical margins. *Oral Oncol, 46*(1), 49-55. doi:10.1016/j.oraloncology.2009.10.011

Hubbard, S. R., & Till, J. H. (2000). Protein tyrosine kinase structure and function. *Annu Rev Biochem, 69*, 373-398. doi:10.1146/annurev.biochem.69.1.373

Jauliac, S., Lopez-Rodriguez, C., Shaw, L. M., Brown, L. F., Rao, A., & Toker, A. (2002). The role of NFAT transcription factors in integrin-mediated carcinoma invasion. *Nat Cell Biol, 4*(7), 540-544. doi:10.1038/ncb816

Johnson, S., Chen, H., & Lo, P. K. (2013). In vitro Tumorsphere Formation Assays. *Bio Protoc, 3*(3).

Kamran, M. Z., Patil, P., & Gude, R. P. (2013). Role of STAT3 in cancer metastasis and translational advances. *Biomed Res Int, 2013*, 421821. doi:10.1155/2013/421821

Kang, M. K., & Park, N. H. (2001). Conversion of normal to malignant phenotype: telomere shortening, telomerase activation, and genomic instability during immortalization of human oral keratinocytes. *Crit Rev Oral Biol Med, 12*(1), 38-54.

Kim, H. S., Pearson, A. T., & Nor, J. E. (2016). Isolation and Characterization of Cancer Stem Cells from Primary Head and Neck Squamous Cell Carcinoma Tumors. *Methods Mol Biol, 1395*, 241-249. doi:10.1007/978-1-4939-3347-1_14

Kohno, N., Kitahara, S., Tamura, E., Tanabe, T., Nakanoboh, M., Kawada, M., & Shirasaka, T. (2000). The role of low dose cisplatin plus 5-fluorouracil for treatment of recurrent and/or advanced squamous cell carcinoma of the head and neck. *Gan To Kagaku Ryoho, 27 Suppl 2*, 592-599.

Kottakis, F., Polytarchou, C., Foltopoulou, P., Sanidas, I., Kampranis, S. C., & Tsichlis, P. N. (2011). FGF-2 regulates cell proliferation, migration, and angiogenesis through an NDY1/KDM2B-miR-101-EZH2 pathway. *Mol Cell, 43*(2), 285-298. doi:10.1016/j.molcel.2011.06.020

Kumar, M., Nanavati, R., Modi, T. G., & Dobariya, C. (2016). Oral cancer: Etiology and risk factors: A review. *J Cancer Res Ther, 12*(2), 458-463. doi:10.4103/0973-1482.186696

Kurabayashi, N., Nguyen, M. D., & Sanada, K. (2015). DYRK1A overexpression enhances STAT activity and astrogliogenesis in a Down syndrome mouse model. *EMBO Rep, 16*(11), 1548-1562. doi:10.15252/embr.201540374

Laguna, A., Aranda, S., Barallobre, M. J., Barhoum, R., Fernandez, E., Fotaki, V., . . . Arbones, M. L. (2008). The protein kinase DYRK1A regulates caspase-9-mediated apoptosis during retina development. *Dev Cell, 15*(6), 841-853. doi:10.1016/j.devcel.2008.10.014

Larsen, S. R., Johansen, J., Sorensen, J. A., & Krogdahl, A. (2009). The prognostic significance of histological features in oral squamous cell carcinoma. *J Oral Pathol Med, 38*(8), 657-662. doi:10.1111/j.1600-0714.2009.00797.x

Lau, M. T., So, W. K., & Leung, P. C. (2013). Fibroblast growth factor 2 induces E-cadherin down-regulation via PI3K/Akt/mTOR and MAPK/ERK signaling in ovarian cancer cells. *PLoS One, 8*(3), e59083. doi:10.1371/journal.pone.0059083

Lee, S. B., Frattini, V., Bansal, M., Castano, A. M., Sherman, D., Hutchinson, K., . . . Lasorella, A. (2016). An ID2-dependent mechanism for VHL inactivation in cancer. *Nature, 529*(7585), 172-177. doi:10.1038/nature16475

Lee, S. H., Rigas, N. K., Lee, C. R., Bang, A., Srikanth, S., Gwack, Y., . . . Shin, K. H. (2016). Orai1 promotes tumor progression by enhancing cancer stemness via NFAT signaling in oral/oropharyngeal squamous cell carcinoma. *Oncotarget, 7*(28), 43239-43255. doi:10.18632/oncotarget.9755

Litovchick, L., Florens, L. A., Swanson, S. K., Washburn, M. P., & DeCaprio, J. A. (2011). DYRK1A protein kinase promotes quiescence and senescence through DREAM complex assembly. *Genes Dev, 25*(8), 801-813. doi:10.1101/gad.2034211

Liu, H., Patel, M. R., Prescher, J. A., Patsialou, A., Qian, D., Lin, J., . . . Clarke, M. F. (2010). Cancer stem cells from human breast tumors are involved in spontaneous metastases in orthotopic mouse models. *Proc Natl Acad Sci U S A, 107*(42), 18115-18120. doi:10.1073/pnas.1006732107

Liu, J. C., Deng, T., Lehal, R. S., Kim, J., & Zacksenhaus, E. (2007). Identification of tumorsphere- and tumor-initiating cells in HER2/Neu-induced mammary tumors. *Cancer Res, 67*(18), 8671-8681. doi:10.1158/0008-5472.CAN-07-1486

Liu, J. F., Crepin, M., Liu, J. M., Barritault, D., & Ledoux, D. (2002). FGF-2 and TPA induce matrix metalloproteinase-9 secretion in MCF-7 cells through PKC activation of the Ras/ERK pathway. *Biochem Biophys Res Commun, 293*(4), 1174-1182. doi:10.1016/S0006-291X(02)00350-9

Liu, Q., Liu, N., Zang, S., Liu, H., Wang, P., Ji, C., & Sun, X. (2014). Tumor suppressor DYRK1A effects on proliferation and chemoresistance of AML cells by downregulating c-Myc. *PLoS One, 9*(6), e98853. doi:10.1371/journal.pone.0098853

Liu, W., Wu, L., Shen, X. M., Shi, L. J., Zhang, C. P., Xu, L. Q., & Zhou, Z. T. (2013). Expression patterns of cancer stem cell markers ALDH1 and CD133 correlate with a high risk of malignant transformation of oral leukoplakia. *Int J Cancer, 132*(4), 868-874. doi:10.1002/ijc.27720

Longley, D. B., & Johnston, P. G. (2005). Molecular mechanisms of drug resistance. *J Pathol, 205*(2), 275-292. doi:10.1002/path.1706

Maehara, O., Suda, G., Natsuizaka, M., Ohnishi, S., Komatsu, Y., Sato, F., . . . Sakamoto, N. (2017). Fibroblast growth factor-2-mediated FGFR/Erk signaling supports maintenance of cancer stem-like cells in esophageal squamous cell carcinoma. *Carcinogenesis, 38*(11), 1073-1083. doi:10.1093/carcin/bgx095

Mancini, M., & Toker, A. (2009). NFAT proteins: emerging roles in cancer progression. *Nat Rev Cancer, 9*(11), 810-820. doi:10.1038/nrc2735

Matsuo, R., Ochiai, W., Nakashima, K., & Taga, T. (2001). A new expression cloning strategy for isolation of substrate-specific kinases by using phosphorylation site-specific antibody. *J Immunol Methods, 247*(1-2), 141-151.

Meng, R. D., Shelton, C. C., Li, Y. M., Qin, L. X., Notterman, D., Paty, P. B., & Schwartz, G. K. (2009). gamma-Secretase inhibitors abrogate oxaliplatin-induced activation of the Notch-1 signaling pathway in colon cancer cells resulting in enhanced chemosensitivity. *Cancer Res, 69*(2), 573-582. doi:10.1158/0008-5472.CAN-08-2088

Meyer, M. J., Fleming, J. M., Lin, A. F., Hussnain, S. A., Ginsburg, E., & Vonderhaar, B. K. (2010). CD44posCD49fhiCD133/2hi defines xenograft-initiating cells in estrogen receptor-negative breast cancer. *Cancer Res, 70*(11), 4624-4633. doi:10.1158/0008-5472.CAN-09-3619

Mimoto, R., Taira, N., Takahashi, H., Yamaguchi, T., Okabe, M., Uchida, K., . . . Yoshida, K. (2013). DYRK2 controls the epithelial-mesenchymal transition in breast cancer by degrading Snail. *Cancer Lett, 339*(2), 214-225. doi:10.1016/j.canlet.2013.06.005

Monroe, M. M., Anderson, E. C., Clayburgh, D. R., & Wong, M. H. (2011). Cancer stem cells in head and neck squamous cell carcinoma. *J Oncol, 2011*, 762780. doi:10.1155/2011/762780

Mori, S., Chang, J. T., Andrechek, E. R., Matsumura, N., Baba, T., Yao, G., . . . Nevins, J. R. (2009). Anchorage-independent cell growth signature identifies tumors with metastatic potential. *Oncogene, 28*(31), 2796-2805. doi:10.1038/onc.2009.139

Moroni, M., Soldatenkov, V., Zhang, L., Zhang, Y., Stoica, G., Gehan, E., . . . Mueller, S. C. (2004). Progressive loss of Syk and abnormal proliferation in breast cancer cells. *Cancer Res, 64*(20), 7346-7354. doi:10.1158/0008-5472.CAN-03-3520

Muller, M. R., & Rao, A. (2010). NFAT, immunity and cancer: a transcription factor comes of age. *Nat Rev Immunol, 10*(9), 645-656. doi:10.1038/nri2818

Nayak, S., Goel, M. M., Makker, A., Bhatia, V., Chandra, S., Kumar, S., & Agarwal, S. P. (2015). Fibroblast Growth Factor (FGF-2) and Its Receptors FGFR-2 and FGFR-3 May Be Putative Biomarkers of Malignant Transformation of Potentially Malignant Oral Lesions into Oral Squamous Cell Carcinoma. *PLoS One, 10*(10), e0138801. doi:10.1371/journal.pone.0138801

Nishikawa, R., Ji, X. D., Harmon, R. C., Lazar, C. S., Gill, G. N., Cavenee, W. K., & Huang, H. J. (1994). A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc Natl Acad Sci U S A, 91*(16), 7727-7731.

Noda, T., Nagano, H., Takemasa, I., Yoshioka, S., Murakami, M., Wada, H., . . . Monden, M. (2009). Activation of Wnt/beta-catenin signalling pathway induces chemoresistance to interferon-alpha/5-fluorouracil combination therapy for hepatocellular carcinoma. *Br J Cancer, 100*(10), 1647-1658. doi:10.1038/sj.bjc.6605064

O'Brien, C. A., Kreso, A., & Jamieson, C. H. (2010). Cancer stem cells and self-renewal. *Clin Cancer Res, 16*(12), 3113-3120. doi:10.1158/1078-0432.CCR-09-2824

Ota, N., Ohno, J., Seno, K., Taniguchi, K., & Ozeki, S. (2014). In vitro and in vivo expression of aldehyde dehydrogenase 1 in oral squamous cell carcinoma. *Int J Oncol, 44*(2), 435-442. doi:10.3892/ijo.2013.2188

Pallini, R., Ricci-Vitiani, L., Banna, G. L., Signore, M., Lombardi, D., Todaro, M., . . . De Maria, R. (2008). Cancer stem cell analysis and clinical outcome in patients with glioblastoma multiforme. *Clin Cancer Res, 14*(24), 8205-8212. doi:10.1158/1078-0432.CCR-08-0644

Park, J., Song, W. J., & Chung, K. C. (2009). Function and regulation of Dyrk1A: towards understanding Down syndrome. *Cell Mol Life Sci, 66*(20), 3235-3240. doi:10.1007/s00018-009-0123-2

Peter, M. E. (2010). Regulating cancer stem cells the miR way. *Cell Stem Cell, 6*(1), 4-6. doi:10.1016/j.stem.2009.12.006

Pozo, N., Zahonero, C., Fernandez, P., Linares, J. M., Ayuso, A., Hagiwara, M., . . . Sanchez-Gomez, P. (2013). Inhibition of DYRK1A destabilizes EGFR and reduces EGFR-dependent glioblastoma growth. *J Clin Invest, 123*(6), 2475-2487. doi:10.1172/JCI63623

Pramanik, K. K., Singh, A. K., Alam, M., Kashyap, T., Mishra, P., Panda, A. K., . . . Mishra, R. (2016). Reversion-inducing cysteine-rich protein with Kazal motifs and its regulation by glycogen synthase kinase 3 signaling in oral cancer. *Tumour Biol, 37*(11), 15253-15264. doi:10.1007/s13277-016-5362-x

Prescott, D. M. (1972). Biology of cancer and the cancer cell: normal and abnormal regulation of cell reproduction. *CA Cancer J Clin, 22*(4), 262-272.

Prince, M. E., Sivanandan, R., Kaczorowski, A., Wolf, G. T., Kaplan, M. J., Dalerba, P., . . . Ailles, L. E. (2007). Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci U S A, 104*(3), 973-978. doi:10.1073/pnas.0610117104

Prince, M. E. P., Zhou, L., Moyer, J. S., Tao, H. M., Lu, L., Owen, J., . . . Li, Q. (2016). Evaluation of the immunogenicity of ALDH(high) human head and neck squamous cell carcinoma cancer stem cells in vitro. *Oral Oncology, 59*, 30-42. doi:10.1016/j.oraloncology.2016.05.013

Radhakrishnan, A., Nanjappa, V., Raja, R., Sathe, G., Puttamallesh, V. N., Jain, A. P., . . . Chatterjee, A. (2016). A dual specificity kinase, DYRK1A, as a potential therapeutic target for head and neck squamous cell carcinoma. *Sci Rep, 6*, 36132. doi:10.1038/srep36132

Reguart, N., & Remon, J. (2015). Common EGFR-mutated subgroups (Del19/L858R) in advanced non-small-cell lung cancer: chasing better outcomes with tyrosine kinase inhibitors. *Future Oncol, 11*(8), 1245-1257. doi:10.2217/fon.15.15

Richard, V., Sebastian, P., Nair, M. G., Nair, S. N., Malieckal, T. T., Kumar, T. R. S., & Pillai, M. R. (2013). Multiple drug resistant, tumorigenic stem-like cells in oral cancer. *Cancer Letters, 338*(2), 300-316. doi:DOI 10.1016/j.canlet.2013.06.011

Rozen, E. J., Roewenstrunk, J., Barallobre, M. J., Di Vona, C., Jung, C., Figueiredo, A. F., . . . de la Luna, S. (2018). DYRK1A Kinase Positively Regulates Angiogenic Responses in Endothelial Cells. *Cell Rep, 23*(6), 1867-1878. doi:10.1016/j.celrep.2018.04.008

Ruan, S., Jia, F., & Li, J. (2017). Potential Antitumor Effect of Harmine in the Treatment of Thyroid Cancer. *Evid Based Complement Alternat Med, 2017*, 9402615. doi:10.1155/2017/9402615

Sano, D., & Myers, J. N. (2007). Metastasis of squamous cell carcinoma of the oral tongue. *Cancer Metastasis Rev, 26*(3-4), 645-662. doi:10.1007/s10555-007-9082-y

Schatton, T., Frank, N. Y., & Frank, M. H. (2009). Identification and targeting of cancer stem cells. *Bioessays, 31*(10), 1038-1049. doi:10.1002/bies.200900058

Seifert, A., Allan, L. A., & Clarke, P. R. (2008). DYRK1A phosphorylates caspase 9 at an inhibitory site and is potently inhibited in human cells by harmine. *FEBS J, 275*(24), 6268-6280. doi:10.1111/j.1742-4658.2008.06751.x

Shaw, J. P., Utz, P. J., Durand, D. B., Toole, J. J., Emmel, E. A., & Crabtree, G. R. (1988). Identification of a putative regulator of early T cell activation genes. *Science, 241*(4862), 202-205.

Shchemelinin, I., Sefc, L., & Necas, E. (2006). Protein kinases, their function and implication in cancer and other diseases. *Folia Biol (Praha), 52*(3), 81-100.

Sheridan, C., Kishimoto, H., Fuchs, R. K., Mehrotra, S., Bhat-Nakshatri, P., Turner, C. H., . . . Nakshatri, H. (2006). CD44+/CD24- breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res, 8*(5), R59. doi:10.1186/bcr1610

Shiozawa, Y., Nie, B., Pienta, K. J., Morgan, T. M., & Taichman, R. S. (2013). Cancer stem cells and their role in metastasis. *Pharmacol Ther, 138*(2), 285-293. doi:10.1016/j.pharmthera.2013.01.014

Siegel, R. L., Miller, K. D., & Jemal, A. (2018). Cancer statistics, 2018. *CA Cancer J Clin, 68*(1), 7-30. doi:10.3322/caac.21442

Smolensky, D., Rathore, K., Bourn, J., & Cekanova, M. (2017). Inhibition of the PI3K/AKT Pathway Sensitizes Oral Squamous Cell Carcinoma Cells to Anthracycline-Based Chemotherapy In Vitro. *J Cell Biochem, 118*(9), 2615-2624. doi:10.1002/jcb.25747

Soltysova, A., Altanerova, V., & Altaner, C. (2005). Cancer stem cells. *Neoplasma, 52*(6), 435-440.

Sullivan, J. P., Spinola, M., Dodge, M., Raso, M. G., Behrens, C., Gao, B., . . . Minna, J. D. (2010). Aldehyde dehydrogenase activity selects for lung adenocarcinoma stem cells dependent on notch signaling. *Cancer Res, 70*(23), 9937-9948. doi:10.1158/0008-5472.CAN-10-0881

Takahashi, J. A., Fukumoto, M., Igarashi, K., Oda, Y., Kikuchi, H., & Hatanaka, M. (1992). Correlation of basic fibroblast growth factor expression levels with the degree of malignancy and vascularity in human gliomas. *J Neurosurg, 76*(5), 792-798. doi:10.3171/jns.1992.76.5.0792

Tejedor, F. J., & Hammerle, B. (2011). MNB/DYRK1A as a multiple regulator of neuronal development. *FEBS J, 278*(2), 223-235. doi:10.1111/j.1742-4658.2010.07954.x

Uhlen, M., Fagerberg, L., Hallstrom, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., . . . Ponten, F. (2015). Proteomics. Tissue-based map of the human proteome. *Science, 347*(6220), 1260419. doi:10.1126/science.1260419

Uhlen, M., Zhang, C., Lee, S., Sjostedt, E., Fagerberg, L., Bidkhori, G., . . . Ponten, F. (2017). A pathology atlas of the human cancer transcriptome. *Science, 357*(6352). doi:10.1126/science.aan2507

Vermeulen, L., Sprick, M. R., Kemper, K., Stassi, G., & Medema, J. P. (2008). Cancer stem cells--old concepts, new insights. *Cell Death Differ, 15*(6), 947-958. doi:10.1038/cdd.2008.20

Visvader, J. E., & Lindeman, G. J. (2012). Cancer stem cells: current status and evolving complexities. *Cell Stem Cell, 10*(6), 717-728. doi:10.1016/j.stem.2012.05.007

Wainwright, L. J., Lasorella, A., & Iavarone, A. (2001). Distinct mechanisms of cell cycle arrest control the decision between differentiation and senescence in human neuroblastoma cells. *Proc Natl Acad Sci U S A, 98*(16), 9396-9400. doi:10.1073/pnas.161288698

Walte, A., Ruben, K., Birner-Gruenberger, R., Preisinger, C., Bamberg-Lemper, S., Hilz, N., . . . Becker, W. (2013). Mechanism of dual specificity kinase activity of DYRK1A. *FEBS J, 280*(18), 4495-4511. doi:10.1111/febs.12411

Warnakulasuriya, S. (2009). Global epidemiology of oral and oropharyngeal cancer. *Oral Oncol, 45*(4-5), 309-316. doi:10.1016/j.oraloncology.2008.06.002

Wilken, J. A., Badri, T., Cross, S., Raji, R., Santin, A. D., Schwartz, P., . . . Maihle, N. J. (2012). EGFR/HER-targeted therapeutics in ovarian cancer. *Future Med Chem, 4*(4), 447-469. doi:10.4155/fmc.12.11

Woo, T., Okudela, K., Mitsui, H., Yazawa, T., Ogawa, N., Tajiri, M., . . . Masuda, M. (2010). Prognostic value of CD133 expression in stage I lung adenocarcinomas. *Int J Clin Exp Pathol, 4*(1), 32-42.

Wortzel, I., & Seger, R. (2011). The ERK Cascade: Distinct Functions within Various Subcellular Organelles. *Genes Cancer, 2*(3), 195-209. doi:10.1177/1947601911407328

Yamanaka, Y., Friess, H., Buchler, M., Beger, H. G., Uchida, E., Onda, M., . . . Korc, M. (1993). Overexpression of acidic and basic fibroblast growth factors in human pancreatic cancer correlates with advanced tumor stage. *Cancer Res, 53*(21), 5289-5296.

Yang, E. J., Ahn, Y. S., & Chung, K. C. (2001). Protein kinase Dyrk1 activates cAMP response element-binding protein during neuronal differentiation in hippocampal progenitor cells. *J Biol Chem, 276*(43), 39819-39824. doi:10.1074/jbc.M104091200

Yoshida, K. (2008). Role for DYRK family kinases on regulation of apoptosis. *Biochem Pharmacol, 76*(11), 1389-1394. doi:10.1016/j.bcp.2008.05.021

Zhang, H., Sun, K., Ding, J., Xu, H., Zhu, L., Zhang, K., . . . Sun, W. (2014). Harmine induces apoptosis and inhibits tumor cell proliferation, migration and invasion through down-regulation of cyclooxygenase-2 expression in gastric cancer. *Phytomedicine, 21*(3), 348-355. doi:10.1016/j.phymed.2013.09.007

Zhang, L., Zhang, F., Zhang, W., Chen, L., Gao, N., Men, Y., . . . Jiang, Y. (2015). Harmine suppresses homologous recombination repair and inhibits proliferation of hepatoma cells. *Cancer Biol Ther, 16*(11), 1585-1592. doi:10.1080/15384047.2015.1078021

Zhang, X., Ibrahimi, O. A., Olsen, S. K., Umemori, H., Mohammadi, M., & Ornitz, D. M. (2006). Receptor specificity of the fibroblast growth factor family. The complete

mammalian FGF family. *J Biol Chem, 281*(23), 15694-15700. doi:10.1074/jbc.M601252200

Zhao, J. (2016). Cancer stem cells and chemoresistance: The smartest survives the raid. *Pharmacol Ther, 160*, 145-158. doi:10.1016/j.pharmthera.2016.02.008

Zheng, X., Carstens, J. L., Kim, J., Scheible, M., Kaye, J., Sugimoto, H., . . . Kalluri, R. (2015). Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. *Nature, 527*(7579), 525-530. doi:10.1038/nature16064

Zou, B., Sun, S., Qi, X., & Ji, P. (2012). Aldehyde dehydrogenase activity is a cancer stem cell marker of tongue squamous cell carcinoma. *Mol Med Rep, 5*(4), 1116-1120. doi:10.3892/mmr.2012.781