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# Cell-Specific Mechanisms of Microrna Modulation of Anoctamin-1 (ANO1) in Oral Cancer

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# CELL-SPECIFIC MECHANISMS OF MICRORNA MODULATION OF ANOCTAMIN-1 (ANO1) IN ORAL CANCER

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# Abstract


**CELL-SPECIFIC MECHANISMS OF MICRORNA MODULATION OF ANOCTAMIN-1 (ANO1) IN ORAL CANCER**

By

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Anoctamin-1 (ANO1) is a calcium-gated chloride ion channel with multiple physiological roles, including transepithelial ion transport, smooth muscle contraction, salivary secretion, and nociception. Recently, ANO1 has been identified to play a key role in cancer progression. ANO1 is overexpressed in different types of cancer, including oral cancer. ANO1 overexpression is commonly the result of ANO1 gene amplification on the chromosome 11q13 locus. In cancer cells, ANO1 upregulation has been reported to promote cell proliferation and migration of tumor cells, whereas ANO1 downregulation induces apoptosis through multiple signaling pathways. As such, ANO1 is now considered a useful biomarker for cancer progression and a prognostic indicator for poor outcomes. Reducing ANO1 expression is a therapeutic approach to suppress ANO1 activity in promoting tumor cell proliferation, invasion, and metastasis. Specific miRNAs that directly target ANO1 mRNA and negatively regulate ANO1 gene expression have been reported. The role of miRNA influencing the expression of ANO1 in oral cancer cells is not understood.

The aims of this study were to determine if microRNAs that specifically target ANO1 are expressed in oral squamous cell carcinoma and to evaluate if the introduction of miRNA that target ANO1 to oral cancer cells will influence proliferation and apoptosis of oral cancer cells.

We performed quantitative PCR (qPCR) of miRNA expression in various cultured OSCC cell lines (SCC4, SCC15, SCC25, CAL27), and the non-malignant OKF4 cell line to examine endogenous microRNA expression. SCC9 and SCC15 cells were transfected with miR-144 or miR-381 mimics to determine the effect of upregulating miRNA that target ANO1 transcripts. Experimental growth assays of SCC-9 and SCC-15 were performed to evaluate proliferation following miRNA targeting of ANO1 and changes in the expression of ANO1 and apoptotic caspase genes, CASP3, CASP8 and CASP9, were assessed by qPCR.

We determined that miR-9, miR-132, and miR-381 that specifically target ANO1 transcripts for downregulation are expressed in SCC4, SCC9, SCC15, SCC25 and CAL27 cells. miR-144, which also targets ANO1 transcripts for downregulation, is only expressed in SCC9 and SCC15. Compared to the non-malignant OKF4 cells, miR-132, miR-144 and miR-381 appear to be dysregulated in oral cancer cells. The introduction and transfection of miR-144 and miR-381 mimics to SCC9 and SCC15 was confirmed by qPCR. miR-144 and miR-381 transfection in SCC9 cells resulted in a similar and significant reduction in SCC9 proliferation ($p = 0.001$ and $p = 0.002$, respectively). An anti-proliferative effect relative to controls was observed following miR-144 and miR-381 transfection in SCC15 cells. miR-144 significantly reduced SCC15 proliferation compared with non-transfected cells ($p = 0.03$). miR-381 did slow SCC15 proliferation, however, the reduction in proliferation was not significant. In both SCC9 and SCC15 cells transfected with miR-144, ANO1 transcripts were significantly decreased ($p = 0.02$; $p = 0.007$, respectively), compared to non-transfected controls. Transfection of miR-381 in

SCC9 also decreased ANO1 transcripts significantly ($p = 0.03$), but no significant decrease in ANO1 expression was observed in SCC15 cells that were transfected with miR-381. Significant increases in CASP3, CASP8 and CASP9 apoptotic gene expression (2.8 to 9.2-fold) were found when miR-144 and miR-381 targeting ANO1 were transfected in SCC9 and SCC15 cells.

miR-9, miR-132, miR-144, miR-381 that specifically target ANO1 transcripts for downregulation are expressed in oral squamous cell carcinoma. Transfecting miR-144 and miR-381 in oral squamous cell carcinoma reduced ANO1 expression and influenced proliferation and apoptotic gene expression in these oral cancer cells. This study provides novel information regarding the cellular mechanisms through which ANO1 channel function may modulated in oral cancer.

# Acknowledgements

I would like to give a very big thank you to Dr. Fiona Britton for her invaluable help with this project. Her many hours spent in the lab assisting me with this project as well as her overall support not just with this project but in general made my research experience so much more enjoyable. I am very grateful for her meticulous ways and ensuring things were done properly. Additionally, I would like to thank Shelley Williams for her help in the lab. I would also like to thank my committee members, Drs. Karl Kingsley, Katherine Howard, Brian Chrzan, and Maxim Gakh for their help and support. Lastly, another heartfelt thank you to all of my co-residents, UNLV faculty, and family for all of their encouragement during this process.

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# Chapter 1: Introduction

Anoctamin-1 (ANO1) is a transmembrane, voltage- and calcium-activated ion channel, whose function is to mediate chloride ion transport in many cells and tissues. ANO1 has important roles in transepithelial ion transport, smooth muscle contraction, salivary secretion, nociception, and neuronal excitability (Pedemonte and Galietta, 2014). Recently, ANO1 has been identified to play a pivotal role in cancer progression (Zhang et al, 2021). ANO1 is overexpressed in many cancers including head and neck cancer, gastrointestinal, and colorectal cancer (Guo et al, 2022). The overexpression of ANO1 is due to ANO1 gene amplification of the genomic chromosome 11q13 locus (Wang et al., 2017). Numerous studies have indicated that ANO1 overexpression is associated with classical hallmarks of cancer such as uncontrolled proliferation, resistance to cell death, and activation of invasion and metastasis (Guo et al., 2022). In cancer cells, ANO1 upregulation promotes cell proliferation and migration of tumor cells (Qu et al., 2014), whereas ANO1 downregulation induces apoptosis through multiple signaling pathways, including TGF-$\beta$, NF-$\kappa$B, EGFR/MAPK and CaMKII/MAPK signaling pathways (Liu Y et al., 2021).

Given the important link between ANO1 overexpression in cancer progression, metastasis and poor prognosis, research has focused on the identification of inhibitors of ANO1 activity *in vivo* and *in vitro* with the goal of developing therapeutic approaches to reduce ANO1 expression in cancer cells. MicroRNAs are single stranded non-coding RNA molecules that are involved in post-transcriptional RNA silencing and could potentially be a therapeutic mechanism to downregulate ANO1 gene expression in cancer cells (Feng et al., 2016). Researchers have uncovered specific miRNAs that directly target ANO1 mRNA and negatively regulate ANO1 gene expression (Cao et al, 2017, Jiang et al, 2019, Zhen et al, 2019). For

example, miR-144 was recently identified as directly targeting ANO1 expression and when miR-144 was overexpressed in colorectal cancer, the aggressive cancer phenotype was suppressed (Jiang et al, 2019). In other studies, miR-132 and miR-381 directly target ANO1 and influenced cell proliferation and metastasis in gastric and colorectal cancers, respectively (Cao et al, 2017).

Our understanding of the fundamental concepts of ANO1 channel function in oral cancer is still limited. The role of miRNA downregulating ANO1 expression in oral cancer has not been reported and it is unknown whether miRNA targeting ANO1 will alter the cellular phenotype of oral cancer cells.

**Research Questions and Hypotheses**

Research question 1

Are microRNAs (miR-9, -132, -144, -381) that specifically target ANO1 expressed in oral squamous cell carcinomas?

- Null hypothesis ($H_0$): microRNAs (miR-9, -132, -144, -381) that specifically target ANO1 are not expressed in oral cancers.
- Alternative hypothesis ($H_A$): microRNAs (miR-9, -132, -144, -381) that specifically target ANO1 are expressed among oral cancers.

Research question 2.

Does the modulation of specific microRNAs (miR-144, miR-381) which target ANO1 influence proliferation and apoptosis of oral cancer cells?

- Null hypothesis ($H_0$): Modulating specific microRNAs (miR-144, miR-381) which target ANO1 does not affect proliferation or apoptosis of oral cancer cells.
- Alternative hypothesis ($H_A$): Modulating specific microRNAs (miR-144, miR-381) which target ANO1 does affect proliferation or apoptosis of oral cancer cells.

# Chapter 2: Background and Literature Review

## Oral Cancer

Oral squamous cell carcinoma (OSCC) is one of the most common head and neck malignancies, accounting for approximately 90% of all head and neck cancers. OSCC ranks sixth in overall cancer mortality according to GLOBOCAN 2020 reports (Sung et al., 2020). The five-year survival rate has been reported at approximately 66%, and the death rate is estimated annually to be 10,000 people in the United States (Sung et al., 2020). With OSCC, the prognosis and survival rates are poor, since this cancer frequently metastasizes to lymph nodes, and there are no effective targeted therapies (Ling et al., 2021). According to multiple studies, smoking or chewing tobacco and alcohol consumption are the predominant risk factors to the development of oral carcinogenesis (Vigneswaran and Williams, 2014). Infection with the human papilloma virus, mainly types 16 and 18, is also associated with OSCC pathogenesis (Candotto et al., 2017). Although OSCC etiology, clinical presentation, histopathological features, and progression are well elucidated, OSCC remains a life-threatening pathology, often due to late staging of the cancer at diagnosis and aggressive features, including metastasis and recurrence. The main treatment usually involves surgical excision, often with adjuvant radiotherapy (Watters et al., 2021).

## Genetic Abnormalities in Oral Cancer

One of the hallmarks of carcinogenesis is the uncontrolled potential for cancer cells to replicate and proliferate (Hanahan and Weinberg, 2011). A genetic abnormality that is responsible for this phenotype in several cancers, including oral cancer and other cancers of the head and neck, is the amplification of a genomic locus on human chromosome 11q13 (Ramos-

Garcia et al., 2017). Chromosomal abnormalities involving the 11q13 locus are associated with poor prognosis in patients with oral squamous cell carcinoma (Åkervall et al., 1995). The 11q13 genomic locus contains several genes that are dysregulated in cancer cells, including cyclin D1 (*CCND1*), a cyclin protein required for the progression of $G_1$ of the cell cycle, fibroblast growth factors (*FGF3*, *FGF4*, *FGF19*), and FAS-associated death domain protein (*FADD*), a molecule that regulates cell apoptotic signals (Ramos-Garcia et al., 2017). Importantly, the 11q13 region is the genomic location of the ANO1 gene that encodes the Anoctamin-1 ion channel (Ramos-Garcia et al., 2017), the focus of the current study. Figure 1 highlights the genomic information for chromosome 11q13 including ANO1 that is frequently overamplified in cancer.

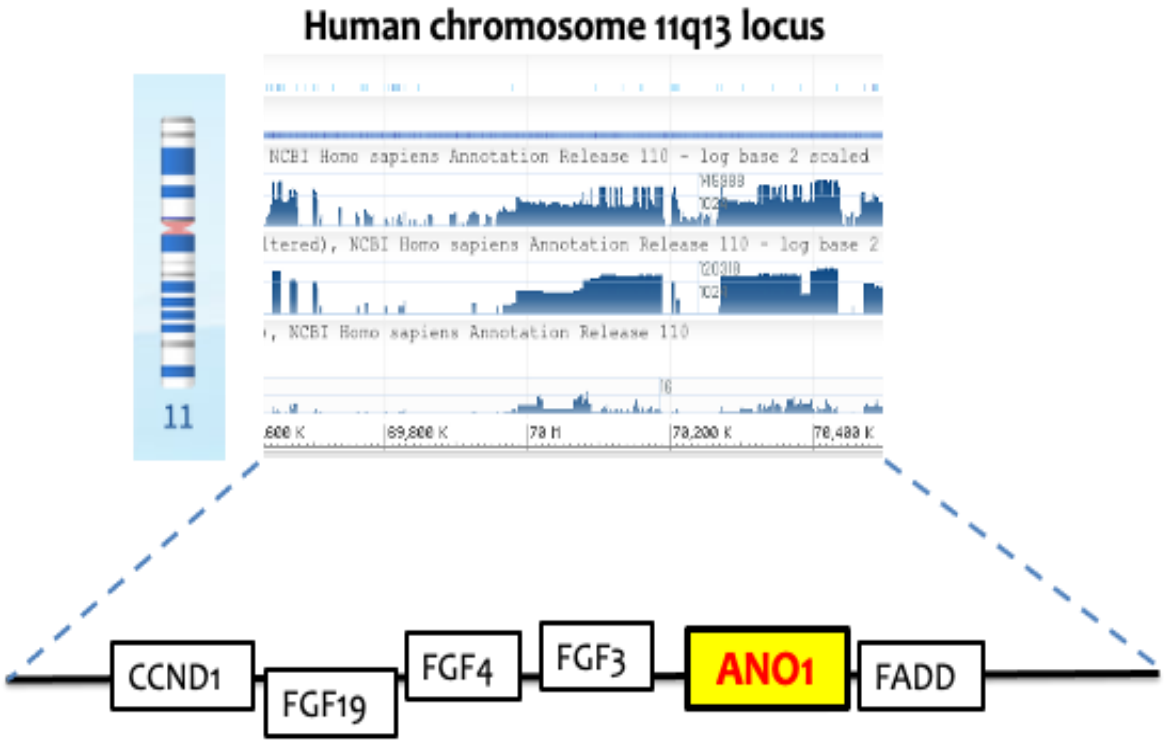

Figure 1: The Anoctamin-1 (ANO1) gene is located on human chromosome 11q13 a genomic locus frequently overamplified in cancer. The genomic information depicted for chromosome 11q13 was accessed from the Human Genome Resources (https://www.ncbi.nlm.nih.gov/) at the National Center for Biotechnology Information (NCBI). Other cancer associated genes in this 11q13 locus are depicted and include cyclin D1 (CCND1), a cell cycle protein required for progression through G1; fibroblast growth factors-19, -4, and -3 (FGF19, FGF4, FGF3) and Fas-associated death domain protein (FADD), an adapter molecule that mediates cell apoptotic signals.

**Anoctamin-1 (ANO1) Channels**

ANO1 is a voltage-sensitive calcium-activated chloride channel that is located in the cell membrane of many different tissues including epithelial, smooth muscle, myocardium, neurons and endothelial cells (Pedemonte and Galietta, 2014). In 2008, it was determined that the function of ANO1 is to mediate transmembrane ion transport (Caputo et al., 2008; Schroeder et al., Yang et al, 2008). Since then, many critical physiological roles have been attributed to ANO1 including the regulation of airway fluid secretion, gastrointestinal tract motility, salivary gland secretion, vascular smooth muscle contraction and nociception in neurons (reviewed in Oh and Jung, 2016; Ji et al., 2019). In line with the varied distribution and functions of ANO1 in different tissues, abnormal ANO1 gene expression or dysfunction of ANO1 channels is linked to several pathological processes, including asthma, diarrhea, xerostomia, hypertension, neuropathic pain, cystic fibrosis, and various cancers (Liu Y et al., 2021).

**ANO1 and Cancer**

ANO1 has been classified as an influential biomarker when it comes to the malignant potential, stage progression of cancer malignancies and prognosis (Guo et al, 2022). ANO1 is overexpressed in many cancers including, gastric, breast, colon, hepatic, esophageal, head and neck squamous cell carcinoma, and pancreatic ductal carcinoma (Zhang et al, 2021). Studies have examined the gene copy number of ANO1 in these cancers and found that ANO1 gene amplification at the genomic chromosome 11q13 locus commonly accounts for ANO1 overexpression in these cancers (Wang et al., 2017). Numerous studies (reviewed in Guo et al, 2022), have found the overexpression of ANO1 is associated with several classical hallmarks of cancer (Hanahan and Weinberg, 2011), including proliferative signaling, evasion of growth suppressors, resistance to cell death, induction of angiogenesis, and activation of invasion and metastasis (Guo et al., 2022). In cancer cells, ANO1 upregulation promotes cell proliferation and migration of tumor cells (Qu et al., 2014), whereas ANO1 downregulation induces apoptosis through multiple signaling pathways, including TGF-$\beta$ (Song et al, 2018), NF-$\kappa$B (Liu J et al., 2021, EGFR/MAPK and CaMKII/MAPK signaling pathways (Britschgi et al., 2013, Liu Y et al., 2021). For instance, studies have shown that ANO1 engages in the activation of the Ras-Raf-MEK-ERK1/2 proliferative signaling pathway for the promotion of tumor cell growth and invasion in head and neck squamous cell carcinoma (Duvvuri et al., 2012). ANO1 is also involved in the protein network pathway capable of engaging in the regulation of EGFR constitutive protein phosphorylation and SRC, AKT, and enhancement of the metastasis of the cancer tissues and cells (Britschgi et al., 2013). Anti-apoptotic proteins MCL-1 and BCL-2 are increased with the overexpression of ANO1 in breast cells, indicating that ANO1 has evasion and cell death resistant properties (Britschgi et al., 2013). In terms of activating invasion and

metastasis, ANO1 is involved in the EGFR-STAT3 signaling pathway feedback loop that aids tumorigenesis and cell propagation in breast cancer (Wang et al., 2019).

Given the important link between ANO1 overexpression in cancer progression, metastasis and poor prognosis, researchers focused on the identification and characterization of inhibitors of ANO1 activity *in vivo* and *in vitro* (Guo et al, 2022), with the goal of developing therapeutic compounds that reduce ANO1 expression and activity. Another area of growing interest is whether microRNA can be potentially used as a therapeutic mechanism to downregulate ANO1 gene expression in cancer cells (Feng et al., 2016).

**microRNA**

A microRNA is a single stranded non-coding RNA molecule that is involved in post-transcriptional negative regulation of gene expression and RNA silencing. Genes for miRNAs are transcribed to a primary miRNA and processed within the nucleus to a precursor miRNA (pre-miRNA) by Drosha, a class 2 RNase III enzyme (Wahid et al., 2014). Pre-miRNAs are then exported to the cytoplasm, where they are further processed by Dicer enzyme, an RNase III type protein, to become mature miRNAs, which are usually 21-25 nucleotides in length. Mature miRNA are loaded onto the Argonaute protein in the cytoplasm to produce the RNA-induced silencing complex (RISC) which can then target specific mRNA transcripts (Wahid et al, 2014). RISC uses the miRNA nucleotide sequence as a template for recognizing complementary sequences on target mRNA molecules (Gregory et al, 2005). When miRNA binds a complementary strand, RISC activates RNase and cleaves the RNA (Gregory et al, 2005). miRNAs can inhibit translation and control the expression of roughly 30% protein-coding genes (Jiang et al, 2019).

**miRNA downregulation of ANO1 in Cancer**

Existing studies indicate that specific miRNAs can directly target ANO1 mRNA and negatively regulate ANO1 gene expression. Such microRNAs include miR-9, miR-144, miR-132 and miR-381 (Guo et al, 2022). miRNAs downregulate ANO1 expression by attenuating ANO1 mRNA transcripts and inhibiting translation via binding of the miRNA to complementary sequences in the 3'-untranslated region of ANO1. Therefore, reducing ANO1 expression is a promising approach to suppress ANO1 pro-cancerous activity by promoting tumor cell proliferation, invasion, and metastasis. In a recent study, the transfection of miR-9 and miR-144 in colorectal cancer cells, were shown to bind to ANO1 transcripts and reduce colorectal cancer cell proliferation, migration, and promote cell death (Jiang et al, 2019). Additionally, proteins associated with ANO1 in colorectal cancer cells such as P-AKT, P-ERK, and CD1 have been inhibited when miR-9 targeting ANO1 has been upregulated (Park et al, 2019). In a gastric cancer study, miR-381 targeted ANO1 and modulated the TGF-β pathway, which influenced gastric cancer cell proliferation and metastasis (Cao et al., 2017). In a breast cancer study, miR-144 also inhibits the expression of PTEN in order to stimulate the Ras-Raf-MEK-ERK1/2 pathway, which impedes breast cell survival and growth (Jiang et al, 2019). There is a need for the continued assessment of microRNAs influence on ANO1. An understanding of the fundamental concepts of how ANO1 functions in cancer and the role of miRNA inhibitory effects on ANO1 gene expression is still limited. There are studies indicating, for example, microRNA downregulation of ANO1 expression in colorectal cancers, but there are few studies indicating its role in oral cancer.

It is evident that ANO1 is overexpressed in many types of cancers and this overexpression is associated with poor prognosis in cancer patients. Our purpose of study is to

understand the role of microRNA and whether it can influence the expression of ANO1 in oral cancer. In this study, we will first determine whether microRNAs that have been reported to specifically target ANO1 are expressed in oral cancer cell lines. Secondly, we will introduce miRNA mimics, specifically miR-144 and miR-381 to oral cancer cells to determine the effect of miRNA downregulation of ANO1 expression and on cancer cell proliferation and apoptosis.

# Chapter 3: Materials and Methods

## Cell culture

Several human oral squamous cell carcinoma (OSCC) cell lines were used in this project including, SCC-4, SCC-9, SCC-15, SCC-25, and Cal-27. All cell lines were originally obtained from the American Type Culture Collection (Manassas, VA), and were thawed and recovered from inventoried frozen stocks. Cal-27 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum and 1% penicillin-streptomycin (Hyclone Laboratories, Inc.). The SCC-4, SCC-9, SCC-15, and SCC-25 cell lines were cultured in DMEM/F12 Ham's growth media supplemented with 10% bovine calf serum and penicillin-streptomycin (1%). A non-malignant oral cell line, OKF4 was included for comparison and was kindly provided by Dr. Howard and Shelley Williams at the UNLV School of Dental Medicine Department of Biomedical Sciences. OKF4 cells were cultured in keratinocyte–serum-free medium (Ker-SFM) supplemented with 0.2 ng/ml epidermal growth factor. Cells were maintained at 37°C in a 5% $CO_2$ tissue culture chamber. All monolayer cells were passaged using 0.25% trypsin/EDTA solution (Fisher Scientific) following standard cell culture procedures in a biological Class II safety level cabinet (NuAire). Cell visualization was by light microscopy on an Olympus CK2 inverted microscope. Cell counts and percentage viability were measured using dual chamber counting slides and a BioRad TC10 automatic cell counter (BioRad Laboratories). Cell viability was determined by dye exclusion following staining with 0.4% Trypan blue (Fisher Scientific).

**miRNA mimics**

*mir*Vana® miRNA-144 and miRNA-381 mimics targeting ANO-1 were purchased from ThermoFisher Scientific. mirVana miRNA mimics are chemically modified double-stranded RNA molecules designed to mimic endogenous microRNAs (miRNAs), resulting in down-regulation of target mRNA translation due to mRNA sequestration or degradation. Like native miRNAs, these mimics have two strands, the mature strand, which is functional and used by the Argonaute protein to specifically target mRNAs, and the passenger strand. The miRNA mimics were dissolved in molecular grade nuclease-free water to generate a stock solution of 10 μM. The miRNA mimics were aliquoted and stored at -20OC. Figure 2 shows the region of complementary base-pairing of miR-144 or miR-381 to the ANO-1 transcript that are targeted for down-regulation.

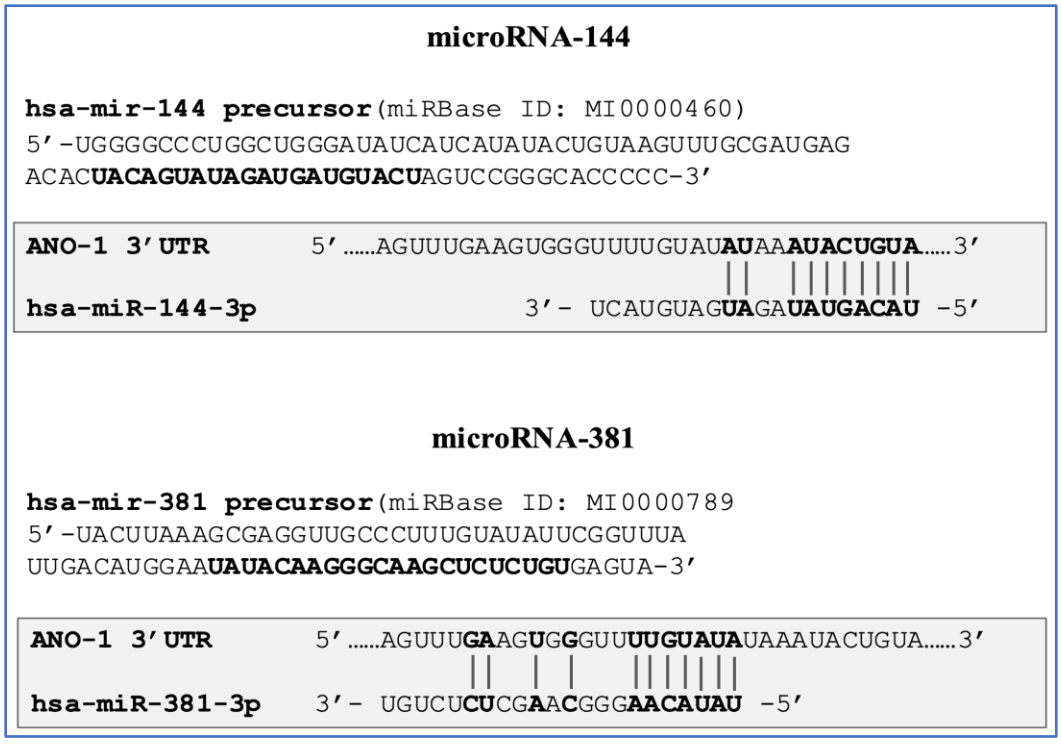

**Figure 2: miR-144 and miR-381 target ANO-1 transcripts for down-regulation.** The miRNA precursor sequences for miR-144 and miR-381 were obtained from miRBase database (https://www.mirbase.org). The mature hsa-miR-144-3p and mature hsa-miR-381-3p sequences are highlighted in bold. The targeted 3'-untranslated region (3'UTR) of ANO-1 messenger transcript where miR-144 and miR-381 mimics bind by complementary base pairing are depicted.

**OSCC cell transfection with miRNA mimics**

The OSCC cell lines SCC-9 and SCC-15 were transfected with either miR-144 mimic, miR-381 mimic, or a non-silencing scrambled miRNA negative control using Lipofectamine™ 2000 transfection reagent (ThermoFisher Scientific). Cells were seeded at a density of $1x10^4$ in 96-well plates (for cell proliferation assays) or at $0.3x10^6$ cells/mL in 6 well plates (for quantitative gene expression analysis), 24 hours prior to transfection. Culture plates were examined by light microscopy to confirm adequate cell confluency (~70-80%). Each culture well received 10pmol of each miRNA. Briefly, miRNA mimics and Lipofectamine 2000 reagent were freshly diluted in serum free Opti-MEM medium (Fisher Scientific) in separate tubes, following the manufacturer's instructions (ThermoFisher Scientific). Each diluted miRNA mimic was then mixed with diluted lipofectamine reagent and incubated at room temperature for at least 20 min to allow complex formation. The culture media from each plate was removed before adding the miRNA-lipofectamine complex dropwise to the wells (50uL to 96-well plates, 300uL to 6 well plates). Transfected cells were incubated for 72 hours at 37°C, with the addition of fresh DMEM/F12 growth medium to the cells after 4 hours. All treatments were performed in

triplicate with each experiment repeated three times. The efficiency of miRNA transfection was evaluated by qPCR. Negative controls included untreated cells, lipofectamine-only treated cells, or cells transfected with non-silencing scrambled miRNA.

**Proliferation Assays**

OSCCs were plated at a seeding density of 1 x $10^4$ cells/mL in 96-well flat bottom tissue culture plates (Fisher Labs). Prior to miRNA treatment, cell confluency was evaluated after 24 hours to ensure 70-80% confluence for optimum transfection. OSCCs were treated with miRNA mimics to evaluate proliferation over 3 days. Three replicates of each experiment were performed, which included three rows of n=8, for a total n=24. After 72 hours, cells were fixed with 10% buffered formalin, stained with a 1% aqueous solution of Gentian Violet (Fisher Labs) and washed with 1X PBS. Each plate was analyzed for absorbance at 600 nm with a BioTek Synergy HTX Multimode Reader and BioTek Gen5 software (Agilent). All values are reported with respect to non-transfected controls, lipofectamine only treatment only or scrambled miRNA negative controls.

**RNA isolation**

Prior to RNA isolation, OSCC cell lines were plated in six-well plates at a seeding density of 0.3 x $10^6$ cells/mL. For miRNA expression analysis, cells were in the logarithmic phase prior to RNA isolation. For miRNA transfected cells, triplicates of each cell line were transfected with miRNA and incubated for 3 days at 37°C. Both treated and untreated OSCCs were lysed with TRIzol reagent (Thermofisher Scientific), according to manufacturer's instructions, and total RNA was extracted with chloroform. RNA precipitates were resuspended

in 20 µL of RNAase-free water. The concentration and purity of the RNA samples were determined using a Nanodrop™ spectrophotometer (Thermofisher Scientific), to measure the absorbance at 260nm ($A_{260}$) and 280 nm ($A_{280}$). RNA was stored at -80°C until use in molecular analysis.

**cDNA Synthesis**

cDNA was reverse transcribed from RNA isolated from OSCC cells using the High-Capacity™ cDNA synthesis system as per the manufacturer's instructions (Applied Biosystems). Each reaction consisted of 2 µg total RNA, 1X reaction buffer (50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM $MgCl_2$ and 10mM DT), 500mM dNTP, 25ng random primer, 1 µL reverse transcriptase and RNAase-free $H_2O$ to a total volume of 20uL. cDNA reactions were incubated in a Mini-AmpPlus™ thermocycler (ThermoFisher Scientific) for 10 minutes at 25°C, then 120 minutes at 37°C, followed by 5 minutes at 85°C for enzyme inactivation. Each cDNA reaction product was diluted 5-fold with 80 µL molecular grade RNAase-free water and stored at -20°C until subsequent quantitative analysis by PCR.

**Quantitative real time PCR**

For quantitative real time PCR (qPCR) gene analysis we employed either SYBR Green chemistry for assays to determine miRNA expression in OSCCs, or Taqman™ chemistry assays for evaluation of alterations of gene expression following introduction of miRNA mimics. The primers used for qPCR SYBR™ Green assays are shown in Table 1. Amplification of either the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene or miRNA16 was included to normalize gene expression across the different cDNA samples. qPCR reactions were assembled in optical 96-well plates or 0.2uL 8 sample strip tubes (Applied Biosciences). Each reaction

consisted of 2µL cDNA, 10µL SYBR™ Green master reaction mix, 1µL each of forward and reverse primer, and 6 µl of nuclease-free $H_2O$ to a total volume of 20uL. Standard curves were generated for each primer set using serially diluted solutions of cDNA. Unknown quantities relative to the standard curve for the miRNA primers were calculated and normalized to endogenous GAPDH or miRNA16 within the same sample. Each cDNA sample was tested in triplicate and cDNA was obtained from at least three biological samples. Taqman™ Gene Expression reagent and specific assay primers for ANO-1, CASP3, CASP8 and CASP9 were obtained from ThermoFisher Scientific. Taqman™ assay primers and associated FAM or VIC probes used are listed in Table 2. qPCR reactions were assembled in optical 96-well plates or 0.2uL 8 sample strip tubes (Applied Biosciences). Each reaction consisted of 2µL cDNA, 10µL Taqman™ master reaction mix, 1µL specific Taqman™ primer and 7 µL $H_2O$ to a total volume of 20uL.

| | |
|---|---|
| **miR-9** | **Forward: 5'-CAGTGGCATTGGGATAACATAA-3'**<br>Reverse: 5'-GGAATTGGGAAGGTTTCTACAT3' |
| **miR-16** | Forward: 5′-TGCGGGTGCTCGCTTCGGCAGC-3'<br>Reverse: 5′-CCAGTGCAGGGTCCGAGGT-3′ |
| **miR-132** | Forward: 5′-GGAGAGCGTCAACAGGGAGA-3'<br>Reverse: 5′-CAGCCAGGAGAAATCAAACAGAG-3' |
| **miR-144** | Forward: 5′-TGCGGTACAGTATAGATGAT-3′<br>Reverse: 5′-CCAGTGCAGGGTCCGAGGT-3′ |
| **miR-381** | Forward- CCAAGAAGCTGAGCGAGTGTCT-3'<br>Reverse- AGCTCCATATTGCTGTCCAGTTC-3' |
| **GAPDH** | Forward: 5′-GGCATGGACTGTGGTCATGAG-3'<br>Reverse: 5′-TGCACCACCAACTGCTTAGC-3' |

**Table 1.** PCR primers employed in this study for quantitative SYBR Green assays of relative miRNA expression.

Real-time amplifications were performed on an Applied Biosystems QuantStudio™ 3 thermocycler (ThermoFisher Scientific) with the cycling profile of 2 minutes at 95°C for enzyme activation, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing-extension at 60 °C for 20 seconds. No template controls for each primer set were included in each assay. GAPDH was used as a reference gene. The relative expression in gene expression were calculated using the comparative (ΔΔCt) method (Livak and Schmittgen, 2001), where fold changes in gene expression, relative to a calibrator sample is equal to $2^{-\Delta\Delta Ct}$.

| Gene Transcript | Taqman™ Assay Id | Reporter Probe |
|---|---|---|
| **GAPDH** | huGAPDH1 | VIC |
| **ANO1** | Hs00216121_m1 | FAM |
| **CASP3** | Hs00234387_m1 | FAM |
| **CASP8** | Hs01018151_m1 | FAM |
| **CASP9** | Hs00962278_m1 | FAM |

**Table 2.** TaqMan™ chemistry PCR primers and associated reporter probes employed in this study for quantitative PCR.

**Statistical Analysis**

Data were presented as mean ± standard deviation (SD) of three independent experiments with each miRNA treatment condition completed in triplicate. Data were exported to Microsoft Excel software. Significance among groups was tested by one way analysis of variance (ANOVA) and Comparisons of change in proliferation or gene expression mean values were analyzed by two-tailed Student's t-tests, which are appropriate for parametric data analysis. A $p$ value of $< 0.05$ was considered statistically significant.

# Chapter 4: Results

## Analysis of miRNA expression in OSCCs

This study performed quantitative PCR (qPCR) of miRNA expression in various cultured OSCC cell lines (SCC4, SCC15, SCC25, CAL27), and the non-malignant OKF4 cell line. The key to successful quantitative analysis is the isolation of RNA of sufficient concentration and purity. Table 3 shows representative data of typical RNA yields obtained from OSCC culture cells grown in T-25 flasks. Isolated RNA samples were examined by spectrophotometer analysis to measure RNA absorbance at 260nm and 280nm UV wavelengths. The A260/A280 ratio of >1.8 measured from was considered pure and free from contaminants.

**RNA Isolation: T25 culture flasks**

| OSCC | RNA Concentration (ng/ul) | RNA Purity (A260/A280) | Total Yield (ug) |
|---|---|---|---|
| CAL27 | 4904 | 1.87 | 98.1 |
| SCC4 | 6021 | 1.88 | 120.4 |
| SCC5 | 5266 | 2.01 | 105.3 |
| SCC9 | 4288 | 1.89 | 85.8 |
| SCC15 | 6418 | 2.01 | 128.4 |
| SCC25 | 6764 | 1.94 | 135.2 |
| OKF4 | 4012 | 1.85 | 80.2 |

**Table 3:** Typical RNA yields and purity from RNA samples isolated from various OSCC cell lines cultured in T25 flasks. RNA concentration and purity was determined by

spectrophotometer analysis that measured RNA absorbance in the UV wavelengths of 260nm ($A_{260}$) and 280nm ($A_{280}$ nm).

Each OSCC RNA sample was reverse transcribed into cDNA. Each cDNA reaction contained similar OSCC RNA input amounts to ensure consistency for subsequent quantitative analysis by PCR. The expression of miR-9, miR-132, miR-144, miR-381 or miR-16 in each OSCC was determined by examining PCR amplification curves to obtain the PCR cycle at which the exponential amplification of each miRNA crossed a specified threshold level, a.k.a., the cycle threshold (Ct). miRNA expression in OSCC was then normalized to Ct values calculated for endogenous GAPDH transcripts within the same OSCC sample and this relative expression is expressed as delta Ct (ΔCt). Figure 3 shows the ΔCt expression of miR-9, miR-132, miR-144, miR-381, and miR16 in SCC4 SCC9, SCC15, SCC25 and CAL27 cells, normalized to GAPDH. Analysis of miRNA expression in non-malignant OKF4 cells was included for a comparison and to gauge if there was dysregulation of these miRNAs in OSCC cells.

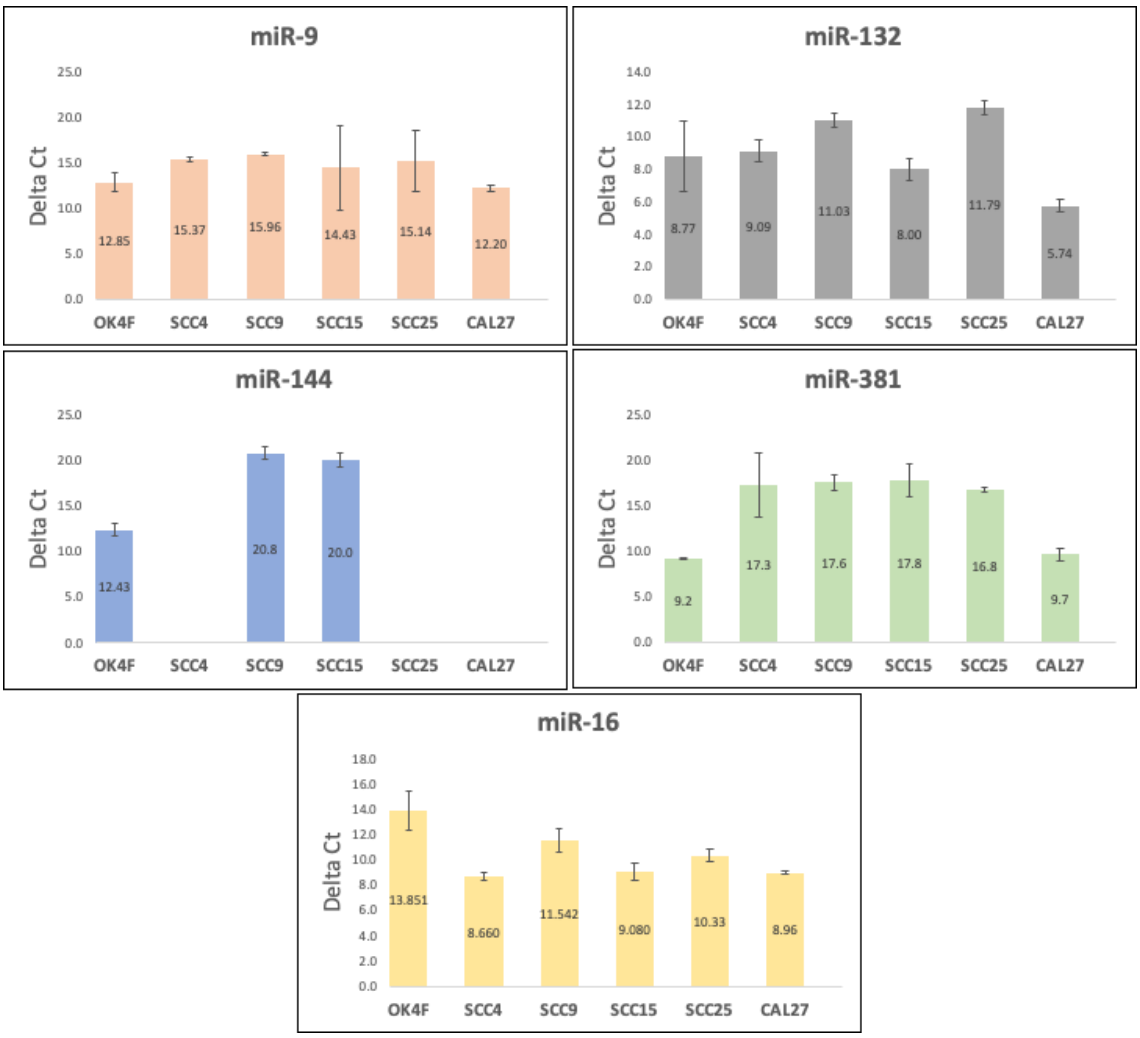


**Figure 3. Analysis of miRNA expression in OSCC by qPCR**. miR-9, miR-132, miR-144, miR-381 and miR16 expression in SCC4, SCC9, SCC15, SCC25 and CAL27 cells was normalized to GAPDH expression. Average gene expression was calculated from triplicate qPCR reactions performed on 3 separate RNA isolations. Data is expressed as the mean ΔCt (relative gene expression) with error bars indicating standard deviation. The non-malignant cell line OK4F was included for comparison.

miR-9 and miR-132 are expressed in all OSCC cells, with the mean ΔCt of miR-9 and miR-132 being consistent across all OSCC, as well as the OK4F cells (Figure 3). miR-16 is expressed at consistent levels in each OSCC, but the relative expression of miR-16 is greater in the OSCC cell lines, the mean ΔCt ranging from 8.6 ± 0.3 to 11.5 ± 0.9, compared to OK4F cells (ΔCt = 13.8 1.5; Figure 3). miR-381 is expressed in all OSCC cells. Compared to miR-381 expression in OK4F cells (ΔCt = 9.2 ± 0.1), miR-381 expression is less in SCC4 (ΔCt =17.3 ± 3.5), SCC9 (ΔCt =17.6 ± 0.8), SCC15 (ΔCt =17.8 ± 1.8), and SSC25 (ΔCt = 16.8 ± 0.3). CAL27 is the only OSCC that had similar miR-381 expression (ΔCt = 9.7 ± 0.6) as OK4F non-malignant cells. We found that miR-144 is expressed in SCC9 and SCC15, but we could not detect expression of miR-144 in SCC4, SCC25 or CAL27 (Figure 3). Compared to miR-16 in OK4F cells (ΔCt = 12.4 ± 0.7) Compared to miR-16 in OK4F cells (ΔCt =17.3 ± 3.5), miR-16 expression is less in SCC9 and SCC15 cells (ΔCt = 20.8 ± 0.7 and 20.0 ± 0.6, respectively).

**Effect of miR-144 and miR-38 targeting ANO-1 transcripts on OSCC proliferation.**

We examined the proliferation of SCC9 and SCC15 cells that were transfected with miR-144 or miR-381 mimics to determine the effect of upregulating miRNA that target ANO1 transcript downregulation. For these experiments OSCC were cultured in 6-well plates for cationic liposome-mediated transfection with miRNA mimics and cellular proliferation was compared after 72 hours. Controls included non-transfected cells, cells transfected with lipofectamine reagent alone, or transfection with a scrambled miRNA.

Figure 4 shows that both miR-144 and miR-381 transfection in SCC9 caused a similar and significant reduction in proliferation. miR-144 significantly reduced SCC9 proliferation (0.52 ± 0.20) compared with non-transfected cells (1.69 ± 0.61, $p = 0.001$, n=3), and miR-381

significantly reduced SCC9 proliferation (0.91 ± 0.45) compared with non-transfected cells ($p$ = 0.002, n=3).

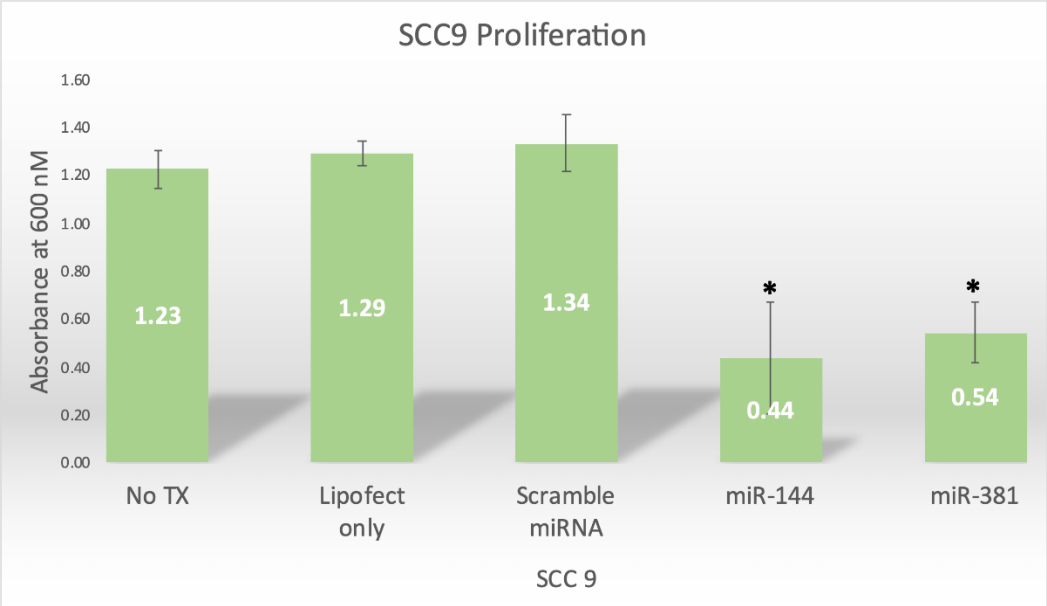


**Figure 4: Effect of miR-144 and miR-381 targeting ANO1 on SCC9 proliferation**. SSC9 proliferation assay showing mean A600 nM values ± SD after 72 hours of miRNA transfection. Average gene expression was calculated from 3 individual experiments. Data was analyzed for statistical significance by t-tests of miR-144 or miR-381transfection vs. non transfected cells (*p>0.05).

Figure 5 shows the effect of miR-144 and miR-381 transfection on SCC15 proliferation. miR-144 and miR-381 both had an anti-proliferative effect on SCC15**.** We found that

transfecting miR-144 significantly reduced SCC15 proliferation (0.44 ± 0.23) compared with non-transfected cells (1.23 ± 0.08, $p$ = 0.03, n=3). miR-381 did result in slowing of SCC15 proliferation. However, this reduction was not significant (p = 0.064).

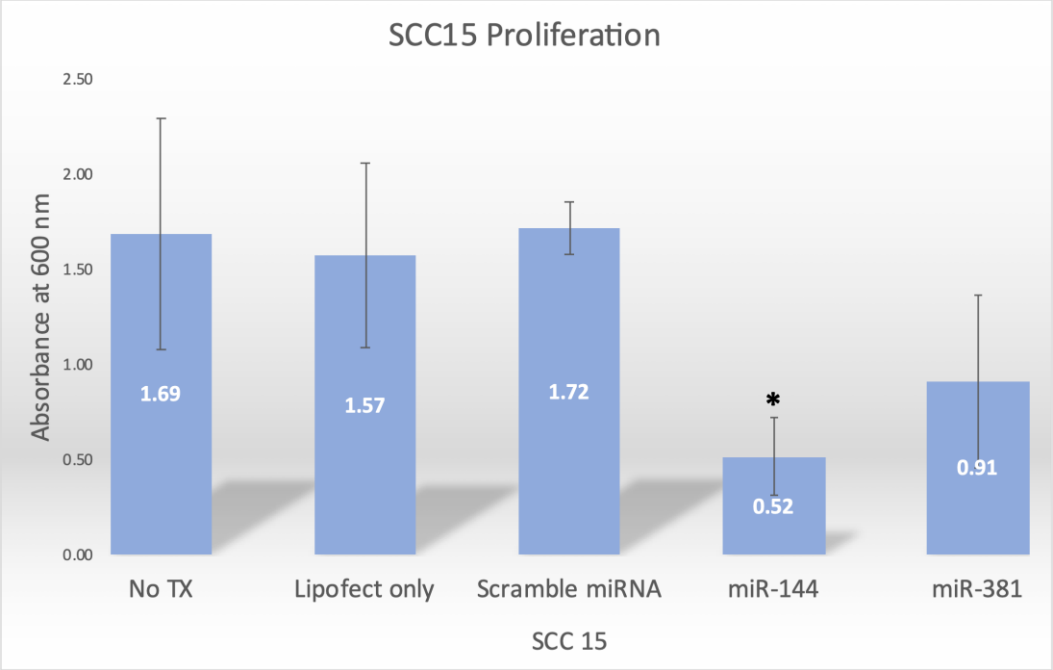


**Figure 5: Effect of miR-144 and miR-381 targeting ANO1 on SCC15 proliferation**. SCC15 proliferation assay showing mean A600 nM values ± SD after 72 hours of miRNA transfection. Average gene expression was calculated from 3 individual experiments. Data generated was analyzed for statistical significance by t-tests of miR-144 or miR-381 transfection vs. non transfected cells (*p>0.05).

**Effect of miR-144 and miR-381 targeting ANO1 transcripts on apoptotic CASP3, CASP8 and CASP9 gene expression in OSCC.**

Quantitative PCR assays of SCC9 and SCC15 cells transfected with either miR-144 mimic or miR-381 mimic were performed to evaluate the effect of these miRNA on ANO1, and CASP3, CASP8 and CASP9 gene expression. For these experiments OSCC were cultured in 6-well plates for cationic liposome-mediated transfection with miRNA mimics and total RNA was isolated 72 hours later. Table 4 shows typical RNA yields obtained from SCC9 and SCC15 cells cultured in 6 well dishes. We obtained RNA of sufficient concentration and purity for subsequent analysis, but it was noted that miR-144 or miR-381 transfected cells yielded less RNA overall.

| OSCC | RNA Concentration (ng/ul) | RNA Purity (A260/A280) | Total Yield (ug) |
|---|---|---|---|
| SCC9 no TXT | 4382 | 2.04 | 87.6 |
| SCC9 miR-144 TXT | 3209 | 2.01 | 64.2 |
| SCC9 miR-381 TXT | 2102 | 2.02 | 42.0 |
| SCC15 no TXT | 2144 | 2.07 | 42.9 |
| SCC15 miR-144 TXT | 21 | 2.08 | 0.42 |
| SCC15 miR-381 TXT | 472 | 2.01 | 9.4 |

**Table 4:** Typical RNA yields and purity of samples isolated from SCC9 or SCC15 cells cultured in 6 well dishes and transfected (TXT) with miR-144 or miR-381 mimics. RNA concentration and purity was determined by spectrophotometer analysis of RNA absorbance at 260nm ($A_{260}$) and 280nm ($A_{280}$ nm) UV wavelengths.

All RNA samples were reverse transcribed into cDNA for qPCR. We determined relative gene expression (ΔCt) as the difference in cycle thresholds (Ct) values obtained from PCR amplification curves normalized to Ct values determined for endogenous GAPDH within the same cDNA sample. Controls included untreated cells, lipofectamine-only treated cells, or cells transfected with non-silencing scrambled miRNA.

Figure 6 shows the relative expression (ΔCt normalized to GAPDH) of miR-144 and miR-381 in SCC9 and SCC15 cells transfected with miRNA mimics. In miR-144 transfected cells, the relative expression of miR-144 was significantly increased in both SCC9 and SCC15 cells (15.7 ± 1.8 and 11.5 ± 0.9, respectively) compared to non-transfected SCC9 or SCC15 cells (21.6 ± 1.9 and 23.1 ± 0.7 respectively; $p = 0.02$ in SCC9 and $p = 0.007$ in SCC15, n=3). In both SCC9 and SCC15 cells transfected with miR-381, the relative expression of miR-381 was also significantly increased. miR-381 expression in SCC9 was 13.3 ± 1.8, compared to non-transfected cells (20.8 ± 3.8; $p = 0.03$, n=3). miR-381 expression in SCC15 was 9.2 ± 4.3, compared to non-transfected SCC15 cells (18.6 ± 1.7; $p = 0.04$, n=3). These results indicate that we achieved efficient transfection of OSCCs with miR-144 and miR-381.

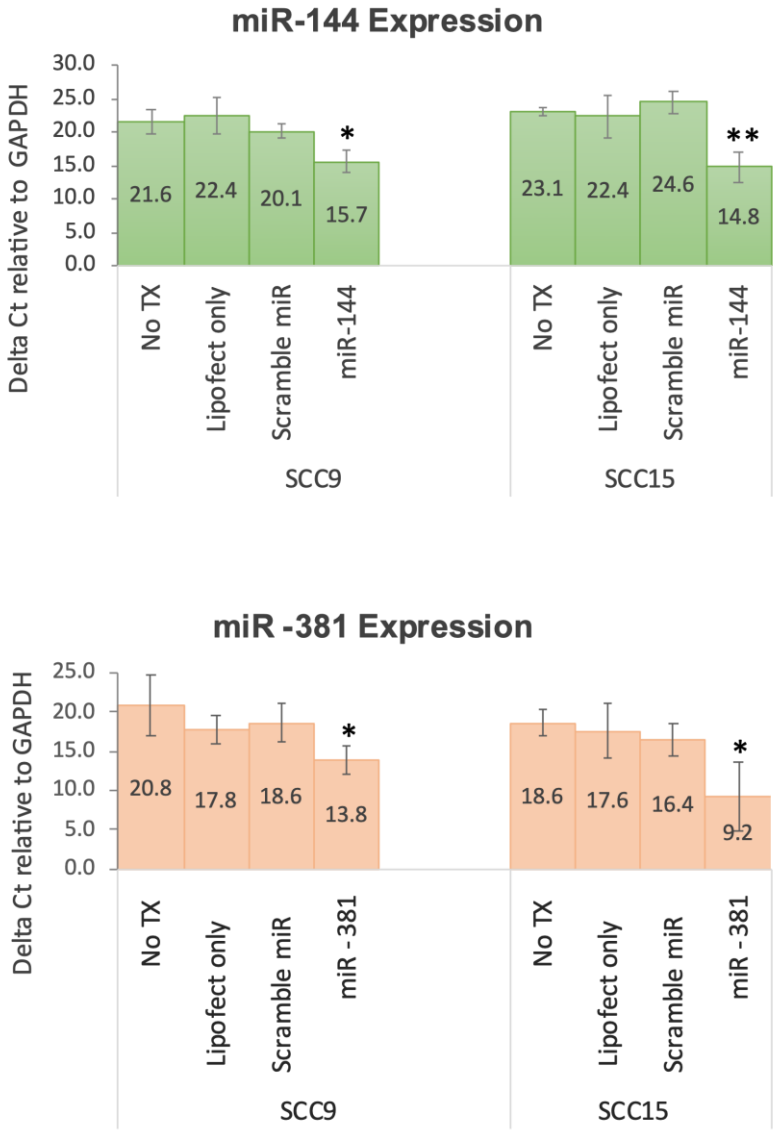


**Figure 6: qPCR analysis of miR-144 and miR-38 expression in SCC9 or SCC15 cells transfected with miRNA mimics.** Average gene expression was calculated from triplicate qPCR reactions with each miRNA treatment condition performed in 3 separate experiments. Data is expressed as the mean ΔCt (relative gene expression) with error bars indicating standard

deviation. * P < 0.05 indicates a significant difference compared to control. Controls are non-transfected cells (No TX), lipofectamine only treated cells, or cells transfected with scrambled miRNA. miR-144 expression was significantly increased in SCC9 ($p$ = 0.02, n=3) and SCC15 ($p$ = 0.007, n=3) in miR-144 transfected vs. non-transfected cells. In miR-381 transfected cells, miR-381 expression was significantly increased in SCC9 ($p$ = 0.03, n=3) and SCC15 cells ($p$ = 0.04, n=3), compared to controls.

Figure 7 shows the relative expression of ANO1 (ΔCt normalized to GAPDH) in SCC9 and SCC15 cells transfected with miR-144 or miR-381 mimics. In miR-144 transfected cells, ANO1 expression in SCC9 cells (9.62 ± 0.81) was significantly decreased compared to non-transfected controls (6.57 ± 0.76, $p$ = 0.02, n=3). A significant decrease in ANO1 expression in SCC15 cells transfected with miR-144 (11.95 ± 1.96) was also observed when compared to controls (7.07 ± 0.21, $p$ = 0.006, n=3). In miR-381 transfected cells, ANO1 expression in SCC9 cells (8.88 ± 0.56) decreased significantly compared to non-transfected controls ($p$ = 0.04, n=3). ANO1 expression in SCC15 cells decreased (10.49 ± 2.71) compared to control (11.95 ± 1.96), but this decrease was not statistically significant. Overall, these results indicate that miR-144 and miR-381 mimics are negatively regulating ANO1 transcription.

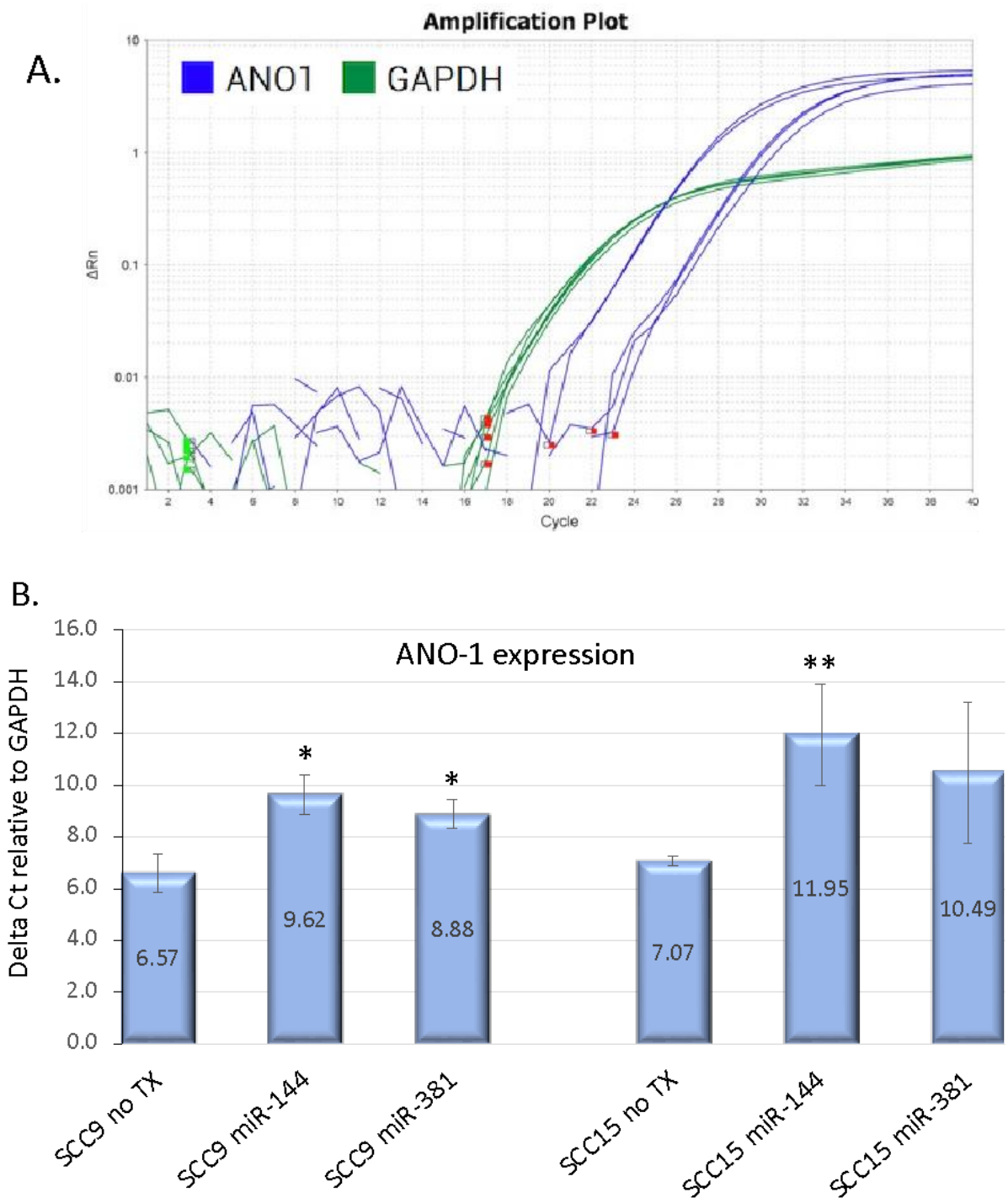


**Figure 7: qPCR analysis of ANO1 expression in OSCC transfected with miR-144 and miR-38 mimics.** Panel A. Representative PCR amplification plot for ANO1 and GAPDH. A decrease

in ANO1 expression is indicated by a right shift in the amplification and corresponding increase in Ct values. Panel B. Average gene expression was calculated from triplicate qPCR reactions with each miRNA treatment condition performed in 3 separate experiments. Data is expressed as the mean ΔCt (relative gene expression) with error bars indicating standard deviation. *p>0.05 or **p>0.005 indicates a significant difference compared to control.

**The effect of miR-144 and miR-381 targeting ANO-1 transcripts on OSCC apoptotic gene expression.**

Figure 8 shows the fold differences in the expression of apoptotic genes CASP3, CASP8 and CASP9 in SCC9 and SCC15 cells that were transfected with miR-144 and miR-381 mimics. Compared to non-transfected cells both miR-144 and miR-381 transfected cells exhibited increases in CASP3, CASP8 and CASP9 expression. We observed fold increases of 5.7 ± 2.4, 8.6 ± 0.6 and 5.0 ± 1.3 for CASP3, CASP8 and CASP9, respectively in miR-144-transfected SCC9 cells. Fold increases of 9.2 ± 1.4, 5.7 ± 1.7 and 5.4 ± 0.7 for CASP3, CASP8 and CASP9 respectively were observed in miR-144-transfected SCC15 cells. In miR-381-transfected SCC9 cells, CASP3, CASP8 and CASP9 exhibited fold increases of 2.8 ± 1.2, 5.1 ± 1.4, and 6.4 ± 2.1, respectively, compared to non-transfected cells. Likewise, in miR-381-transfected SCC15 cells, CASP3, CASP8 and CASP9 exhibited fold increases of 3.9 ± 1.6, 1.8 ± 1.2 and 3.7 ± 0.9, respectively, compared to non-transfected cells. All the fold increases in CASP3, CASP8 and CASP9 were statistically significant ($p<0.05$, $n=3$), except for CASP8 increased expression in miR-381-transfected SCC15 cells. These results indicate that miR-144 transfection of OSCCs increased the expression of pro-apoptotic genes CASP3, CASP8 and CASP9.

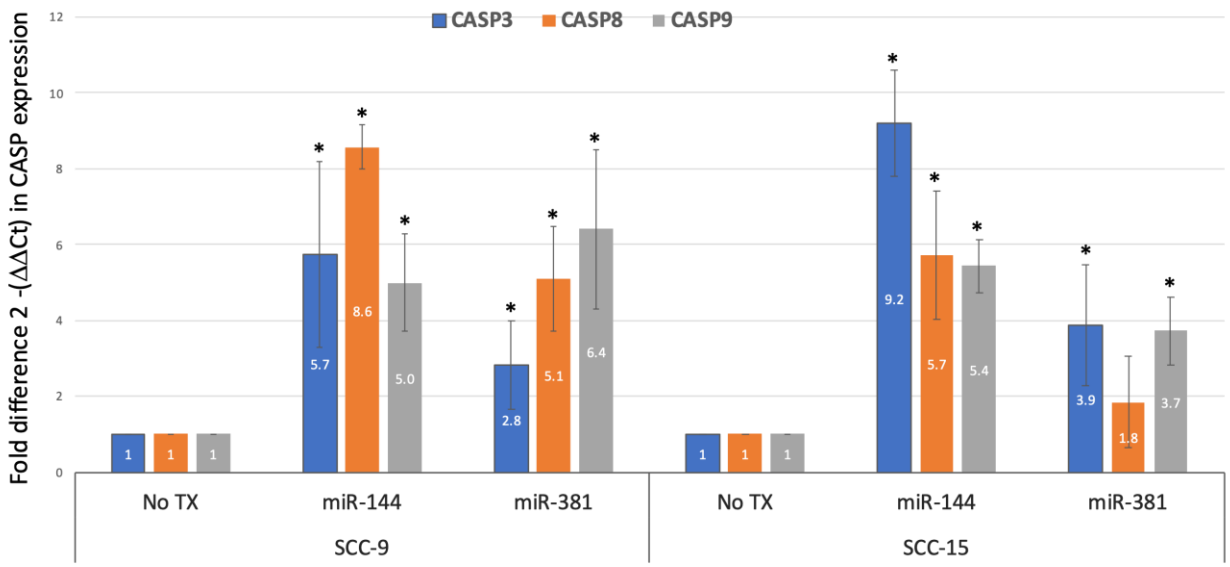


**Figure 8: CASP3, CASP8 and CASP9 apoptotic gene expression in OSCC transfected with miR-144 and miR-38 mimics.** Average gene expression was calculated from triplicate qPCR reactions with each miRNA treatment condition performed in 3 separate experiments. Data is expressed as the mean fold difference in expression compared to controls (No TX). Error bars indicate standard deviation. *$p < 0.05$ indicates a significant increase in CASP expression compared to control.

# Chapter 5: Discussion and Conclusion

The primary objective of this study was to evaluate whether miRNA that downregulate ANO1 are expressed in oral cancer OSCC cell lines and to assess whether transfecting miRNA mimics into oral cancer cell lines can alter cellular phenotypes of proliferation and apoptotic gene expression due to ANO1 downregulation.

Initially we determined that miR-9, miR-132, and miR-381 that specifically target ANO1 transcripts for downregulation are expressed in the OSCC cell lines, SCC4, SCC9, SCC15, SCC25 and CAL27 cells. miR-144, which also targets ANO1 transcripts for downregulation, is only expressed in SCC9 and SCC15. Compared to the non-malignant OKF4 cells, miR-132, miR-144 and miR-381 appear to be dysregulated in OSCCs. Studies from human cancers have shown that miRNA profiles from tumor tissue often differ from normal tissue, including oral cancer (Yete and Saranath, 2020; Wang et al, 2021). We focused subsequent experiments on miR-144 and miR-381 and their modulation of ANO1 expression and cancer cell phenotypes since miR-144 and miR-381 were downregulated in our OSCC expression analysis. In addition, targeting of ANO1 by miR-144 and miR-381 has been extensively validated in studies by Jiang et al., (2019) and Cao et al., (2019), respectively.

SCC9 and SCC15 cells were transfected with miR-144 or miR-381 mimics to determine the effect of miRNA that target ANO1 transcripts. We were able to confirm the successful introduction and transfection of miR-144 and miR-381 mimics to SCC9 and SCC15 as evidenced by the increased expression of miR-144 and miR-381 observed in qPCR analysis. Transfecting miR-144 significantly decreased ANO1 transcripts in both SCC9 and SCC15 cancer cells. Transfecting miR-381 also significantly decreased ANO1 transcripts in SCC9, but

the decrease in ANO1 expression observed in SCC15 cells with miR-381 was not significant. Our evaluation of SCC9 and SCC15 proliferation following miR-144 or miR-381 transfection indicates that miRNA induced downregulation of ANO1 slows proliferation in OSCC cells. miR-144 transfection resulted in similar and significant reductions in SCC9 and SCC15 cellular proliferation. miR-381 transfection resulted in significant reductions in SCC9 cells but the anti-proliferative effect of miR-381 on SCC15 proliferation was not significant. The SCC9 and SCC15 OSCC cell lines did not respond to the same degree when miR-381 is upregulated. The SCC15 cells were not as sensitive to miR-381 modulation. Our experiments to evaluate changes in apoptosis in OSCC cells showed that apoptotic caspase genes, CASP3, CASP8 and CASP9 are significant increased by miR-144 or miR-381 transfection in OSCCs.

Previous research has shown that microRNAs are involved in numerous pathways that promote tumor suppression in OSCC (Wang et al, 2020). For example, microRNAs are involved in the PI3K/Akt signaling pathway in OSCC cells (Myakannan et al, 2016). Growing genetic and cancer biology evidence shows that miRNAs tightly regulate the PI3K/AKT pathway (Naguchi et al, 2013). The PI3K/PTEN/AKT/mTOR signaling pathway is essential for maintaining homeostasis in proliferation, metabolism, migration, apoptosis, and other processes (Martini et al, 2014) and its dysregulation or constitutive activation as a result of mutations has been linked to the development of oral cancer (Murugan et al, 2016). It has been hypothesized that more than 47% of HNSCC samples, and specifically 38% of Indian OSCC samples, carry at least one genetic variation in this pathway (Mayakannan et al, 2016). As a result, losing miRNAs' ability to regulate PI3K/AKT signaling can have serious biological repercussions. In a different study, when miR-381-3p was upregulated, OSCC cell ability to proliferate and advance through the cell cycle was markedly inhibited, and apoptosis was encouraged (Yang et al., 2017). Notably, miR-

381-3p directly interacted with the 3' untranslated region of fibroblast growth factor receptor 2 (FGFR2) to reduce its expression. FGFR2 knockdown replicated miR-381-growth-suppressive 3p's effects (Yang et al., 2017). In contrast, increasing FGFR2 expression reduced the effects of miR-381-3p in OSCC cells (Yang et al, 2017). In OSCC tissues, the expression patterns of miR-381-3p and FGFR2 were negatively associated (Yang et al, 2017).

Not all OSCC cell lines responded to the same degree when microRNAs were upregulated in ANO1, as shown in our data. The complex nature of how miRNAs operate, as well as the unpredictable tumorigenicity of ANO1 in OSCCs, makes it difficult to pinpoint exactly why our proliferative and apoptotic analysis showed variable results. Therefore, there is a need for more studies to evaluate the growing role of miRNA and its effectiveness on ANO1 channels in oral cancer.

**Summary and Conclusions**

In the recent reports, researchers discovered ANO1 functioning as a calcium-activated chloride channel within 11q13 locus elevated in numerous cancers including oral cancer (Ramos-Garcia et al., 2017). The aims of the study were to explore if 1) miRNAs that negatively regulate ANO1 gene expression are expressed in oral cancer and, 2) if overexpressing miRNA that target ANO1 can influence oral cancer cell proliferation and apoptotic status. Utilizing the transfection of miR-144 and miR-144 -381 mimics to downregulate the expression of ANO1, our data present novel findings associated with the role of ANO1 channels in the modulation of the cellular phenotype concerning oral cancer cells.

Based on the findings of this study, we can reject the null hypothesis for research question 1 and 2 and accept the alternative hypotheses for both.

1. Are microRNAs (miR-9, -132, -144, -381) that specifically target ANO1 expressed in oral squamous cell carcinomas?
    - Alternative hypothesis ($H_A$): microRNAs (miR-9, -132, -144, -381) that specifically target ANO1 are expressed among oral cancers.
2. Does the modulation of specific microRNAs (miR-144, miR-381) which target ANO1 influence proliferation and apoptosis of oral cancer cells?
    - Alternative hypothesis ($H_A$): Modulating specific microRNAs (miR-144, miR-381) which target ANO1 does affect proliferation or apoptosis of oral cancer cells.

**Limitations and Future Direction**

Our understanding of miRNA influence on ANO1 transcription and the effect on the cellular phenotype of OSCCs has improved as a result of this study, particularly in relation to the ability to alter and manipulate proliferation and apoptotic gene expression. A limitation in this study was not having a control plasmid available to verify transfection efficiency across experimental repeats. The incorporation of a control such as a plasmid expressing green fluorescent protein or expressing β-galactosidase would identify experimental variation in transfection efficiency.

Another limitation of the study is that we could have included additional OSCC cell lines to acquire more data for our study. Our study focused on SSC9 and SSC15, but the addition of SCC4 and SCC25 would have aided in our understanding of miRNA effect on ANO1 channels.

Our study focused on miRNA-144 and miRNA-381 because they have been validated for specific targeting of ANO1 by previous studies (Jiang et al., 2019; Cao et al., 2019). However, examining the effects of other miRNA that target ANO1 channels should be considered in future studies, such as miRNA-9 and miRNA-132. Proteins associated with ANO1 and colorectal cancer cells such as P-AKT, P-ERK, and CD1 have been inhibited when miRNA-9 has been overexpressed (Park et al, 2019). Makutani et al. (2016) found miRNA-132 in primary colorectal cancer cell tissues with liver metastatic lesions to be significantly downregulated. We found miR-132 directly targets ANO1 and transfection of miR-132 significantly inhibited cell invasion and proliferation (Makutani et al., 2016).

Another hallmark of cancer to potentially explore in the future is the effect of miRNA targeting ANO1 on OSCC invasion and migration properties and if miRNAs down-regulate this aggressive cellular phenotype. ANO1 overexpression in head and neck squamous cell carcinoma (HNSCC) has been shown to increase the potential for distance metastasis (Ayoub et al., 2010). In esophageal cancer, ANO1 overexpression was correlated with advanced clinical stage and lymph node metastasis (Okuyama and Yanamoto, 2022.). Oral cancer etiology and progression are well elucidated, but oral cancer remains a life-threatening pathology, often due to late staging of the cancer at clinical diagnosis. It would thus be beneficial to evaluate whether miRNA can inhibit the contribution of ANO1 to invasion and migration of oral cancer cells that occurs in the later stages of tumor growth.

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