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The Role of SOX5 in the Progression of Oral Squamous Cell Carcinoma

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The Role of SOX5 in the Progression of Oral Squamous Cell Carcinoma

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Oral Biology

by

Jeffrey Brumbaugh

2018
ABSTRACT OF THE THESIS

The Role of SOX5 in the Progression of Oral Squamous Cell Carcinoma

by

Jeffrey Brumbaugh

Master of Science in Oral Biology
University of California, Los Angeles, 2018

Professor Shen Hu, Chair

Background: Cancer of the head and neck, including oral, laryngeal, and pharyngeal sites, is the sixth most common malignancy in the world. Every year, nearly 650,000 patients worldwide receive the diagnosis of head and neck cancer, and around 350,000 die from this disease [1]. More than 95% of these cancers are head and neck squamous cell carcinoma (HNSCC) in histology [1]. Early detection of the cancer and discovery of new targeted therapies are a few of the main approaches that may be able to increase patient survival rates.

SOX5, or the SRY box 5 protein, is a member of the SOX family of transcription factors and has yet to be fully characterized in oral cancer. In cancer biology, SOX5 has been shown to be involved in epithelial to mesenchymal transition (EMT) in breast cancer, hepatocellular cancer, prostate cancer, lung adenocarcinoma, and osteosarcoma [2-6]. SOX5 has also recently
been linked to nasopharyngeal cancer, but no study to date has described the role of SOX5 in oral cancer [7].

Equally significant in the pathogenesis of HNSCC, overexpression of epithelial growth factor (EGF) and its receptor (EGFR) has been found in roughly 90% of HNSCC tumors [8]. The downstream target effectors of EGFR, including the JAK/STAT pathway, have also been shown to be activated in HNSCC [8, 9]. In particular, STAT3 is known to be constitutively activated in many types of cancers, including HNSCC [8, 10].

**Objectives:** This study aims to identify the role and regulating mechanisms of SOX5 in oral cancer. Considering the prominence of EGFR and STAT3 in HNSCC, a link between STAT3 and SOX5 could help to elucidate a pathway of activation and regulation. SOX5 has been shown to be a downstream target of STAT3 in murine Th17 cells, but no relationship has yet been defined in cancer.

**Methods:** Phenotypic studies were conducted in highly invasive oral cancer cell lines (UM1 and UM5) with knockdown of SOX5 and in a low-invasive oral cancer cell line (UM2) with SOX5 upregulation. MTT, migration, and invasion assays were utilized to assess phenotype, and Western blotting and qPCR were used to quantify protein and gene expression levels. Chromatin immunoprecipitation (ChIP) followed by qPCR was used to examine if STAT3 binds to the promoter of SOX5.

**Results:** Endogenous SOX5 is up-regulated in UM1 and UM5 cells, when compared to UM2 and UM6 cells, respectively (p<0.05). Knockdown of SOX5 in UM1 and UM5 cells shows decreased proliferation, migration, and invasion potential. When treated with EGF, expression of SOX5 increased in all cell lines, and UM2 showed an increased ability to migrate and invade. ChIP
assay results indicate that STAT3 binds to the promoter region of SOX5 in both UM1 and UM5 cells.

**Conclusions:** This study has demonstrated that SOX5 may play an important role in head and neck cancer progression by promoting cancer cell growth, migration, and invasion. EGF induces the expression of SOX5 in head and neck cancer cells via the regulation of STAT3 and enhances cancer cell migration and invasion. These findings suggest that EGF-STAT3-SOX5 axis is an important regulatory pathway in head and neck cancer progression.
The thesis of Jeffrey Brumbaugh is approved.

Yong Kim
Diana Messadi
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University of California, Los Angeles
2018
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INTRODUCTION

Cancer of the head and neck, including oral, laryngeal, and pharyngeal sites, is the sixth most common malignancy in the world. Every year, nearly 650,000 patients worldwide receive the diagnosis of head and neck cancer, and around 350,000 die from this disease [1]. More than 95% of these cancers are head and neck squamous cell carcinoma (HNSCC) in histology [1]. These cancers have traditionally been treated with therapies including surgical resection, radiation therapy, and chemotherapy as both primary and adjuvant modalities [1]. On the other hand, more refined target-specific therapies, including small-molecule drugs and monoclonal antibodies, are directed at cancer cells while sparing normal cells and producing fewer side effects [1]. Even with these new techniques, patient survival rates still have not significantly improved, due to tumor recurrence and metastasis [1]. Early detection of cancers as well as discovery of new targeted therapies are a few of the main approaches that may be able to increase patient survival rates.

The SOX gene family was first discovered in 1990 during the search for families of evolutionarily conserved transcription factor genes [11]. Eventually, this search led to the cloning of SRY, the sex-determining region Y gene, which is the mammalian Y-linked testis-determining gene. SRY was the first SOX protein characterized, and now a full 20 SOX proteins have been identified. All SOX proteins share a highly conserved high-mobility-group box domain that was originally identified in SRY [12]. SOX5, or the SRY box 5 protein, is a member of this SOX family, which belongs to the subgroup of the SOXD proteins. Structurally, the SOXD proteins – SOX5, SOX6, and SOX13 – all contain a leucine zipper and a coiled-coil domain which causes them to form constitutive dimers in solution [13]. This dimerization is essential in the ability of the SOX proteins to bind DNA. Functionally, SOX5 is not grouped
with the rest of the SOXD proteins, but is often described as part of a “SOX trio” consisting of SOX5, SOX6, and SOX9 [14-16]. This SOX trio provides a signaling cascade to induce chondrogenesis and chondrocyte differentiation [14-16] (Figure 1). Individually, SOX 5 hinders melanogenesis, promotes neural crest generation, and controls the rate of neurogenesis [17]. In cancer biology, on the other hand, SOX5 has been shown to be involved in epithelial to mesenchymal transition (EMT) in breast cancer, hepatocellular cancer, prostate cancer, lung adenocarcinoma, and osteosarcoma [2-6]. SOX5 has also recently been linked to nasopharyngeal cancer [7], but no study to date has described the role of SOX5 in oral cancer.

As Hanahan and Weinberg first described in 2000, a commonality existed among cancer cell genotypes that could be described by six essential alterations – or hallmarks – in cell physiology [18]. Firstly, cancer cells exhibit the acquired capability of self-sufficiency in growth signals so that growth signals can be produced by the cell itself, eliminating the dependence on growth factors from other cells and tissues. To compound this self-sufficiency, these cancer cells also show an insensitivity to anti-growth signals that normally maintain tissue homeostasis. The next two hallmarks of cancer include the ability to evade apoptosis and a limitless replicative potential. In this way, cancer cells can circumvent senescence and expand into the mass of a tumor. Sustained angiogenesis is another hallmark of cancer that is crucial to supply the oxygen and nutrients needed for cell function and survival. Lastly, tissue invasion and metastasis is one of the most lethal characteristics of cancer, which causes 90% of human cancer deaths [18].

While certainly the causes for cancer are multifactorial, the SOX family plays crucial roles in cell fate and differentiation [12], and SOX5 in particular shows evidence to be a transcription factor implicated in the growth and development of several types of cancer. As regulators of cell fate and differentiation, SOX proteins have myriad roles in cellular growth and development.
Identification of potential binding sites of the SOX5 transcription factor would provide further insight into the mechanisms over which SOX5 and other SOX proteins govern in cancers.

In a similar manner, epidermal growth factor (EGF) and its receptor (EGFR) have also been well-studied in cancers. The EGFR family of receptor tyrosine kinases, also known as ErbBs or HER receptors, consists of four individual receptors: EGFR/ErbB-1, HER2/ErbB-2, HER3/ErbB-3, and HER4/ErbB-4 [9, 19]. They are all structurally related and share a single chain transmembrane domain composed of an extracellular ligand-binding region and an enzymatic cytoplasmic region, a tyrosine kinase domain, and a C-terminal end [9, 19]. Upon ligand binding, EGFR undergoes a conformational change, including receptor dimerization and cross-phosphorylation, which creates binding sites for adapter proteins and signaling complexes [19]. Dissociation then releases the bound effector molecules to stimulate downstream signaling cascades [19]. Functionally, EGFR assumes many different roles involved with the morphogenesis and maintenance of specific types of tissues [9]. It is from aberrations in these crucially important roles that cancers may arise.

Most epithelial malignancies, in fact, exhibit overexpression of EGFR [8]. Especially significant in the pathogenesis of HNSCC, overexpression of EGFR and one of its ligands, transforming growth factor alpha (TGF-α), has been found in roughly 90% of HNSCC tumors [8]. The downstream target effectors of EGFR, including PI3K/mTOR, JAK/STAT, and PLCγ1/PKC pathways, have also been shown to be activated in HNSCC (Figure 2) [8, 9]. In particular, STAT3 and STAT5 are known to be constitutively activated in many types of cancers, including HNSCC [8, 10, 20]. Increased levels of EGFR have been correlated with poor clinical prognosis [8, 21], and upregulation of STAT3 has been linked with HNSCC development and progression [10, 22].
This study aims to identify the role and related mechanisms of SOX5 in oral cancer progression. Considering the prominence of EGFR and STAT3 in HNSCC, a link between STAT3 and SOX5 could help to elucidate a pathway of activation and regulation. SOX5 has been shown to be a downstream target of STAT3 in murine Th17 cells and in B-cell lymphoma [23-25], but no relationship has yet been defined in oral cancer. This study of SOX5 may reveal a novel molecular mechanism underlying oral cancer progression and lead to possible targets for therapeutic intervention.
MATERIALS AND METHODS

Cell culture

Human head and neck squamous cancer cell (HNSCC) lines – UM1, UM2, UM-SCC5 (UM5), and UM-SCC6 (UM6) were cultured and maintained in Dulbecco’s modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Normal human oral keratinocytes (NHOKs) were cultured in keratinocyte basal media with human keratinocyte growth supplement. Cultures were maintained in a humidified chamber at 37°C in an atmosphere of 5% CO₂, and medium was changed every 3 days. Cells were passaged or harvested at 90% confluence with trypsinization. UM1 and UM5 are highly invasive HNSCC lines, while UM2 and UM6 are low-invasive HNSCC cell lines [26-28]. UM1 and UM2 cells originate from tongue cancer of the same patient, while UM5 cells derive from a laryngeal tumor, and the site of origin for UM6 cells is the base of a tongue cancer [26-28].

UM1, UM2, and UM5 cells were treated with epidermal growth factor (EGF) to determine the effect on SOX5 expression levels. Thirty-six hours following passage to a 6-well plate, the medium was changed for the cells, and EGF (Gemini Bio, Sacramento, CA, USA) was added to separate wells of the plate to a final concentration of 10 ng/mL. The cells were incubated for 3 days and subsequently harvested for protein and mRNA analysis.

Western blotting

Sample protein was extracted from cell cultures at 90% confluency, following lysis with rehydration (RB) buffer. Polyacrylamide gels (8%) were cast with Mini-PROTEAN cast plates, stands, and wells (Bio-Rad, Hercules, CA, USA), and proteins were separated in a Tris/glycine/SDS running buffer at 120V for 90 minutes. Proteins were transferred onto a
nitrocellulose membrane (Bio-Rad) at 15V for 60 minutes, and the membrane was subsequently blocked with 5% non-fat milk for 60 minutes at room temperature.

Primary antibodies against SOX5 (MyBioSource, San Diego CA, USA; [MBS8245243]) and GAPDH (GeneTex, Irvine, CA, USA; [GT239]), which served as control, were diluted to a ratio of 1:500 and 1:300 respectively, in 5% milk and incubated with the membrane overnight at 4°C with constant agitation. Secondary rabbit antibodies (GE Healthcare, Pittsburgh, PA, USA) were diluted at 1:7500 to conjugate SOX5 antibody, and secondary mouse antibodies (GE Healthcare) were diluted at a ratio of 1:2000 to conjugate GAPDH antibody.

An enhanced chemiluminescence (ECL) kit (GE Healthcare) was utilized to detect the Western blot signal. The membrane was incubated at room temperature for 90 seconds with the ECL reagents, and duplicate radiograph films were exposed to the membrane in a darkroom for 5 seconds to 30 minutes and then developed. The resultant bands were quantified by the ImageJ software, and p value was calculated based on triplicate results.

**Real-time quantitative polymerase chain reaction (RT-qPCR)**

mRNA was extracted from cell lysates using the Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). Conversion to cDNA was completed using the Invitrogen Superscript III reverse transcriptase kit. SYBR Green (Thermo Fisher Scientific, Waltham, MA, USA) served as the fluorophore, and β-Actin served as control. SOX5 and Actin primers were obtained from Sigma-Aldrich, and primer sequences can be found in Table 1. RT-qPCR was run on a Bio-Rad CFX Real-Time PCR Detection System, with the following sequence: polymerase activation at 95°C for 2 minutes, denaturation at 95°C for 15 seconds and extension at 60°C for 1 minute for 40 cycles, and a final melting curve from 65°C to 95°C. Fold change calculations were based on delta-delta-Ct values, and p value was calculated based on triplicate results.
SOX5 knockdown

UM1 and UM5 cell lines were treated with SOX5 siRNA (Santa Cruz Biotechnology, Dallas, TX, USA) and control siRNA (Santa Cruz) for comparison. Cells were cultured in a 6-well plate, treated with target siRNA or control siRNA once at 70% confluency, and incubated for 60 hours. Lipofectamine RNAiMAX (Thermo Fisher) served as the transfection reagent. After 60 hours, cells were either collected for qPCR or Western blot analysis, or they were passaged for further assays.

Migration assay

After SOX5 knockdown or treatment with EGF, cells were passaged and plated in an ibidi silicone 2-well culture insert (ibidi USA, Fitchburg, WI, USA), in a 6-well plate. Each insert was seeded with 35,000 cells for UM1, UM2, and UM5 lines, with a total volume of 70 µL per insert well. The cells were cultured in FBS-free medium for 48 hours, when the silicone inserts were removed, providing an exact 500 µm width of cell-free growth. The cells were washed once with phosphate buffered saline (PBS), then 1 mL of medium containing 2.5% FBS to prevent starvation was added to each well of the 6-well plate. At this point, considered the initial time point, photos were taken with a microscope camera at 40x and 100x magnification every 6 to 12 hours until the gap was closed. The area remaining between migrating fronts of the cells was quantified from three fields of view using the ImageJ software.

Invasion assay

Invasion assay was performed following SOX5 knockdown or treatment with EGF using the Corning Matrigel invasion chambers (Fisher Scientific). In a 24-well Falcon plate, the Matrigel chambers were first rehydrated for 2 hours with FBS-free medium in a humidified chamber. Complete medium was then added as a chemoattract to the well of the 24-well plate,
while 300,000 cells in FBS-free medium were seeded into the Matrigel chambers, which were suspended over the chemoattractant. After 24 hours of incubation to allow for invasion through the Matrigel, all the medium was removed, and the inserts were stained with a Diff-Quik stain kit. Non-invading cells were scrubbed off the top of the Matrigel with a cotton tipped swab, and cells were visualized at 40x and 100x with a microscope camera. Invading cells were counted and quantified from three fields of view using the ImageJ software.

**MTT assay**

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was utilized to measure the proliferation of UM1 and UM5 cells following SOX5 knockdown. Cells were plated in a 96-well plate, following treatment with either SOX5 siRNA or control siRNA. Five wells were used for each sample, at each time point. The outermost wells of the plates were not utilized but were filled with PBS to minimize evaporation. 2,000 cells were plated to each well, to a total volume of 180 µL, and incubated for 24 hours to allow for attachment. After this incubation, 20 µL of yellow tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (10x) was added to each well at day 1 and incubated for 4 hours, for a final 1x concentration. After 4 hours, medium was carefully removed from each well at day 1, and 200 µL of DMSO was added to dissolve the violet formazan crystals formed. The plate was shaken for 30 seconds on an optical reader, and the absorbance was read at 570 nm. DMSO was removed from the plate and the empty wells filled with 200 µL of PBS. This procedure from the addition of MTT reagent was repeated for 6 days in total, and medium was changed for all remaining wells after absorbance measurement on days 2 and 4.

**Chromatin Immunoprecipitation (ChIP)**
A magnetic bead chromatin immunoprecipitation (ChIP) kit (Millipore, Burlington, MA, USA) was utilized on UM1 and UM5 cells to investigate binding between STAT3 and the promoter of SOX5. Cells were grown to 100% confluence in 10 cm plates and treated with 1% formaldehyde to crosslink proteins and DNA. Unreacted formaldehyde was quenched using glycine, and cells were washed with 20 mL of PBS two times. After the plates were scraped for cell collection, the cells were lysed followed by nuclear lysis. Samples were sonicated using a Tekmar sonic disruptor to shear DNA and create crosslinked fragments of about 200-1000 base pairs in length.

Immunoprecipitation of crosslinked protein and DNA occurred during incubation of each sample with a slurry of protein A magnetic beads and 5 µg of an appropriate primary antibody. Positive control was achieved with an anti-acetyl histone H3 antibody, and negative control was achieved with normal rabbit IgG antibody. STAT3 antibody (Proteintech, Rosemont, IL, USA; [10253-2-AP]) was used for immunoprecipitation, and all samples were incubated overnight at 4°C.

Protein/DNA complexes were eluted from the protein A magnetic beads using a series of elution buffers, and crosslinks were reversed by incubation in ChIP elution buffer with proteinase K at 62°C for 2 hours. DNA was purified using spin columns and another series of elution buffers to produce a purified DNA eluate. DNA samples were subjected to qPCR targeting the promoter sequence of SOX5, with a total of five primers tested (Table 1).

**Statistical analysis**

All experiments were performed in triplicate to provide statistical significance. Student’s t-test calculations resulting in a p-value of less than 0.05 were considered statistically significant. Error bars and standard deviations were measured and provided for each experiment.
software (NIH, Bethesda, MD, USA) was utilized to quantify Western blots, migration, and invasion assays.
RESULTS

Differential expression of SOX5 between HNSCC cells and NHOKs

Differential expression of SOX5 was observed between highly invasive oral cancer cell lines UM1 and UM5 and the low-invasive oral cancer cell lines UM2 and UM6, as well as NHOKs. qPCR results show that UM1 and UM5 cells exhibited increased mRNA levels when compared to UM2 and UM6 cells, with an almost 2-fold difference between UM1 and UM2 cells and a 1.5-fold difference between UM5 and UM6 cells (Figure 3). Among all HNSCC lines, protein levels revealed through Western blot showed similar expression patterns, with UM2 protein levels 51% of UM1 (p<0.05), and UM6 protein levels 19% of UM5 (p<0.01) (Figure 4). However, protein levels of SOX5 in NHOK were significantly increased.

siRNA knockdown of SOX5

Knockdown of SOX5 was performed in UM1 and UM5 cell lines to evaluate phenotypic effects of SOX5 down-regulation on cell proliferation, migration, and invasion. In each cell line, knockdown was confirmed through both qPCR and Western blot analyses. Protein levels showed a reduction in both UM1 cells (Figure 5A) and in UM5 cells (Figure 6A) when compared to their control groups. Quantification of mRNA levels showed a 59% reduction in UM1 cells (p<0.05) (Figure 5B) and 35% in UM5 cells (p<0.01) (Figure 6B) when compared to their control groups.

Knockdown of SOX5 inhibited the proliferation of UM1 and UM5 cells

The proliferation rates of UM1 and UM5 cells were measured following SOX5 knockdown over the course of 6 days through MTT assay. The results illustrated an inhibited proliferative capacity of all cell lines when compared to their respective control groups. SOX5 knockdown resulted in a significant decrease in cell proliferation in UM1 cells (p<0.01) (Figure 7) and a comparable decrease in UM5 cells (p<0.05) (Figure 8).
Knockdown of SOX5 inhibited the migration of UM1 and UM5 cells

Migratory capabilities of UM1 and UM5 cells were similarly affected by knockdown of SOX5. Migration rates were suppressed in both cell lines when compared to control, although to a different extent in each cell line. UM1 control cells achieved full gap closure by 24 hours, with 54% of gap surface area remaining in the knockdown group (p<0.05) (Figure 9). UM5 control cells achieved full gap closure by 72 hours, with 29% of gap surface area remaining in the knockdown group (p<0.01) (Figure 10).

Knockdown of SOX5 inhibited the invasion of UM1 and UM5 cells

As highly invasive cell lines, both UM1 and UM5 cells exhibited diminished capability to invade through Matrigel invasion chambers when SOX5 was knocked down. The number of invading cells was quantified after knockdown of SOX5 and demonstrate a 42% reduction in UM1 cells (p<0.01) (Figure 11) and a 49% reduction in UM5 cells (p<0.05) (Figure 12), when compared to control groups.

SOX5 was upregulated in HNSCC cells when treated with EGF

Western blot analysis showed that when treated with EGF, protein expression of SOX5 significantly increased in UM1, UM2, and UM5 cells (Figure 13), as did gene expression of SOX5. An 8.5-fold increase in mRNA levels of SOX5 was observed in UM1 cells (p<0.05), a 3.7-fold increase in UM2 cells (p<0.01), and a 4.9-fold increase in UM5 cells (p<0.01) (Figure 14).

Treatment with EGF promoted the migration and invasion of UM2 cells

Treatment with EGF enhanced the migratory capacity of UM2 cells. The UM2 treatment group achieved full gap closure in 36 hours, as compared to 41% of the gap area remaining in the control group at the same time point (p<0.01) (Figure 15).
While under normal conditions, UM2 is considered low-invasive phenotypically. However, treatment with EGF promoted the invasive capability of UM2 cells. The number of cells invading through Matrigel invasion chambers was quantified and showed an increase of 56% in UM2 EGF-treated cells (p<0.01) (Figure 16).

**Chromatin Immunoprecipitation (ChIP) in UM1 and UM5**

To determine the potential regulatory role of STAT3 on SOX5, ChIP assay was performed in UM1 and UM5 cells using an anti-STAT3 antibody to immunoprecipitate DNA/protein complexes, and qPCR was subsequently performed using five primer sequences against the promoter of SOX5. In UM1 cells, all five primer sequences showed significant results (p<0.05), with a range from 3 to 3.5-fold increase in the levels of SOX5 promoter present when compared to the negative control (Figure 17A). ChIP analysis of UM5 cells showed comparable results, with all except the fourth primer showing significant results (p<0.01) and a 3.2 to 4.7-fold increase in SOX5 promoter DNA levels (Figure 17B).
DISCUSSION

This study aims to determine the role of SOX5 in the growth and progression of oral cancer and to identify potential mechanisms of activity and regulation. SOX5 has previously been identified as a transcription factor regulating neurogenesis, melanogenesis, and chondrogenesis when considered as part of the “SOX trio” of SOX5, SOX6, and SOX9. In cancer biology, SOX5 has also been described as a regulator of EMT and has been linked to nasopharyngeal cancer. To address the first objective, two highly invasive oral cancer cell lines, UM1 and UM5, were subjected to phenotypic assays following the knockdown of SOX5. As demonstrated, knockdown of SOX5 significantly reduced the proliferation (Figures 7, 8), migration (Figures 9, 10), and invasion (Figures 11, 12) of both highly invasive cell lines. These results imply that SOX5 may play a crucial role in the progression of oral cancer, as it does with other types of cancer.

Based on the Western blot analysis and qPCR measurements, the endogenous expression of SOX5 was significantly higher in highly invasive UM1 and UM5 cells when compared to low-invasive UM2 and UM6 cells (Figures 3, 4). However, SOX5 protein levels are also increased in NHOKs, which could potentially be attributed to the conditions in which NHOKs are cultured. Growth factors, including human EGF and insulin-like growth factor-1 (IGF-1), are present in human keratinocyte growth supplement which is used as part of the culture media. It has been demonstrated that SOX5 may serve as a regulator of IGF-1 [29, 30], but no direct link between EGF and SOX5 has been reported. Investigation of the EGFR signaling pathway (Figure 2) identified several downstream targets, which include the JAK/STAT signaling cascade. STAT3, or signal transducer and activator of transcription 3, is a transcription factor
that is activated when phosphorylated by JAK. It has been linked to SOX5 as a regulator in murine Th17 cells [23] but not in oral cancer.

To determine if EGF regulates the expression of SOX5 via STAT3 in HNSCC cells, all cell lines were treated with EGF for 3 days, and resulting SOX5 expression levels were measured. Indeed, SOX5 protein levels were significantly elevated in all EGF treated cells. After EGF treatment, UM2, a low-invasive cancer line, was subjected to phenotypic assays (proliferation, migration and invasion assays). As illustrated in Figures 15 and 16, the treatment with EGF enhanced the ability of UM2 cells to migrate and invade. These results reinforce the importance of SOX5 in the progression of oral cancer.

Although the effects of EGF on SOX5 expression and the migration/invasion potential of UM2 cells are clear, a chromatin immunoprecipitation assay (ChIP) is required to confirm if STAT3, a downstream target of EGF, transcriptionally regulates SOX5 in HNSCC cells. As confirmed by the ChIP assay, STAT3 significantly binds to the promoter of SOX5 in UM1 and UM5 cells (Figure 17), suggesting that STAT3 may transcriptionally regulate SOX5 expression. As such, it appears that EGF binds to EGFR and stimulates STAT3 activity, which in turn regulates the transcription of SOX5 and ultimately affects the phenotype of HNSCC cells.
CONCLUSION

This study has preliminarily characterized the role of transcription factor SOX5 in the growth and progression of HNSCC. Its impact on the proliferation, migration, and invasion of head and neck cancer cells was studied by means of SOX5 knockdown or SOX5 upregulation by EGF. This study also aimed to identify potential targets of action and regulation of SOX5. A connection has been drawn to link EGF, STAT3, and SOX5 in a regulatory pathway, with the suggestion that EGF induces the expression of SOX5 via the regulation of STAT3.

While the phenotypic effects of SOX5 are clearly perceived, the limitations of this study place constraints on the full characterization of the mechanisms at hand. The suggested interaction between STAT3 and SOX5 should be more fully explored to investigate whether this binding is essential for expression of SOX5 and which cofactors may be involved. Further supplemental studies would also include use of a plasmid to overexpress SOX5 in oral cancer cells, thus eliminating any ambiguity of the EGF-STAT3-SOX5 pathway. Overexpression of SOX5 would prove especially useful in rescue experiments of SOX5 knockdown cells.

The upstream regulation of SOX5 through STAT3 merits additional investigation to characterize the interaction between the two molecules, and the downstream targets of SOX5, as a transcription factor itself, also hold a great potential for study. IGF-1 remains a prime target for examination, as SOX5 has been suggested to be a possible enhancer and trans-activator of IGF-1 [29, 30]. In gastric cancer and in renal cell carcinoma, IGF-1 has been shown to increase tumor proliferation, invasion, and metastasis via STAT3 [31-33]. Considering that IGF-1 signals through the JAK/STAT pathway and is also a possible target of the same pathway, a self-regulating feedback loop may exist. Other downstream targets of SOX5 are well-established in the process of EMT, including Twist1 and Snail1 [2-6], and are worthwhile subjects of study in
oral cancer. Recently, long non-coding RNAs (lncRNAs) have gained attention as epigenetic contributors and mediators of cancer progression, carcinogenesis, and metastasis. lncSOX5 has been associated with oral cancer in broad and with tongue carcinogenesis and colorectal cancer specifically [34-37]. Although a distinct and separate entity, lncSOX5 contributes another layer of complexity that surrounds the SOX5 gene and oral cancer.

The translational and clinical applicability of SOX5 as a prognostic and therapeutic target in oral cancer may be inferred from studies investigating its role in the progression of other types of cancer and in cancer processes such as EMT. miRNAs targeting SOX5 have been shown to decrease the migration, invasion, and growth of tumor cells in pituitary tumors, breast cancer, and glioblastoma [38-40]. In osteosarcoma, knockdown of SOX5 resulted in a reduction of cell proliferation and invasion [41]. The potential of SOX5 as a prognostic target has been identified in lung adenocarcinoma, glioma, breast cancer, and melanoma, and association with SOX5 correlated to poor outcomes in prognosis as well as an increase in metastasis [6, 42, 43].

HNSCC therapies involving anti-EGFR agents are becoming more widespread, although their efficacy and safety are not fully understood [44]. The link suggested in this study between EGFR, STAT3, and SOX5 could potentially provide additional insight into the mechanism of the anti-EGFR agents used in treatment. The elucidation of the detailed pathway of SOX5 in oral cancer may lead to potential new targets for cancer treatment modalities.
Figure 1. Part of the “SOX Trio,” SOX5, in conjunction with SOX6 and SOX9, contributes to chondrogenesis through differentiation of mesenchymal cells to chondrocytes [45].
Figure 2. Signaling Pathway of EGF and STAT3 [46]. EGF binds to EGFR, which activates the receptor to phosphorylate and dimerize. Downstream target, STAT3, is activated as part of the JAK/STAT pathway and promotes the transcription of molecules largely involved with cell survival, proliferation, and oncogenesis.
**Figure 3.** qPCR analysis of the gene expression levels of SOX5 among UM1, UM2, UM5, and UM6 cells. Expression levels of SOX5 are significantly higher in UM1 and UM5 cells when compared to UM2 and UM6 cells, respectively (p<0.05).

(* indicates p<0.05; ** indicates p<0.01)
Figure 4. Western blot analysis of the endogenous protein expression levels of SOX5. (A) Western blots of SOX5 in UM1, UM2, UM5, UM6 cells and NHOKs. (B) Quantification of Western blot results showing significantly higher levels of SOX5 in UM1 vs UM2 cells (p<0.05) and in UM5 vs UM6 cells (p<0.01).

(* indicates p<0.05; ** indicates p<0.01)
**Figure 5. siRNA knockdown of SOX5 in UM1 cells.** (A) Western blot analysis showing knockdown of SOX5 with siSOX5 vs siControl. (B) qPCR analysis of SOX5 gene expression levels following siRNA knockdown.

(* indicates p<0.05; ** indicates p<0.01)
Figure 6. siRNA knockdown of SOX5 in UM5 cells. (A) Western blot analysis showing knockdown of SOX5 with siSOX5 vs siControl. (B) qPCR analysis of SOX5 gene expression levels following siRNA knockdown.

(* indicates p<0.05; ** indicates p<0.01)
Figure 7. MTT Assay for UM1 cells. Quantification of proliferation of UM1 cells treated with siSOX5 or siControl for 6 days. SOX5 knockdown cells are shown in yellow and control cells in blue, with a significant difference by the end of day 6 (p<0.01).

(* indicates p<0.05; ** indicates p<0.01)
Figure 8. MTT Assay for UM5 cells. Quantification of proliferation of UM5 cells treated with siSOX5 or siControl for 6 days. SOX5 knockdown cells are shown in yellow and control cells in blue, with a significant difference by the end of day 6 (p<0.05).

(* indicates p<0.05; ** indicates p<0.01)
Figure 9. Migration Assay for UM1 cells. (A) Visualization of gap closure at initial time of insert removal, 12 hours following, and 24 hours following (100x). (B) Quantification of the area of the gap remaining at each time point.

(* indicates p<0.05; ** indicates p<0.01)
Figure 10. Migration Assay for UM5 cells. (A) Visualization of gap closure at initial time of insert removal, 36 hours following, and 72 hours following (100x). (B) Quantification of the area of the gap remaining at each time point.

(* indicates p<0.05; ** indicates p<0.01)
Figure 11. Invasion Assay for UM1 cells. (A) Visualization of invasion of UM1 cells through Matrigel chambers (100x and 40x). (B) Quantification of the number of invading cells, showing a nearly 50% reduction of invasion in the cells with SOX5 knockdown.

(* indicates p<0.05; ** indicates p<0.01)
Figure 12. Invasion Assay for UM5 cells. (A) Visualization of invasion of UM5 cells through Matrigel chambers (100x and 40x). (B) Quantification of the number of invading cells, showing more than a 50% reduction of invasion in the cells with SOX5 knockdown.

(* indicates p<0.05; ** indicates p<0.01)
Figure 13. Western blot analysis of SOX5 in UM1, UM2, and UM5 cells. (A) Protein expression levels of SOX5 are induced by EGF. (B) Quantification of protein expression showing significantly higher levels of SOX5 in EGF treated UM1, UM2 and UM5 cells compared to the corresponding control cells. 

(* indicates p<0.05; ** indicates p<0.01)
Figure 14. qPCR analysis showing SOX5 levels following treatment with EGF in UM1, UM2, and UM5 cells. Expression levels of SOX5 are significantly higher in the EGF treated UM1, UM2 and UM5 cells compared to the corresponding control cells.
(* indicates p<0.05; ** indicates p<0.01)
Figure 15. Migration Assay for UM2 cells. (A) Visualization of gap closure at initial time of insert removal, 18 hours following, and 36 hours following (100x). (B) Quantification of the area of the gap remaining at each time point.

(* indicates p<0.05; ** indicates p<0.01)
Figure 16. Invasion Assay for UM2 cells. (A) Visualization of invasion of UM2 cells through Matrigel chambers (100x and 40x). (B) Quantification of the number of invading cells, showing more than a 50% increase of invasion in the EGF-treated cells.

(* indicates p<0.05; ** indicates p<0.01)
Figure 17. ChIP Assay for UM1 and UM5 cells. (A) qPCR analysis of levels of the promoter of SOX5 following immunoprecipitation with anti-STAT3 antibody in UM1 cells (p<0.01) and (B) in UM5 cells, with negative control as immunoprecipitation with normal rabbit IgG.

(* indicates p<0.05; ** indicates p<0.01)
Table 1. List of primer sequences used for qPCR analysis

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<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>SOX5</td>
<td>ATTGCCACTGCTGGTGTTGT</td>
<td>TGCTAGACACGCTTGAGTGC</td>
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<td>Promoter of SOX5</td>
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<tr>
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<td>TAGGCTCTCTCCGTTCACAC</td>
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<td>Actin</td>
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References


