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Molecular regulation of SOX5 and the role of its aberrant expression in epithelial to mesenchymal transition in human head and neck squamous cell carcinoma cells.

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A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Oral Biology

by

Wroocha Shyam Kadam

2019

ABSTRACT OF THE THESIS

Molecular regulation of *SOX5* and the role of its aberrant expression in epithelial to mesenchymal transition in human head and neck squamous cell carcinoma cells.

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Master of Science in Oral Biology

University of California, Los Angeles, 2019

Professor Shen Hu, Chair

Background: Head and neck cancer includes various tumors which originate in oral cavity, laryngeal, and pharyngeal sites. Globally, it is the sixth most common neoplasia with 6% patient cases diagnosed for this disease.[1] Histologically, more than 95% of head and neck cancer is classified as squamous cell carcinoma (SCC).[2] These are aggressive tumors with a mortality rate between 1-2%.[1] To increase patient survival, it is important to discover new relevant biomarkers for early detection, targeted therapies and screening for relapse or secondary lesions for improved cancer management.

Sex-determining region Y-gene (*SRY*) box 5 protein (SOX5), member of the subgroup D of *SOX* superfamily.[3] Recent literature in the cancer biology revealed that they are highly conserved transcription factors family and *SOX5* has been involved in epithelial to mesenchymal transition (EMT) in cancers like breast cancer, hepatocellular cancer, prostate cancer, lung adenocarcinoma, osteosarcoma and nasopharyngeal cancer.[4-9] Our

preliminary research has shown higher endogenous SOX5 expression in head and neck squamous cell carcinoma (HNSCC) cell lines. Also, *SOX5* knockdown has a regressive effect on the cancer progression in HNSCC.

Our earlier study also demonstrated that among the growth factors only epithelial growth factor (EGF) treatment to the cancer cells evidently upregulated SOX5 expression. EGF and its receptor epidermal growth factor receptor (EGFR) are expressed at much higher levels in HNSCC than in normal epithelial tissue and correlate with poor prognosis.[10, 11] The downstream target effectors of EGFR include the signal transducers and activators of transcription-3 (*STAT3*) which is constitutively activated in HNSCC.[10-13] *SOX5* has also been found as a downstream target of STAT3 in murine Th17 cells and in B-cell lymphoma.[14-16] Thus, investigating the EGF and EGFR signaling *via* STAT3 is very important to identify their role in *SOX5* regulation.

TWIST-1 and *SNAIL-1* are already well-established downstream targets of SOX5 aiding in the process of EMT in some cancers. *TWIST-1* play key roles in embryonic development, while mostly undetectable in healthy adult tissues. It is frequently reactivated in a wide array of human cancers[17-20]. It is also correlated with more metastatic lesions in head and neck cancer.[17] However, it's unclear about the mechanism of *TWIST-1* activation and its upstream signaling pathway during tumorigenesis.

Objectives: This study aims to identify a mechanism of regulation of *SOX5* in head and neck cancer progression. As higher expression of both EGF, EGFR and SOX5 separately correlated with poor clinical prognosis in cancer research. Also, *SOX5* has been found as a downstream target of STAT3 in few cancers. Establishing a link between active form of STAT3, *i.e.* phosphorylated STAT3 (phospho-STAT3), and *SOX5* will be crucial to illustrate a pathway of *SOX5* activation and regulation. Moreover, in some cancers *TWIST-1* and

SNAIL-1 are found as downstream targets of SOX5 aiding in the process of EMT. It could be interesting to identify the downstream targets of SOX5 in HNSCC.

Methods: Western blotting and qPCR were used to quantify SOX5 levels and some EMT markers across *SOX5* knockdown, EGF treatment and *SOX5* overexpression in UM1 and UM5 cells as the pathways potentiating the invasiveness are prominent in these two highly invasive head and neck cancer cell lines. Phenotypic studies were performed with EGF treatment and *SOX5* overexpression. MTT, migration, and invasion assays were utilized to assess phenotypes. Chromatin immunoprecipitation (ChIP) assay was used to examine the regulatory potential of phospho-STAT3 with *SOX5* promoter and of SOX5 with *TWIST-1* promoter. Luciferase assay was used to examine t regulation of *TWIST-1* promoter by SOX5.

Results: With EGF treatment, UM1 and UM5 exhibited an increased ability to proliferate, migrate and invade. With higher levels of *SOX5* after the overexpression of *SOX5*, there was an increase in proliferation, migration, and invasion potential in UM1 and UM5. ChIP assay results suggested binding of phospho-STAT3 to *SOX5* promoter following STAT3 immunoprecipitation and qPCR analysis of the *SOX5* promoter in UM1 and UM5. ChIP assay and Luciferase assay results indicated binding of SOX5 to *TWIST-1* promoter.

Conclusions: This study observes the impact of transcription factor *SOX5* in the progression of head and neck squamous cell carcinoma. *SOX5* has been found to be critical in EMT causing progression of HNSCC, as it has been shown in other types of cancers. Both upregulation and knockdown of *SOX5* have provided evidence to characterize its oncogenic effect in HNSCC cells. This study also found a few of the potential targets of action and regulation of *SOX5*. A link between EGF, STAT3, SOX5 and *TWIST-1* in a regulatory pathway is established suggesting that STAT3 may regulate *SOX5* which may further regulate *TWIST-1*. Recognizing this mechanism of *SOX5* in head and neck cancer provides

additional insightful knowledge about the role of SOX family in cancer. With more validating research, *SOX5* could be used as a prognostic biomarker in HNSCC.

The thesis of Wroocha Shyam Kadam is approved.

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Yong Kim

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2019

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INTRODUCTION

Head and neck cancers include various tumors which originate in oral, laryngeal, and pharyngeal sites. Globally, it is the sixth most common neoplasia with 6% patient cases diagnosed for this disease.[1] Histologically, more than 95% of head and neck cancer is classified as squamous cell carcinoma (SCC).[2] These are aggressive tumors with a mortality rate between 1-2%.[1] They are conventionally been treated with multimodality approach which includes surgery, radiation therapy and chemotherapy as well as supportive care.[2] There is a dire need to explore potential molecular biomarkers relevant for diagnosis, treatment and prognosis of head and neck cancers. Latest research advances concerning molecular characterization of the cancer has helped in better perception of the molecular mechanisms involved in head and neck squamous cell carcinoma (HNSCC) progression. But even with development of some new therapies like small-molecule drugs and monoclonal antibodies, mortality rates still have not significantly improved especially due to tumor recurrence and metastasis.[2] To increase patient survival, discovery of new relevant biomarkers for early detection, targeted therapies and screening for relapse or secondary lesions is essential in addition to the standard treatment.

SOX5 is a member of the subgroup D of *SOX* superfamily.[3] The *SOX* gene family consists of a total 20 SOX proteins which have been identified and are being studied since 1990.[21] *SRY,* which is the mammalian Y-linked testis-determining gene, was the first of the SOX proteins to be characterized. All SOX proteins are evolutionarily conserved transcription factor genes contains a highly conserved high-mobility-group (HMG) box domain that was formerly identified in SRY protein.[22] The other SOXD proteins are SOX6 and SOX9 and often these proteins are categorized as “SOX trio”.[23-25] (Figure 1) Their structure contains a leucine zipper and a coiled-coil domain which causes them to form constitutive dimers, making SOX proteins capable to bind DNA.[26] As the regulators of cell

fate and differentiation, SOX proteins have myriad roles in normal cellular growth & development. SOX trio mediates a signaling cascade to induce chondrogenesis and chondrocyte differentiation.[23-25] (Figure 1). SOX5 is not functionally same as the rest of the SOXD proteins. *SOX5* deters melanogenesis, encourages neural crest generation and regulates the rate of neurogenesis.[3] Recent literature in the cancer biology has revealed that *SOX5* has been involved in epithelial to mesenchymal transition (EMT) in a few cancer types namely breast cancer, hepatocellular cancer, prostate cancer, lung adenocarcinoma, osteosarcoma and nasopharyngeal cancer.[4-9]

A recent study conducted by our research laboratory about *SOX5* has shown that *SOX5* expression is overexpressed in few of HNSCC cell lines compared to the normal oral keratinocytes. Also, we performed SOX5 knockdown studies on UM1 and UM5 cell lines and different assays were performed to characterize its role in HNSCC. It was demonstrated that knockdown of *SOX5* significantly reduced the potential for proliferation, migration and invasion of the cancer cells. Thus, it suggests that *SOX5* may play a crucial role in presenting a distinct change in the phenotype of both highly invasive cancer cell lines. However, the molecular mechanism underlying SOX5 regulation in HNSCC remains unclear. Therefore, this study is intended to identify potential upstream and downstream targets involved in the mechanism of action and regulation of *SOX5* in progression of HNSCC.

Our earlier study also demonstrated that among the growth factors only EGF treatment to the cancer cells evidently upregulated *SOX5* expression. According to comprehensive cancer studies, EGF and EGFR are shown to be overexpressed in most epithelial malignancies compared to than in normal epithelial tissue.[10] EGFR and one of its ligands, transforming growth factor alpha (TGF-α), are expressed at much higher levels in HNSCC in approximately 90% of HNSCC tumors.[10, 11] Increased levels of EGFR is correlated with more aggressive, invasion and metastatic lesions thus leading to poor clinical

prognosis.[10, 27] The EGFR family consists of four receptor tyrosine kinases: EGFR/ErbB-1, HER2/ErbB-2, HER3/ErbB-3 and HER4/ErbB-4.[12, 28] They are composed of three substructures, a single chain transmembrane domain, an extracellular ligand-binding region and an enzymatic cytoplasmic region with a tyrosine kinase domain and a C-terminal end.[12, 28] The diversity in its receptors and ligands offers EGFR undertake versatile roles involving the morphogenesis and conservation of specific types of tissues.[12] Any anomalies in these important roles may lead to tumorigenesis.

What remains especially significant in the pathogenesis of HNSCC are the downstream effects of EGFR mediated through pro-survival pathways like PI3K/mTOR, JAK-STAT, and PLCγ1/PKC pathway.(Figure 2) The downstream target effectors of EGFR include the STAT3 and STAT5, which are constitutively activated in HNSCC and others cancers.[10-13, 29] (Figure 2) The upregulation of STAT3 has been connected with HNSCC development and progression.[13, 30] *SOX5* has been found as a downstream target of STAT3 in murine Th17 cells and in B-cell lymphoma,[14-16] but no relationship has yet been delineated in head and neck cancer. Thus, investigation of EGF and EGFR signaling via STAT3 is very important to identify their role in *SOX5* regulation in HNSCC.

The trademark of cancerous cell genotype could be explained by six physiological alterations.[31]. First two alteration are that the cancer cells develop an insensitivity to anti-growth signals and acquire self-sufficiency to independently produce growth signals like growth factors, including EGF, insulin-like growth factor-1 (IGF-1) and other factors for sustained angiogenesis needed for cell function and survival. It has been confirmed that *SOX5* may serve as a regulator of IGF-1.[32, 33] The next two hallmarks are the ability of cancer cells to circumvent senescence and develop a significant mass due to its self-renewal potential. Lastly, the most fatal features of cancer are tissue invasion and metastasis causing death.[31] On the terminal route of metastasis from primary tumor, cancer cells are required

to adapt changing and often hostile environmental conditions. This plasticity of tumor cells is reproduced by back-and-forth transitions from differentiated to undifferentiated or partial EMT-associated cancer cell phenotypes, gauged by various EMT markers.[34] (Figure 3) EMT is accomplished by the EMT-activating transcription factors (EMT-TFs), mainly of the *SNAIL-1*, TWIST and ZEB families. They play important roles in every stage of cancer progression right from initiation, primary tumor growth, invasion, dissemination and metastasis to colonization as well as in resistance to therapy.[34]

TWIST-1 is a basic helix-loop-helix protein that is transcriptionally active during in embryonic development, while mostly undetectable in healthy adult tissues, it is frequently reactivated in a wide array of human cancers[17-20]. *TWIST-1* play key roles in lineage determination and cell differentiation. In the metastatic cancer, some proposed upstream molecules in activation of *TWIST-1* are AKT1, STAT3, NOTCH1, MAPK, BMP7, RAS, TGF-B, WNT1, WNT2 and NF-KB mediating invasiveness and metastasis.[35] (Figure 4) TWIST-1 can sometimes act independently of SNAIL-1 to repress *E-CADHERIN* and to upregulate fibronectin and *N-CADHERIN*.[36] (Figure 5) Thus, it is usually correlated with more aggressive, invasive and metastatic lesions in head and neck cancer.[17] *TWIST-1* and *SNAIL-1* are already well-established downstream targets of SOX5 aiding in the process of EMT in some cancers. However, little is known about the mechanism of *TWIST-1* activation and its upstream signaling pathway during tumorigenesis and this study could link *SOX5* and *TWIST-1* in their regulation in HNSCC.

Though the etiology for cancer is multifaceted, the SOX family regulate crucial mechanisms in cell fate and differentiation.[22] In particular, *SOX5* is implicated in the growth and development of several types of cancer. Recognizing potential binding sites of the *SOX5* would afford us an insightful knowledge about the mechanisms over which SOX5 and other SOX proteins govern in cancer. Thus, this study aims to investigate the role of *SOX5*

and its regulating mechanisms in head and neck cancer progression. Since, there is a link between EGFR and STAT3 in HNSCC, establishing a connection between active form of STAT3 that is phosphorylated STAT3 and *SOX5* could help to elucidate a pathway of activation and regulation. In addition, finding the downstream targets and the role of *SOX5* EMT will help us understand the salient biochemical activities underlies this devastating cancer disease.

MATERIALS AND METHODS

Cell culture

UM1 cell line- originated from tongue cancer and UM-SCC5 (UM5) cell line was derived from a laryngeal tumor [37-39]. Both lines were cultured and maintained in antibiotics free Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum. Cultures were maintained in a humidified chamber at 37^{o}C in an atmosphere of 5% CO_2, and medium was changed every 3 days. Cells were passaged or harvested at 80% confluency with trypsinization. UM1 and UM5 cell lines were chosen for this study as the pathways potentiating the invasiveness will be prominent in these two highly invasive head and neck cancer cell lines and intervention with regards to this property could be distinctly pronounced.

EGF treatment

UM1 and UM5 cells were treated with EGF to determine the effect on SOX5 expression levels. Twenty-four 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 phenotypic assays.

SOX5 overexpression

UM1 and UM5 cells were cultured in a 6-well plate, once at 70% confluency they were transfected using the manufacturer's protocol. The final solution prepared had 500 ng *SOX5* CRISPR activation plasmid (SC-401854-ACT, Santa Cruz Biotechnology, Dallas, TX, USA) or 500 ng Control CRISPR activation plasmid (SC-437275, Santa Cruz Biotechnology, Dallas, TX, USA) for comparison using UltraCruz® transfection reagent (SC-395739, Santa Cruz Biotechnology, Dallas, TX, USA) and plasmid transfection medium. (SC-108062, Santa

Cruz Biotechnology, Dallas, TX, USA) Incubate the cells for 48 hours, cells were either harvested for qPCR or Western blot analysis or passaged for further assays.

Western blotting

Sample proteins was extracted by lysis of cells with rehydration buffer (RB) and the Bradford assay was performed to calculate their final concentrations. For protein separation, 4-8% polyacrylamide gels were cast with the Mini-PROTEAN cast plates and wells (Bio-Rad, Hercules, CA, USA), and 15 μg of each sample protein were separated in a Tris/glycine/SDS running buffer at 120V for 90 minutes. Proteins were semi-dry transferred onto a nitrocellulose membrane 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 (MBS8245243, MyBioSource, San Diego CA, USA), N-Cadherin (sc-271386, Santa Cruz Biotechnology, Dallas, TX, USA), E-Cadherin (SC-7870, Santa Cruz Biotechnology, Dallas, TX, USA), TWIST-1 (SC-81417, Santa Cruz Biotechnology, Dallas, TX, USA) and GAPDH (GT239, GeneTex, Irvine, CA, USA) were used in this study. All the antibodies were used at the dilution of 1:300 in 5% milk and incubated with the membrane overnight at 4^{o}C with constant agitation. Secondary rabbit antibodies (GE Healthcare, Pittsburgh, PA, USA) were diluted at 1:7500 to conjugate SOX antibody and secondary mouse antibodies (GE Healthcare) were diluted at a ratio of 1:2000 to conjugate N-Cadherin, E-Cadherin, TWIST-1 and GAPDH antibodies. An enhanced chemiluminescence (ECL) kit (GE Healthcare) was utilized to detect the signal. The membrane was incubated at room temperature for 3 minutes with the ECL reagents and duplicate radiograph films were exposed to the membrane in a darkroom for 2 minutes to 30 minutes and then developed. The resultant bands were quantified by the ImageJ, and p value was calculated based on triplicate results by Student's t-test.

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

Total 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. Total mRNA and cDNA final concentration were calculated using the Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). Samples for RT-qPCR were prepared using 10 µl Power up SYBR Green (Thermo Fisher Scientific, Waltham, MA, USA) served as the fluorophore, primers (Sigma-Aldrich St. Louis, MO, USA), 200 ng cDNA and DNAase, and the RNAase free water was used to bring final volume to 20 µl. RT-qPCR was run on a Quant studio 3 Real-Time PCR Detection System machine (Applied Biosystems) with the following sequence: polymerase activation at 95ºC for 2 minutes, denaturation at 95ºC for 15 seconds and extension at 60ºC for 1 minute for 40 cycles, and a final melting curve from 60ºC to 95ºC. Fold change calculations based on delta-delta-Ct values, and p value was calculated based on triplicate results by Student's t-test.

Migration wound healing assay

After *SOX5* overexpression or treatment with EGF, the cells were passaged and plated in a 2-well silicone insert (ibidi USA, Fitchburg, WI, USA), in a 12-well plate. Each insert was seeded with 70,000 cells for UM1 and UM5, with a total volume of 100 µL per insert well. The cells were cultured in FBS-free medium for 24 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 0.5 mL of medium containing 5% FBS was added to each well of the 12-well plate to prevent starvation. At this point, considered the initial time point, photos were taken with a microscope camera at 40x and 100x magnification every 12 hours until the gap was closed. The area remaining between migrating

fronts of the cells was quantified based on triplicate results using the ImageJ software and p value was calculated by Student's t-test.

Migration transwell assay

Following *SOX5* overexpression or treatment with EGF, transwell chambers (Costar) were used for migration assays. In a 24-well Falcon plate, the chambers were first rehydrated for 2 hours with FBS-free medium in humidified chamber. Complete medium was then added as a chemoattract to the well of the 24-well plate, while 50,000 cells in FBS-free medium were seeded into the Matrigel chambers which were suspended over the chemoattractant. After 36 hours of incubation to allow for invasion through the Matrigel, all the medium was removed, and the inserts were stained with crystal violet. Non-migrating cells were scrubbed off the top of the transwell with a cotton tipped swab, and cells were visualized at 40x and 100x with a microscope camera. The migrating cells were counted and quantified based on triplicate results using the ImageJ software and p value was calculated by Student's t-test.

Invasion assay

Invasion assay was performed following *SOX5* overexpression 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 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 36 hours of incubation to allow for invasion through the Matrigel, all the medium was removed, and the inserts were stained with crystal violet. Non-invading cells were scrubbed off the top of the Matrigel with a cotton tipped swab, and invaded cells were visualized at 40x and 100x with a microscope camera. Invading cells were counted and quantified based on triplicate results using ImageJ software and p value was calculated by Student's t-test.

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* overexpression or EGF treatment. 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. In total, 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 a 10x yellow tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well of timepoint day 1 and incubated for 4 hours, for a final 1x concentration. After 4 hours, medium was carefully removed from each well of timepoint day 1, and 200 µL of DMSO was added to dissolve the violet formazan crystals formed. The plate was gently 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 day 2 and day 4.

Chromatin Immunoprecipitation (ChIP)

A magnetic bead chromatin immunoprecipitation (ChIP) kit (Millipore, Burlington, MA, USA) was utilized to perform ChIP assay on UM1 and UM5 cells to investigate the binding between phospho-STAT3 and the promoter of *SOX5* and between SOX5 and promotor of *TWIST-1*. Cells were grown to 100% confluency 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 10 µg of an appropriate primary antibody. The positive control was achieved with an anti-acetyl histone H3 antibody, and the negative control was achieved with rabbit IgG antibody. The protein of interest was immunoprecipitated with phospho-STAT3 antibody (44-384G, Thermo Fisher Scientific, Waltham, MA, USA) or SOX5 antibody (MBS8245243, MyBioSource, San Diego CA, USA), and all samples were incubated overnight at 4ºC.

Protein/DNA complexed 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 62ºC for 2 hours. DNA was purified using spin columns and another series of elution buffers to produce a purified DNA eluate. To quantify the enrichment fold, 3µg genomic DNA samples immunoprecipitated by phospho-STAT3 antibody were subjected to qPCR targeting the promoter sequence of *SOX5*, with a total of five primers tested and 3µg DNA samples immunoprecipitated by SOX5 antibody were used for targeting the promoter sequence of *TWIST-1*, with a total of three primers.

Luciferase reporter assay

UM1 and UM5 cells were cultured in a 6-well plate to reach 70% confluency, for transfection. The final solutions were prepared in four different ways by diluting (I) 500 ng *SOX5* CRISPR activation plasmid and 300ng of *TWIST-1* promoter reporter clone (S717559 Active Motif, Carlsbad, USA) (II) 500 ng and Control CRISPR activation plasmid and 300ng of *TWIST-1* promoter reporter clone (III) 300ng of *TWIST-1* promoter reporter clone (IV) 300ng of empty reporter vector according to manufacturer's protocol using the UltraCruz® transfection reagent (SC-395739, Santa Cruz Biotechnology, Dallas, TX, USA) and plasmid

transfection medium. (SC-108062, Santa Cruz Biotechnology, Dallas, TX, USA) Incubate the cells for 48 hours.

After post transfection period, Reporter lysis buffer/ assay buffer were used to obtain respective sample lysates according to the manufacturer's protocol using the LightSwitch Assay Kit (LS010, Switchgear, Carlsbad, USA). The assay solution was prepared by adding reconstituted 100X substrate to assay buffer just prior to use. Afterwards, 20 µL of lysate with 100µL LightSwitch Assay Solution was directly added to each sample well in 96 well plate and incubated 30 minutes at room temperature while preventing light exposure. After 30 minutes of incubation, the luminescence signal in each well was measured for 1.5 seconds in a plate luminometer (SpectraMax L). All experiments were repeated in quadruplicates results and p value was calculated by Student's t-test.

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. The ImageJ software (NIH, Bethesda, MD, USA) was utilized to quantify the results of Western blots, migration, and invasion assays.

RESULTS

Treatment with EGF promotes the proliferation of UM1 and UM5 cells

To characterize the proliferation of the head and neck cancer cell lines, we performed the MTT assay. The proliferation rates of UM1 and UM5 cells with EGF treatment or no treatment were measured by absorbance following treatment with MTT over 6 days. As shown in Figure 6, UM1 and UM5 showed significantly higher proliferation rates with EGF treatment ($p<0.05$). The results demonstrated an enhanced proliferative ability of EGF treated cell groups when compared to their respective control groups.

Treatment with EGF increases the migration of UM1 and UM5 cells

To investigate the impact of EGF treatment on the motility of head and neck cancer cells, we conducted a wound healing assay of UM1 and UM5 cancer cells with EGF treatment or no treatment. We observed that both UM1 and UM5 cells, with EGF treatment, had faster rates of migration than the untreated cells. The EGF treatment group achieved full gap closure in 36 hours, as compared to 26.58% and 65.56% of the gap area remaining in the control group at the same time point of UM1 and UM5 cells, respectively ($p<0.001$) (Figure 7 and 8). Also, the migration transwell assay results show more migrating cells with EGF treatment than the no treatment group (UM1, $p<0.05$; UM5, $p<0.01$) (Figure 9 and 10).

Treatment with EGF stimulates the invasion of UM1 and UM5 cells

To investigate the effect of treatment with EGF in the invasion potential of UM1 and UM5 cells, we performed transwell invasion assay on UM1 and UM5 cells with or without EGF treatment. After EGF treatment, the number of EGF treated cells invading through Matrigel invasion chambers was found significantly increased when compared to the cells with no treatment (both UM1 and UM5, $p<0.01$) (Figure 11 and 12). Treatment with EGF promoted the invasive capability of these cell lines.

ChIP for *SOX5* promoter

To investigate if phospho-STAT3 binds to *SOX5* promoter in HNSCC cells, a ChIP assay was performed in UM1 and UM5 cells using an anti-phospho-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 very significant results ($p<0.01$) with a range from 10.5 to 13.3-fold increase in the levels of *SOX5* promoter present when compared to the negative control (Figure 13A). ChIP analysis of UM5 cells showed comparable results, with all primer showing significant results ($p<0.001$) with 8.5 to 16.89-fold increase in *SOX5* promoter levels (Figure 13B). These results suggest that phospho-STAT3 binds to the promoter of *SOX5*.

Plasmid overexpression of *SOX5*

EGF treatment was found to upregulate *SOX5* at both protein and mRNA expression levels in our earlier study. Here we investigated the effect of *SOX5* overexpression by using the *SOX5* overexpression (*SOX5* OE) plasmid. Western blotting and qPCR were used to analyze protein and mRNA expression changes, respectively, in UM1 and UM5 cells, which were transfected with *SOX5* overexpression plasmid or control plasmid vector (CTRL vector). Protein levels showed a distinct increase in both UM1 and UM5 cells (Figure 14A) when compared to their respective controls. Quantification of mRNA levels by qPCR showed significant increase in UM1 cells ($p<0.01$) and UM5 cells ($p<0.001$) (Figure 14B) when compared to their controls.

Overexpression of *SOX5* promotes the proliferation of UM1 and UM5 cells

The MTT assay was performed to characterize the proliferation of UM1 and UM5 cells with SOX5 overexpression. The proliferation rates of UM1 and UM5 cells transfected with *SOX5* overexpression plasmid or control plasmid vector were measured by absorbance following treatment with MTT over 6 days. As shown in Figure 15, UM1 and UM5 cells with

SOX5 overexpression showed significantly higher proliferation rates ($p<0.05$) than the control cells. The results demonstrated *SOX5* overexpression promotes the proliferation of HNSCC cells.

Overexpression of *SOX5* promotes the migration ability of UM1 and UM5 cells

To investigate the impact of *SOX5* overexpression on the motility of head and neck cancer cells, we conducted a wound healing assay of UM1 and UM5 cancer cells transfected with *SOX5* overexpression plasmid treatment or control plasmid vector. We observed that *SOX5* overexpressing cells had faster rates of migration than the control plasmid vector treated cells. *SOX5* overexpressing UM1 cells achieved full gap closure by 36 hours, with 80.7% of gap surface area remaining in control group ($p<0.0001$) (Figure 16). *SOX5* overexpressing UM5 cells achieved full gap closure by 60 hours, with 89.92% of gap surface area remaining in the control group ($p<0.0001$) (Figure 17). Migration transwell assay results also showed more migrating cells in *SOX5* overexpressing group than control group (UM1, $p<0.05$; UM1, $p<0.01$) (Figure 18 and 19)

Overexpression of *SOX5* enhances the invasion of UM1 and UM5 cells

To investigate the effect of *SOX5* overexpression on the invasion potential of UM1 and UM5 cells, we performed transwell invasion assay after transfecting UM1 and UM5 cells with *SOX5* overexpression plasmid or the control plasmid vector. The number of *SOX5* overexpressing cells invading through Matrigel invasion chambers was significantly increased when compared to the control cells (both UM1 and UM5, $p<0.001$) (Figure 20 and 21). These results suggested that SOX5 overexpression promoted the invasion of UM1 and UM5 cells.

Changes in the expression of EMT markers

We further investigated the relationship between *SOX5* and EMT in HNSCC cells by confirming the how different interventions of *SOX5* affect the expression of EMT markers. In

UM1 and UM5 cells, the expression of 10 distinct EMT markers was evaluated through qPCR and the expression of three EMT markers was measured with Western blotting. Samples from knockdown, EGF treatment and *SOX5* overexpression were used along with their respective control groups. Shown in Figure 22 A and B are Western blotting and qPCR analysis results from the UM1 and UM5 cells transfected with si*SOX5* (*SOX5* KD) or control scramble siRNAs (siCTRL). In both UM1 and UM5 cells, the expected results of increased E-Cadherin level and suppressed expression of N-Cadherin and TWIST-1 in *SOX5* knockdown compared to its control, suggest that there is gain of epithelial marker E-Cadherin and loss of mesenchymal marker N-Cadherin. Similar assays were performed on UM1 and UM5 cells upon EGF treatment. As we expected, in UM1 and UM5 cells, the expression of EMT markers, N-Cadherin and TWIST-1, were acquired while E-cadherin was attenuated after treatment with EGF (Figure 23A). Likewise, the quantification of mRNA expression of EMT markers shows an increased *N-Cadherin* and *TWIST-1* level and *E-Cadherin* level is reduced in EGF treatment group when compared to control (Figure 23B). We also performed Western blotting and qPCR of the EMT markers in UM1 and UM5 cells transfected with *SOX5* overexpression plasmid or control plasmid vector. Similarly, in both UM1 and UM5 cells with *SOX5* overexpression, the expression of N-Cadherin and TWIST-1 was found to be increased whereas the expression of f E-Cadherin was decreased (Figure 24A). qPCR analysis also demonstrated similar results at mRNA expression levels (Figure 24B), suggesting that there is change to mesenchymal nature by gain of N-Cadherin and loss of epithelial marker E-Cadherin due to *SOX5* overexpression.

ChIP assay for *TWIST-1* promoter

To determine if SOX5 binds to *TWIST-1* promoter region, we performed a ChIP assay in UM1 and UM5 cells using a SOX5 antibody to immunoprecipitate DNA/protein complexes and qPCR was subsequently performed using three different primers for the

promoter of *TWIST-1*. In UM1 cells, all three primer sequences showed significant results ($p<0.01$), with a range from 21.4 to 27.4-fold increase in the levels of *TWIST-1* promoter present when compared to the negative control (Figure 25A). ChIP analysis of UM5 cells showed comparable results, with three primers showing significant results ($p<0.01$), with a 19.9 to 23.7-fold increase in *TWIST-1* promoter compared to the negative control (Figure 25B).

Luciferase reporter assay in UM1 and UM5 cells for *TWIST-1* promoter

Luciferase reporter assays were performed to further verify if SOX5 regulates the expression of *TWIST-1* in HNSCC cells. Our results showed a higher activity luciferase activity in sample with *SOX5* OE plasmid and the *TWIST-1* reporter construct in both UM1 and UM5 cells. The luciferase expression in the *TWIST-1* promoter reporter construct by itself and control plasmid vector and *TWIST-1* promoter reporter construct, showed slightly higher activity than the empty vector but much lower than the *SOX5* overexpression plasmid and *TWIST-1* promoter reporter construct. Overall, both UM1 and UM5 cells showed a similar pattern of luciferase activities under the four different conditions (Figure 26). These results suggest that SOX5 may transcriptionally regulate the expression of *TWIST-1* in HNSCC cells.

DISCUSSION

Our preliminary studies have shown that higher endogenous SOX5 expression is found in HNSCC cells when compared to the normal oral keratinocytes. Also, the *SOX5* knockdown has a regressive effect on the cancer progression in HNSCC. It was also demonstrated that among the growth factors only EGF treatment to the cancer cells evidently upregulates *SOX5* expression. But there is no direct link between EGF and SOX5 has been suggested yet. The JAK/STAT signaling cascade is one of the several pathways mediated by EGFR. In order to closely examine SOX5 interaction with the potential upstream signaling molecules like EGF and STAT3. The effect of EGF treatment was investigated phenotypically in UM1 and UM5 cells (Figure 6 - Figure 12).

Also, our ChIP assays have demonstrated that phosphorylated STAT3 binds to *SOX5* promoter in HNSCC cells. In fact, the enrichment fold for phosphorylated STAT3 was significantly higher than those for STAT3 (Figure 13), suggestive of a regulatory relationship between phospho-STAT3 and SOX5 in HNSCC cells.

Our studies also demonstrate that *SOX5* overexpression promotes the proliferation, migration and invasion potential of UM1 and UM5 cells (Figure 14 - Figure 21). Collectively, our findings suggest that EGF binds to EGFR and stimulates STAT3 activity, which in turn upregulates SOX5 transcriptionally and ultimately affects the aggressive nature of these cancer cells.

SOX5 has also been described as a regulator of EMT and linked to nasopharyngeal cancer [9]. In this study, to determine the influence of *SOX5* in EMT process, UM1 and UM5 cells were treated with three separate conditions, including knockdown of *SOX5*, EGF treatment and *SOX5* overexpression. Our results revealed that higher levels of SOX5, N-Cadherin and TWIST-1 while reduced level of E-Cadherin in both EGF treated and *SOX5* overexpressing cells whereas an opposite result was obtained in the *SOX5* knockdown cells

(Figure 22 - Figure 24). Our findings indicate that *SOX5* indeed plays a crucial role in the EMT and progression of HNSCC.

Since *SOX5* and *TWIST-1* expression levels are concurrently increased, ChIP and luciferase assays were performed to confirm this connection between SOX5 and *TWIST-1*. The results verified that there is direct interaction and activation between SOX5 and *TWIST-1* promoter (Figure 25 - Figure 26). Collectively, all these findings conclude that EGF upregulates the expression of SOX5 in HNSCC cells via STAT3 and subsequently activate the TWIST-1 mediated EMT process and may promote the progression of HNSCC.

CONCLUSION

This study has satisfactorily identified a potential pathway related to the role of transcription factor SOX5 in the progression of HNSCC. It also identified the potential targets of action and regulation of *SOX5*. A connection linking EGF, STAT3, SOX5 and *TWIST-1* in a regulatory pathway was established, which suggests that STAT3 may regulate SOX5 and SOX5 may in turn regulate *TWIST-1*, in HNSCC cells The phenotypic effects of *SOX5* overexpression are very supportive of already known *SOX5* correlation with poor prognosis of certain cancers[7, 40, 41]. Our studies represent a step further uncovering the potential role of *SOX5* role in cancer biology.

Among the limitations of this study, screening the endogenous levels of EGF, EGFR, STAT3 and phospho-STAT3 in HNSCC tissues and normal oral epithelium could be utilized to develop more meticulous experiments like using high *SOX5* expression vs low *SOX5* expression SCCs or high EGFR expression vs low EGFR expression SCCs or invasive SCC vs non-invasive SCCs to validate the proposed pathway and establish a strong connection between EGF and SOX5 and their involvement in EMT process. Furthermore, the successful replication of this potential mechanism in vivo will provide a more comprehensive evidence. This study has identified a few regulatory components of pathway which may play pivotal role in cancer progression. It's important to discover the other potential target of SOX5 and cofactors associated with this suggested pathway to understand how *SOX5* plays such an intensive effect on the progression of HNSCC. Furthermore, rescue experiments of *SOX5* knockdown cancer cells will provide more additional insight.

It would be useful to study to established link to other downstream targets of SOX5 in the process of EMT, including *SNAIL-1* [8] in HNSCC. The long non-coding RNAs (lncRNAs) are recently explored as the epigenetic contributors and mediators of cancer progression, carcinogenesis, and metastasis. There is unambiguously evidence associating

lncSOX5 with colorectal cancer and tongue carcinogenesis[42-45]. It suggests there is a complex signaling network that *SOX5* may be a part of in HNSCC. There have been therapies such as miRNAs targeting of *SOX5* shown to decline the migration, invasion, and growth of cancerous cells in pituitary tumors, breast cancer, and glioblastoma[46-48]. The knockdown of SOX5 in osteosarcoma resulted in a hampering cell proliferation and invasion[49]. *SOX5* has been identified as a prognostic marker in several cancers like lung adenocarcinoma, glioma, breast cancer and melanoma. Its association with poor prognostic outcomes as well as increased metastatic activity has been reported[7, 40, 41]. These findings have indicated that SOX5 may serve as a potential therapeutic target or prognostic biomarker in HNSCC.

The potential pathways identified in this study among EGFR, STAT3, and SOX5 could potentially provide some insight into the mechanism of the anti-*EGFR* agents used in treatment recommended for HNSCC therapies [50]. For *SOX5* to be considered as a clinically relevant biomarker, it needs to be extensively studied not only in HNSCC but also other types of cancer. The substantiation of connections with established pathways in this study will provide insightful knowledge to achieve potentially successful development of new potential cancer treatment modalities with regard this *SOX* superfamily member.

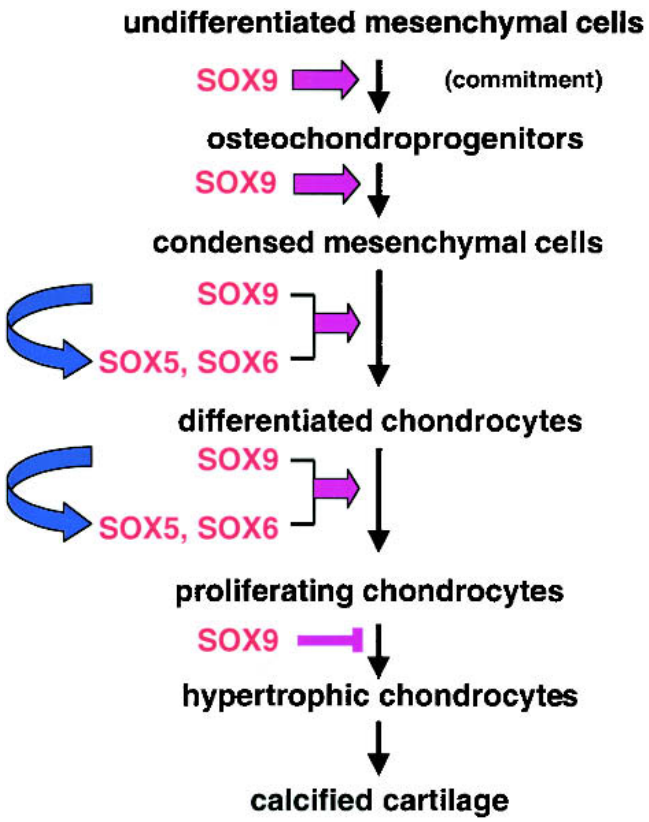


Figure 1. SOX5 in conjunction with SOX6 and SOX9 is the "SOX Trio" which contributes to chondrogenesis through differentiation of mesenchymal cells to chondrocytes. Figure 1 image is adapted from reference[51].

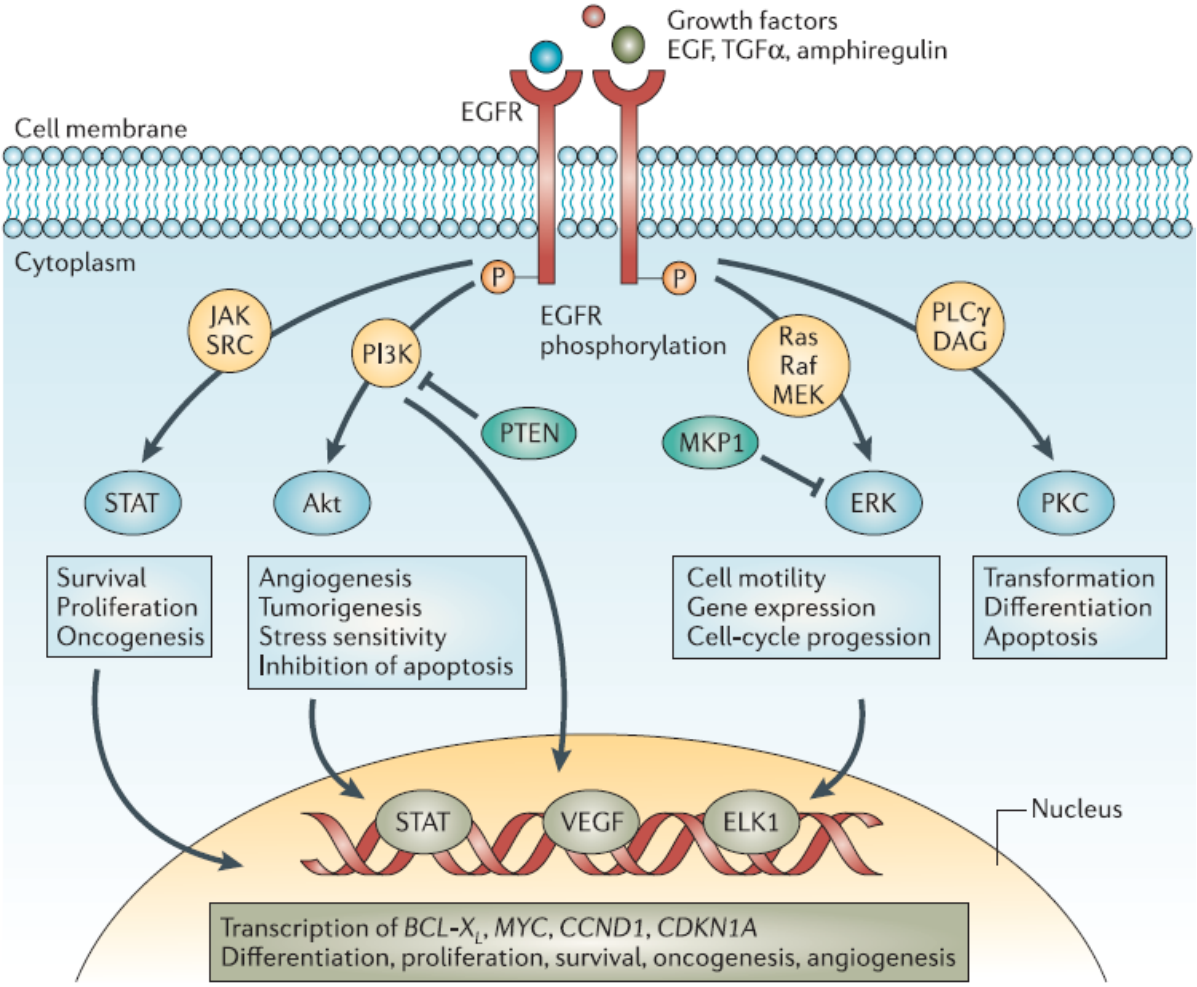


Figure 2. Pathway of EGF and STAT3. EGF binds to EGFR which activates the EGFR receptor to phosphorylate and dimerize. Its downstream effector, STAT3 is activated as part of the JAK-STAT pathway and promotes the transcription of molecules largely involved with cell survival, proliferation and oncogenesis. Figure 2 image is adapted from reference[52].

Markers of EMT

Acquired markers		Attenuated markers	
Name	**EMT type**	**Name**	**EMT type**
Cell-surface proteins			
N-cadherin	1, 2	E-cadherin	1, 2, 3
OB-cadherin	3	ZO-1	1, 2, 3
α5β1 integrin	1, 3		
αVβ6 integrin	1, 3		
Syndecan-1	1, 3		
Cytoskeletal markers			
FSP1	1, 2, 3	Cytokeratin	1, 2, 3
α-SMA	2, 3		
Vimentin	1, 2		
β-Catenin	1, 2, 3		
ECM proteins			
α1(I) collagen	1, 3	α1(IV) collagen	1, 2, 3
α1(III) collagen	1, 3	Laminin 1	1, 2, 3
Fibronectin	1, 2		
Laminin 5	1, 2		
Transcription factors			
Snail1 (Snail)	1, 2, 3		
Snail2 (Slug)	1, 2, 3		
ZEB1	1, 2, 3		
CBF-A/KAP-1 complex	2, 3		
Twist	1, 2, 3		
LEF-1	1, 2, 3		
Ets-1	1, 2, 3		
FOXC2	1, 2		
Goosecoid	1, 2		
MicroRNAs			
miR10b	2	Mir-200 family	2
miR-21	2, 3		

ZEB1, zinc finger E-box binding homeobox 1.

Figure 3. A list of biomarkers panel used to demonstrate EMT, some of which are acquired and some of which are attenuated during transition. Figure 3 table is adapted from reference[36].

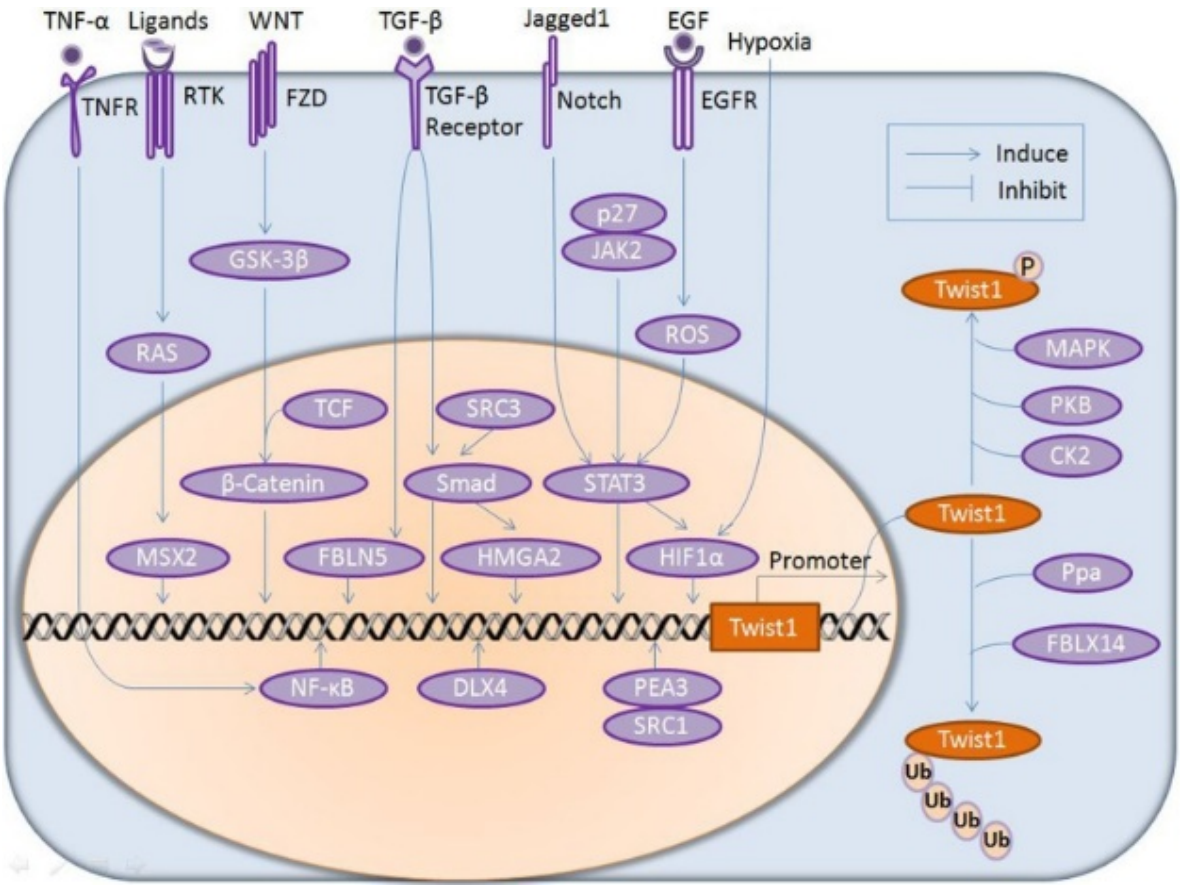


Figure 4. Pathways of activating *TWIST-1* signaling involves a diverse set of upstream targets including EGF, EGFR and intermediate molecules like STAT3 in cancer progression signaling and SOX5 may belong in this pathway. Figure 4 is adapted from reference[20].

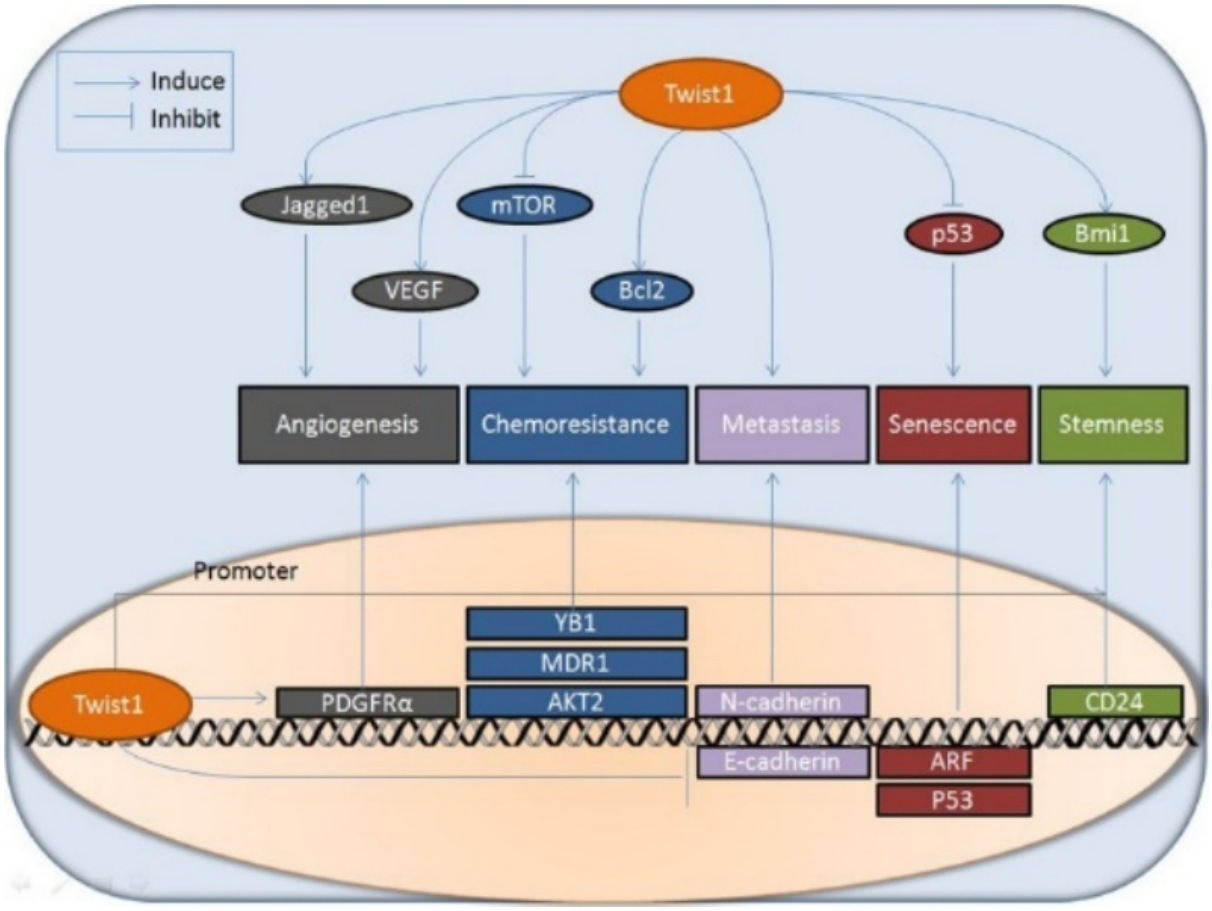


Figure 5. TWIST-1 exerts its multiple biological effects (angiogenesis, chemo-resistance, metastasis, senescence, and stemness) via various downstream pathways, acting as a transcription factor regulating the expression of an array of target genes like by attenuation of E-Cadherin and increasing N-Cadherin during cancer progression. Figure 5 is adapted from reference[20].

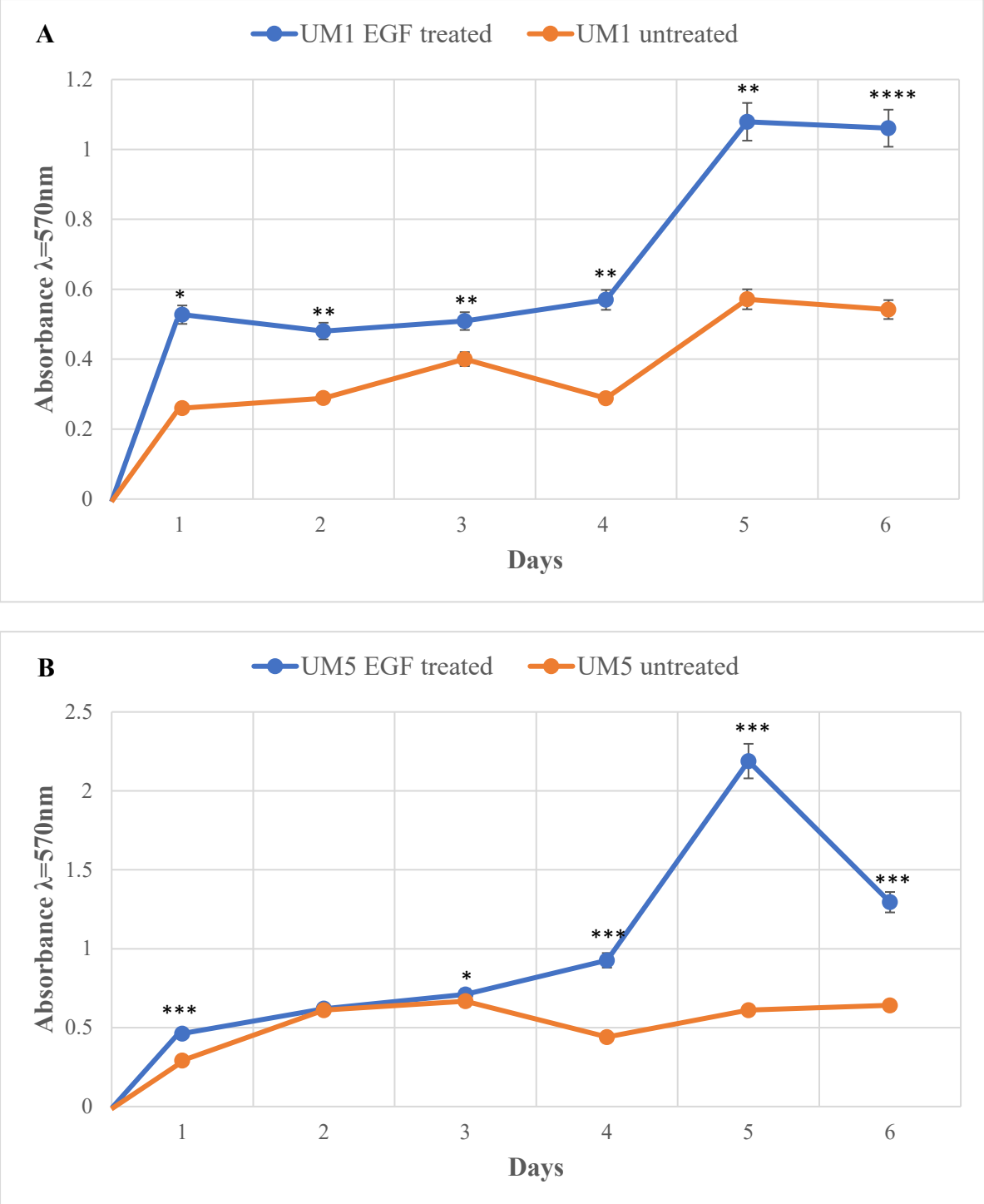


Figure 6. Quantification of MTT assay for cell proliferation. **(A)** in UM1 **(B)** in UM5, comparing EGF treated cells vs untreated shows that there is a significant increase in proliferation in EGF treated groups (blue) compared to untreated groups (orange), measured by absorbance following treatment with MTT over 6 days.

(*, $p<0.05$; **, $p< 0.01$; ***, $p<0.001$; ****, $p<0.0001$)

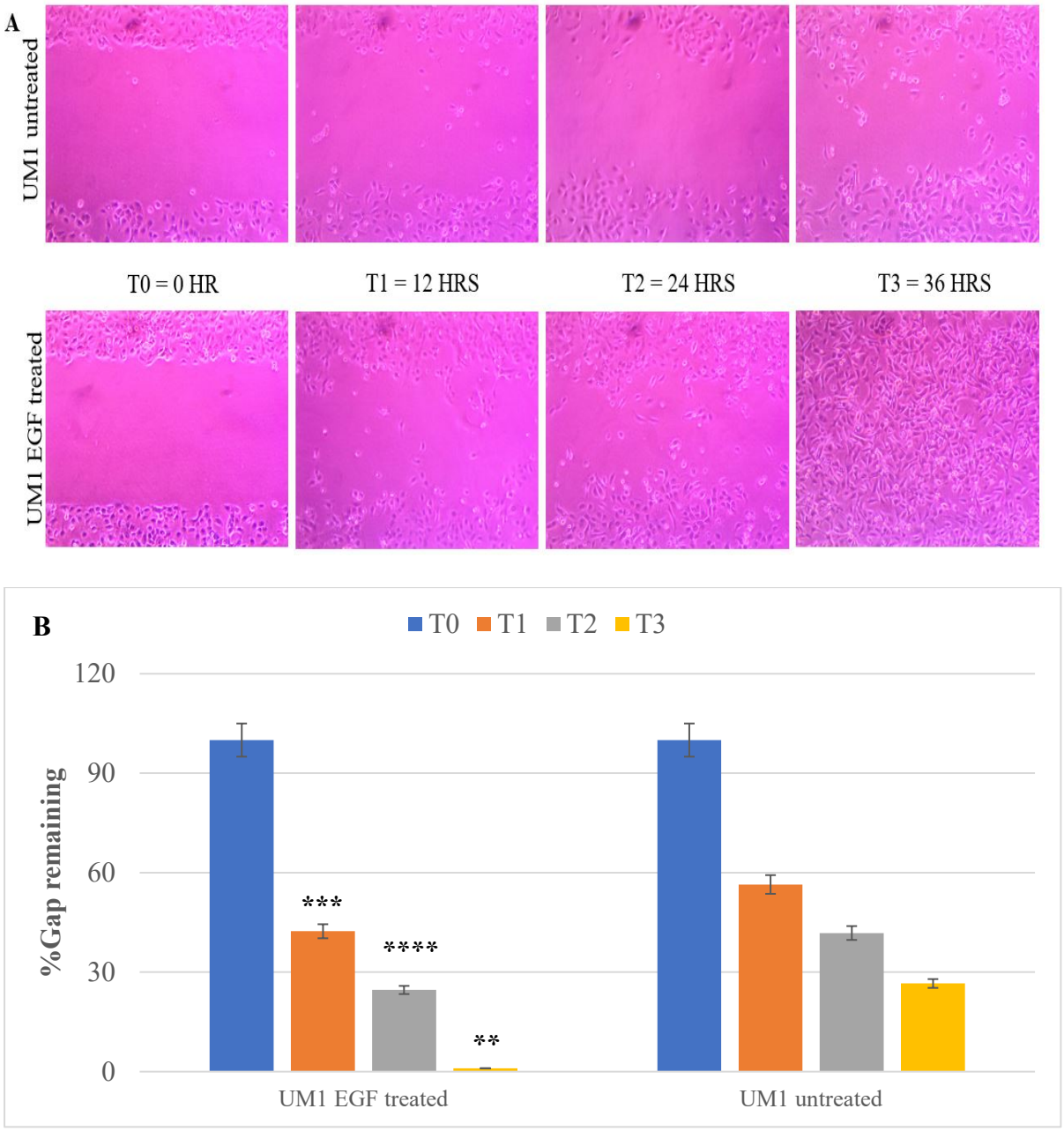


Figure 7. Migration assay for cell migration capability. **(A)** 100x visualization of gap closure at initial time of insert removal, 12 hours following, 24 hours following and 36 hours following. **(B)** Quantification of the area of the gap remaining at each time point in UM1, showing an increase in migration in EGF treated cells vs untreated.

(**, $p<0.01$; ***, $p< 0.001$; ****, $p<0.0001$)

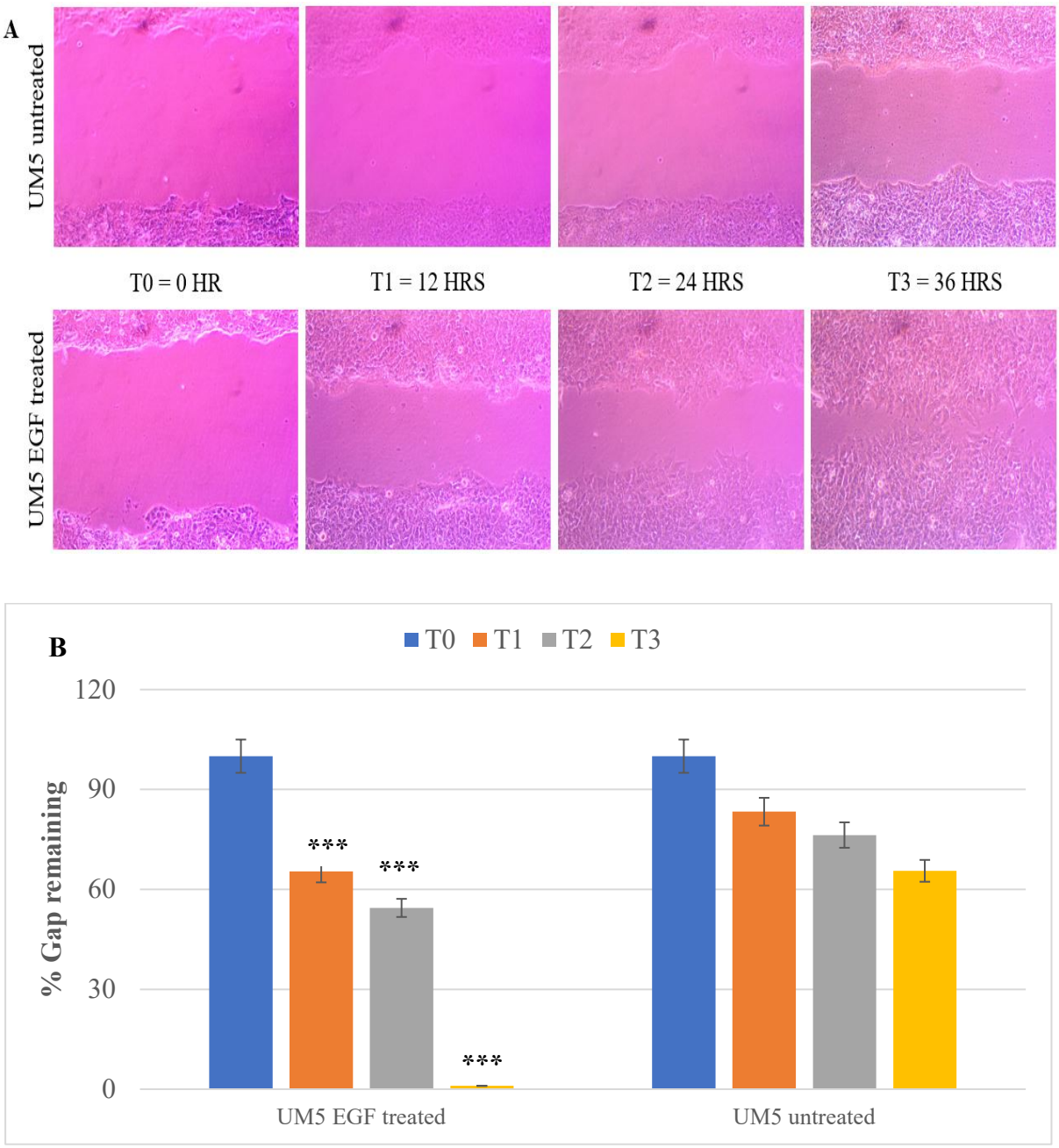
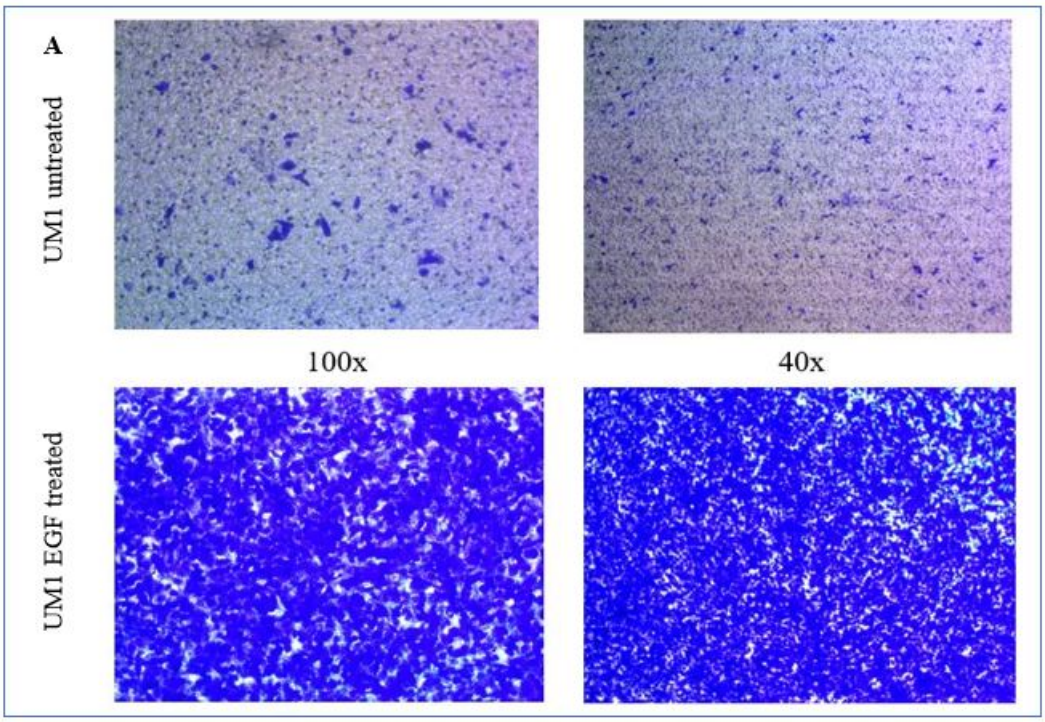


Figure 8. Migration assay for cell migration capability. **(A)** 100x visualization of gap closure at initial time of insert removal, 12 hours following, 24 hours following and 36 hours following. **(B)** Quantification of the area of the gap remaining at each time point in UM5, showing significant increased migration in EGF treated cells vs untreated.

(***, $p < 0.001$)



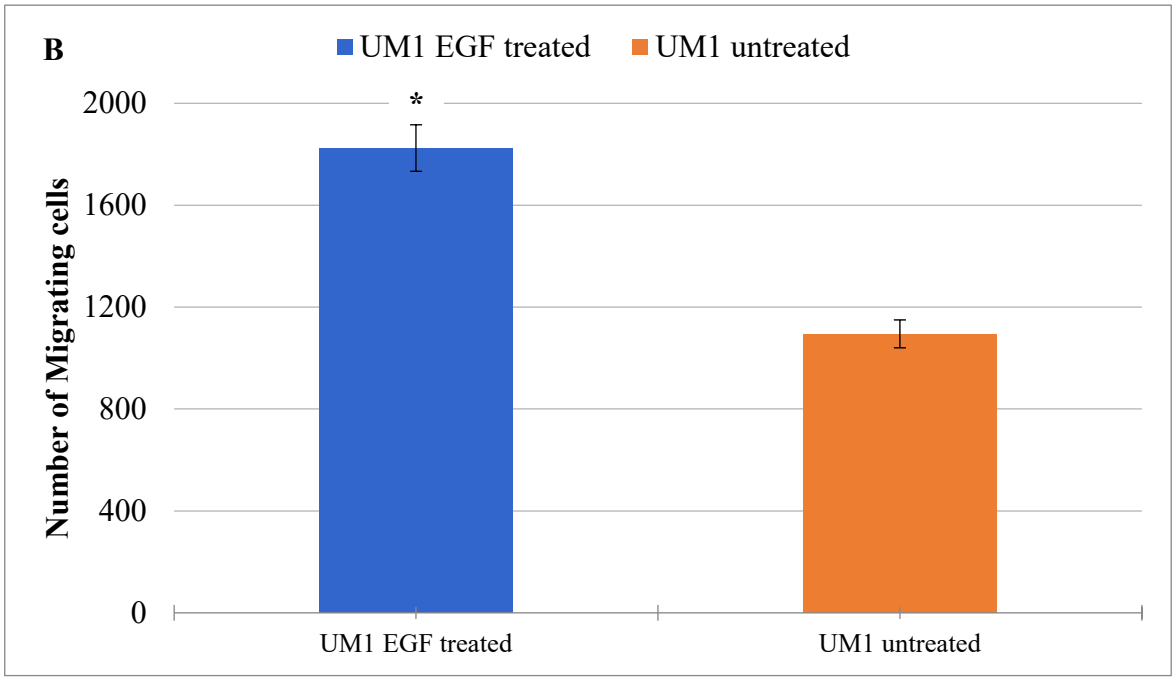
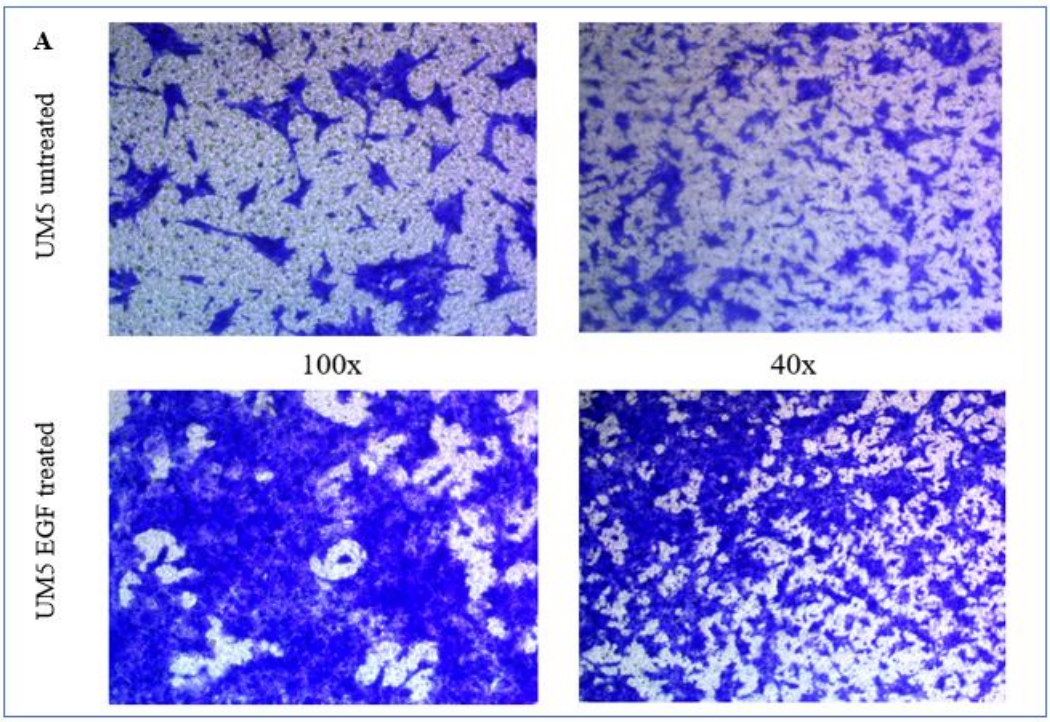


Figure 9. Migration Transwell assay for cell migration capability. **(A)** 100x and 40x visualization of migration of UM1 comparing EGF treated cells vs untreated through migration transwell chambers. **(B)** Quantification of the number of migrating cells, showing increased migration in the UM1 EGF treated group compared to untreated group. (*, $p<0.05$)



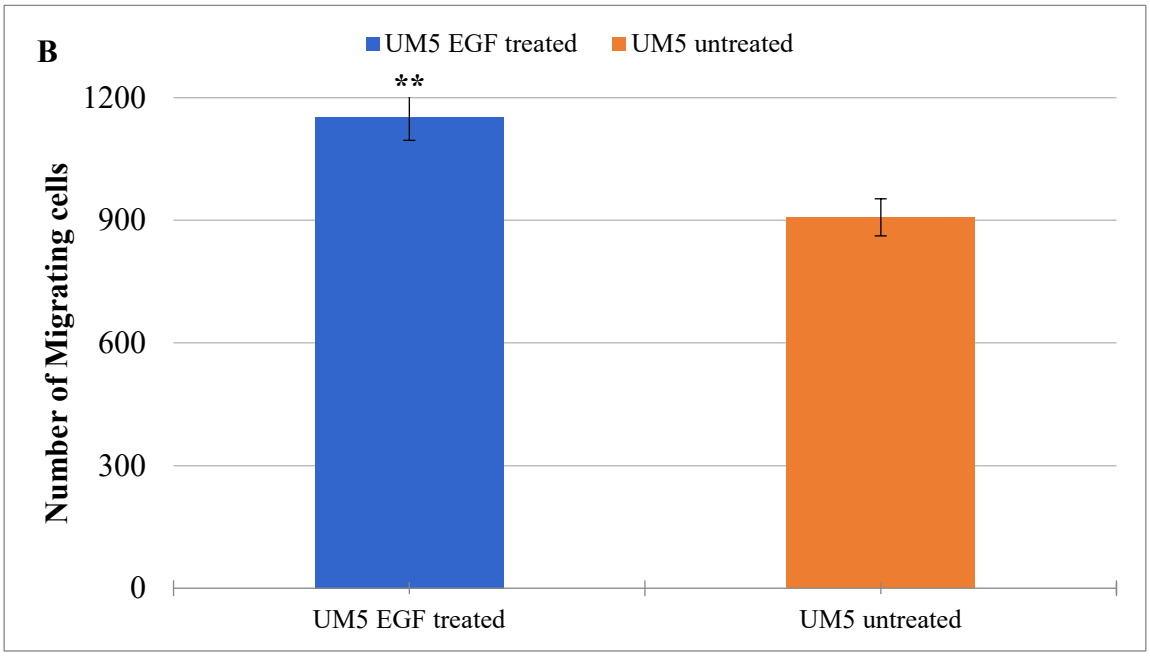
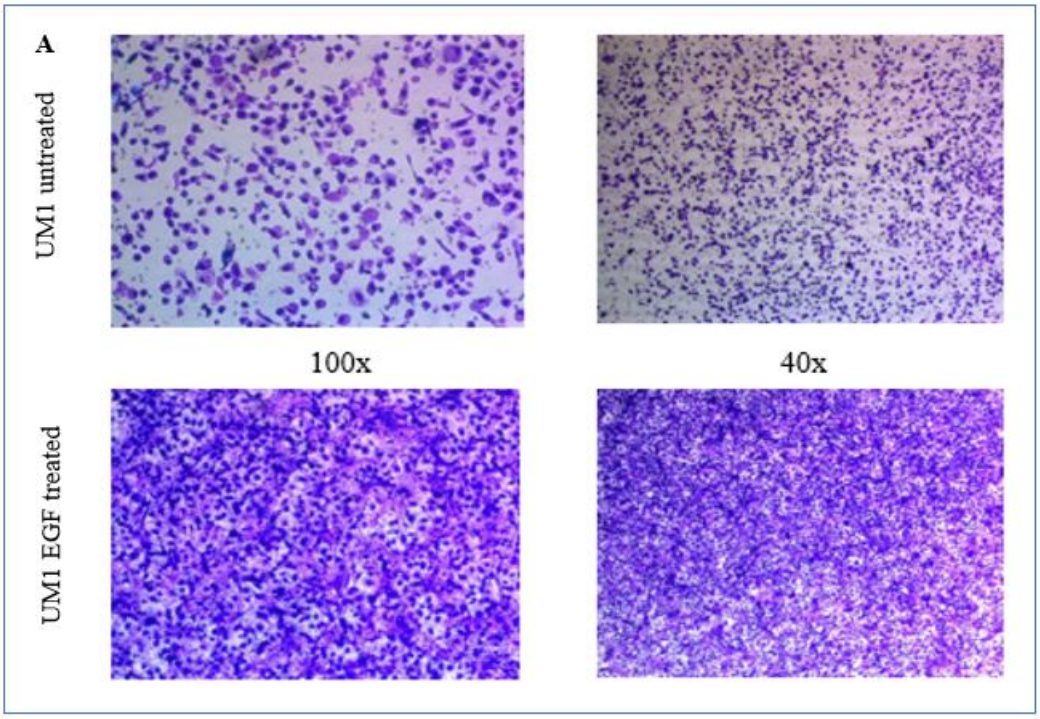


Figure 10. Migration Transwell assay for cell migration capability. **(A)** 100x and 40x visualization of migration of UM5 comparing EGF treated cells vs untreated through migration transwell chambers. **(B)** Quantification of the number of migrating cells, showing increased migration in the UM5 EGF treated group compared to untreated group. (**, $p<0.01$)



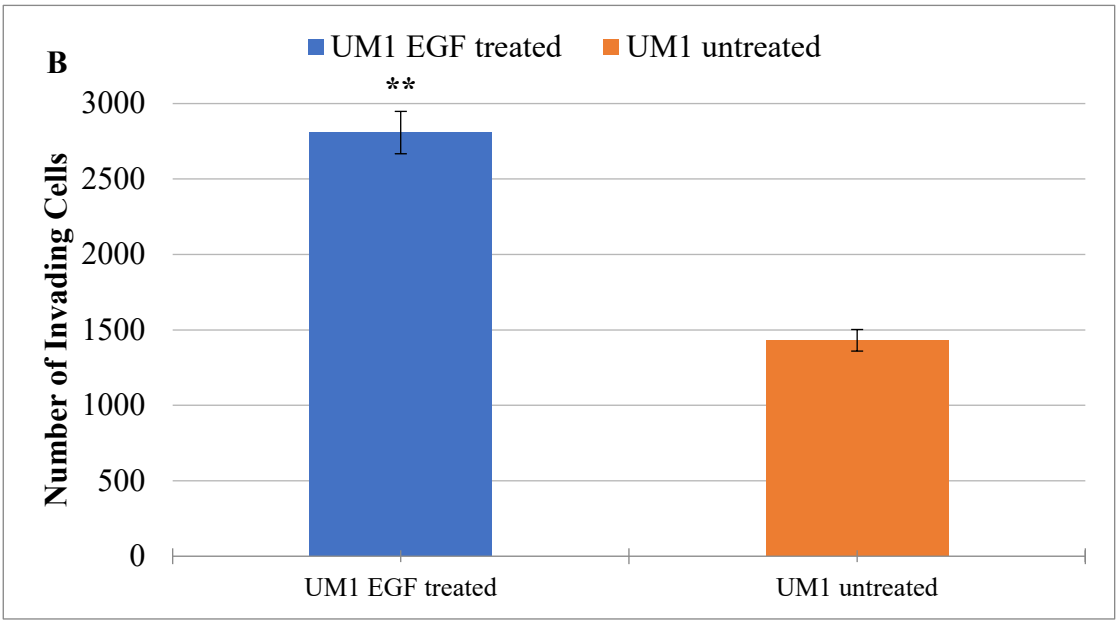
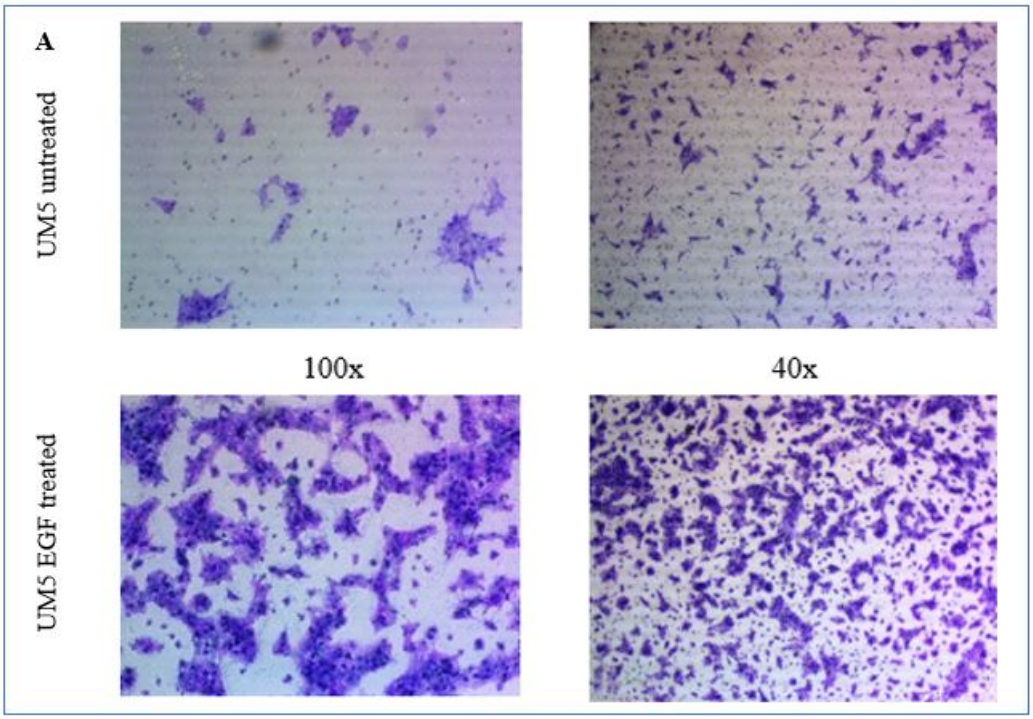


Figure 11. Invasion assay for cell invasion potential. **(A)** 100x and 40x visualization of migration of UM1 comparing EGF treated cells vs untreated through Matrigel invasion chambers. **(B)** Quantification of the number of invading cells, showing increased invasion in the UM1 EGF treated group compared to untreated group.

(**, $p<0.01$)



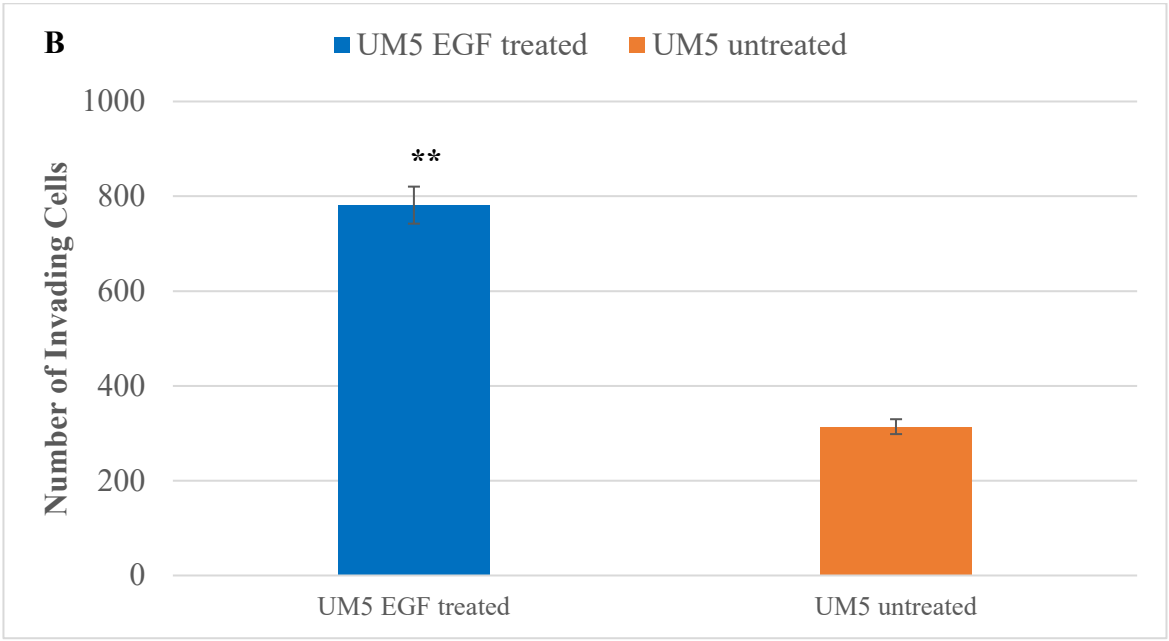


Figure 12. Invasion assay for cell invasion potential. **(A)** 100x and 40x visualization of migration of UM5 comparing EGF treated cells vs untreated through Matrigel invasion chambers. **(B)** Quantification of the number of invading cells, showing increased invasion in the UM5 EGF treated group compared to untreated group.

(**, $p<0.01$)

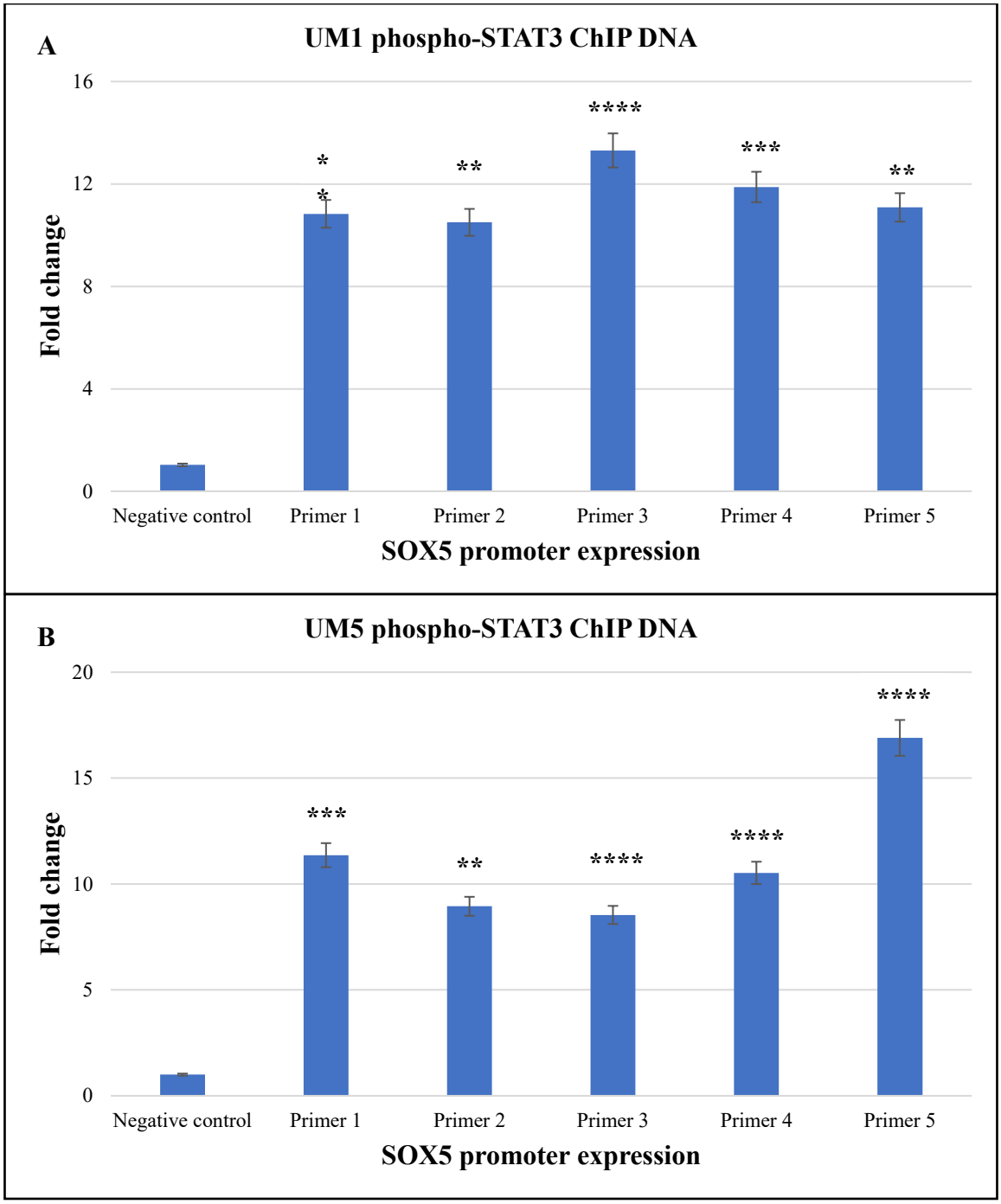


Figure 13. ChIP with qPCR analysis with phospho-STAT3 antibody. **(A)** in UM1 cell and **(B)** in UM5 cells, both showed significant fold increase in the levels of *SOX5* promoter across all five primer sequences compared to negative control as immunoprecipitation with normal rabbit IgG. (**, $p< 0.01$; ***, $p< 0.001$; ****, $p<0.0001$)

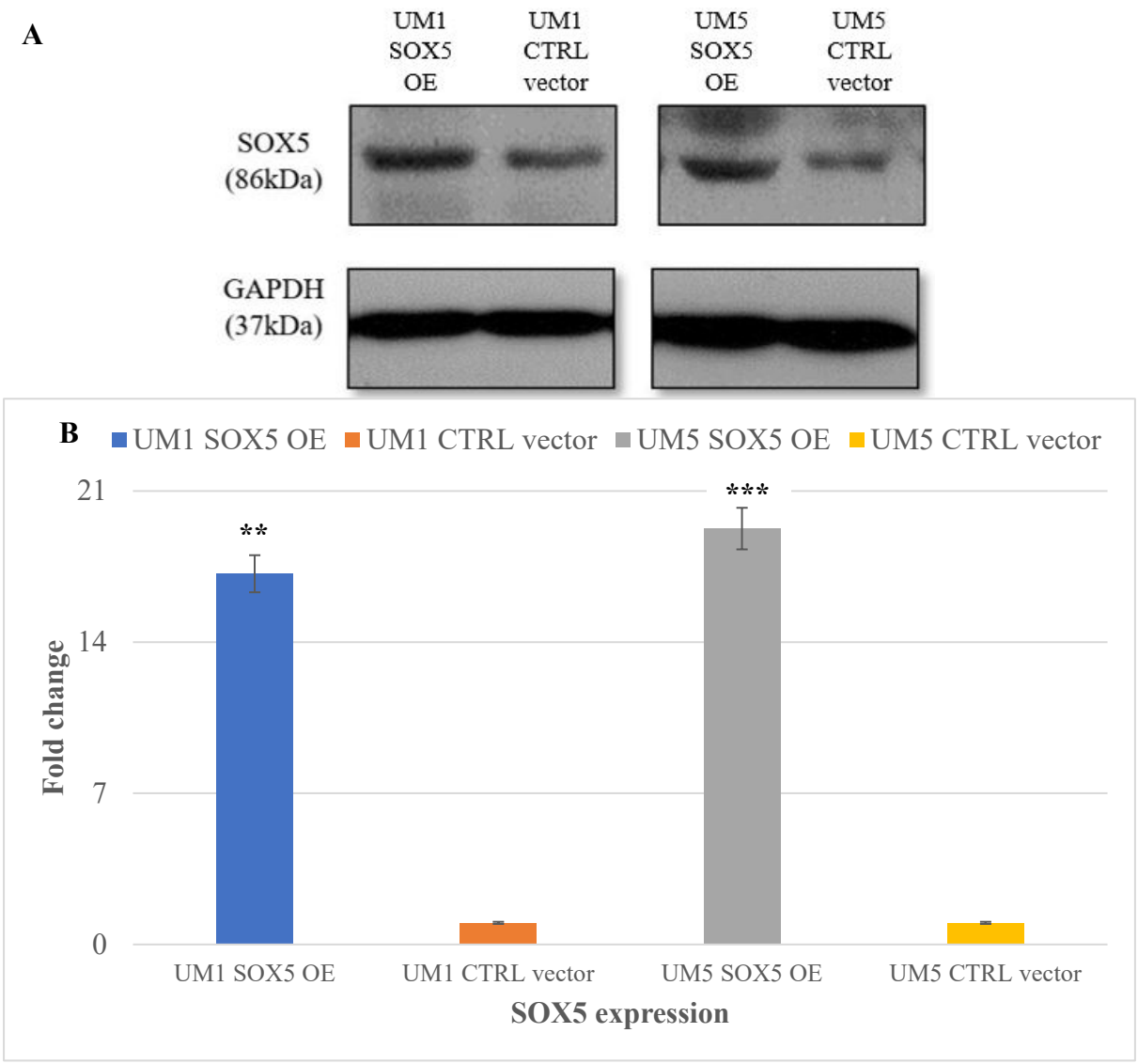


Figure 14. Western blot and qPCR analyses of overexpression of *SOX5* in UM1 and UM5 cells (A) Relative higher protein levels of SOX5, with GAPDH as loading control in *SOX5* OE plasmid vs control vector. **(B)** Quantification of relative higher *SOX5* levels in *SOX5* OE plasmid vs control vector, with Actin as loading control. (** , $p < 0.01$; *** ,$p < 0.001$)

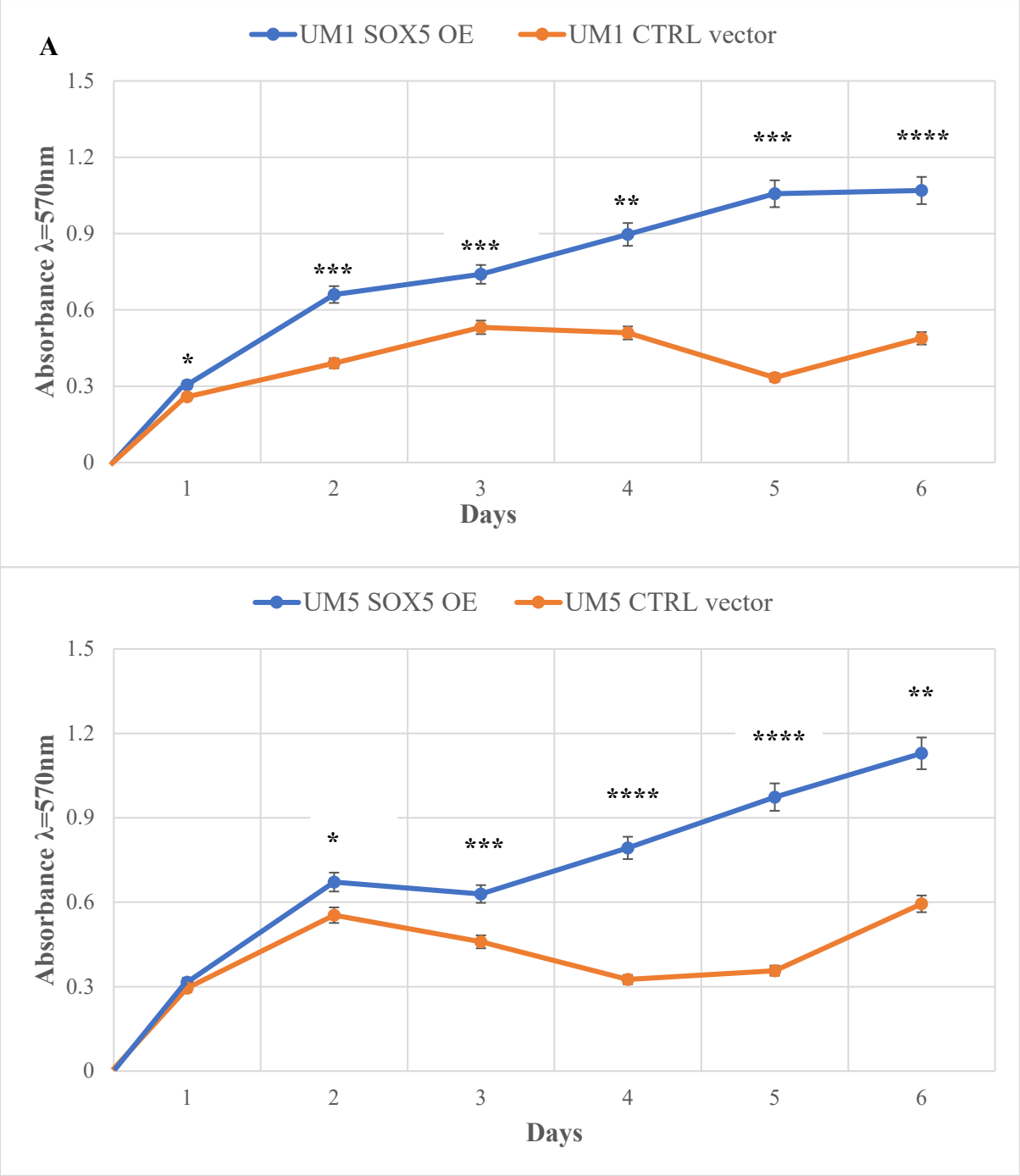


Figure 15. Quantification of MTT assay for cell proliferation. **(A)** in UM1 **(B)** in UM5, comparing *SOX5* overexpression cells vs control vector cells show that *SOX5* overexpressing groups with a significant increase in proliferation in blue compared to control vector groups in orange, measured by absorbance following treatment with MTT over 6 days. (*,$p<0.05$; **,$p< 0.01$; *** , $p<0.001$; ****, $p<0.0001$)

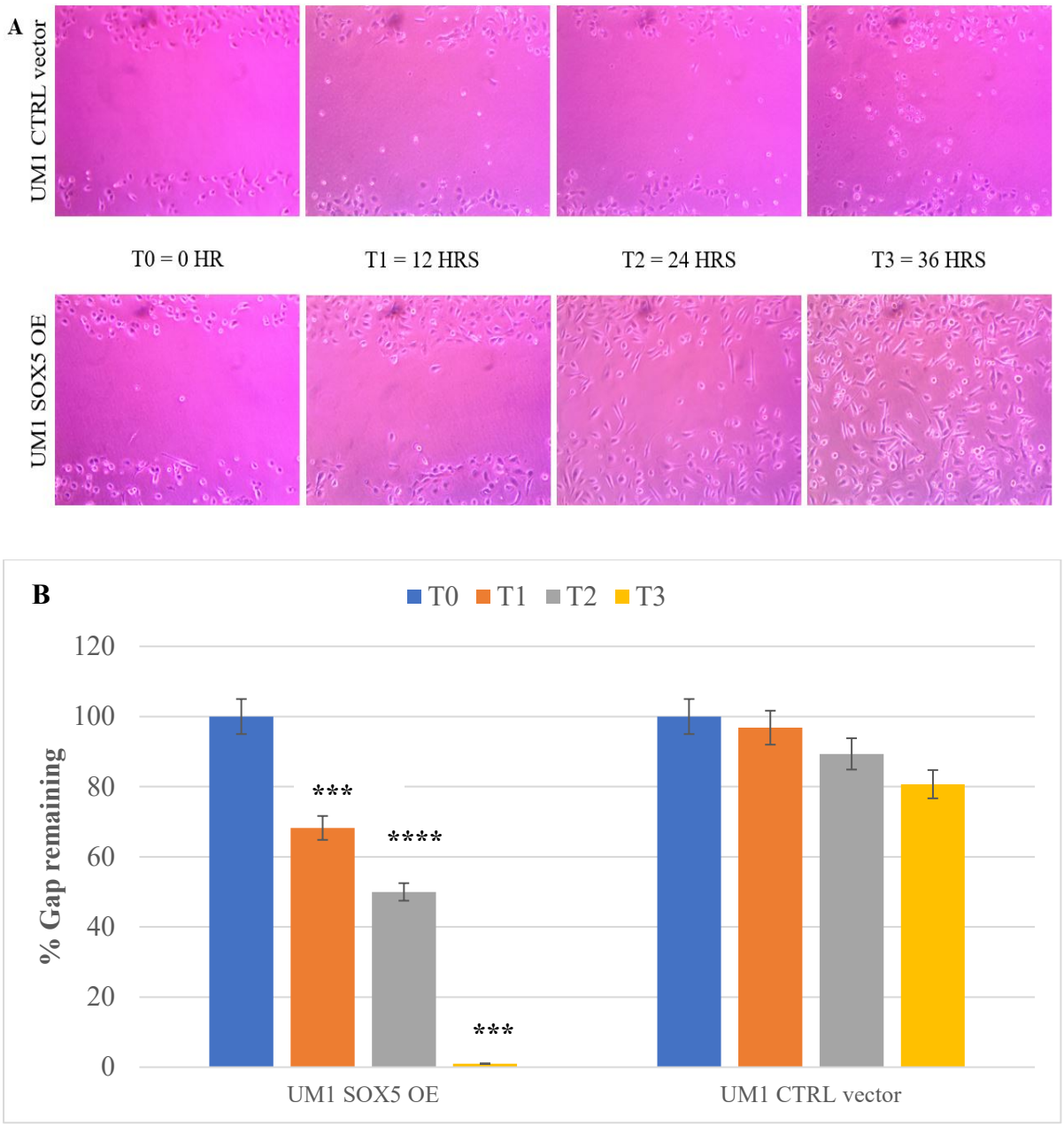


Figure 16. Migration assay for cell migration capability. **(A)** 100x visualization of gap closure at initial time of insert removal, 12 hours following, 24 hours following and 36 hours following. **(B)** Quantification of the area of the gap remaining at each time point in UM1, showing significant increased migration in *SOX5* overexpressing cells vs CTRL vector cells. (***, $p < 0.001$, ****, $p < 0.0001$)

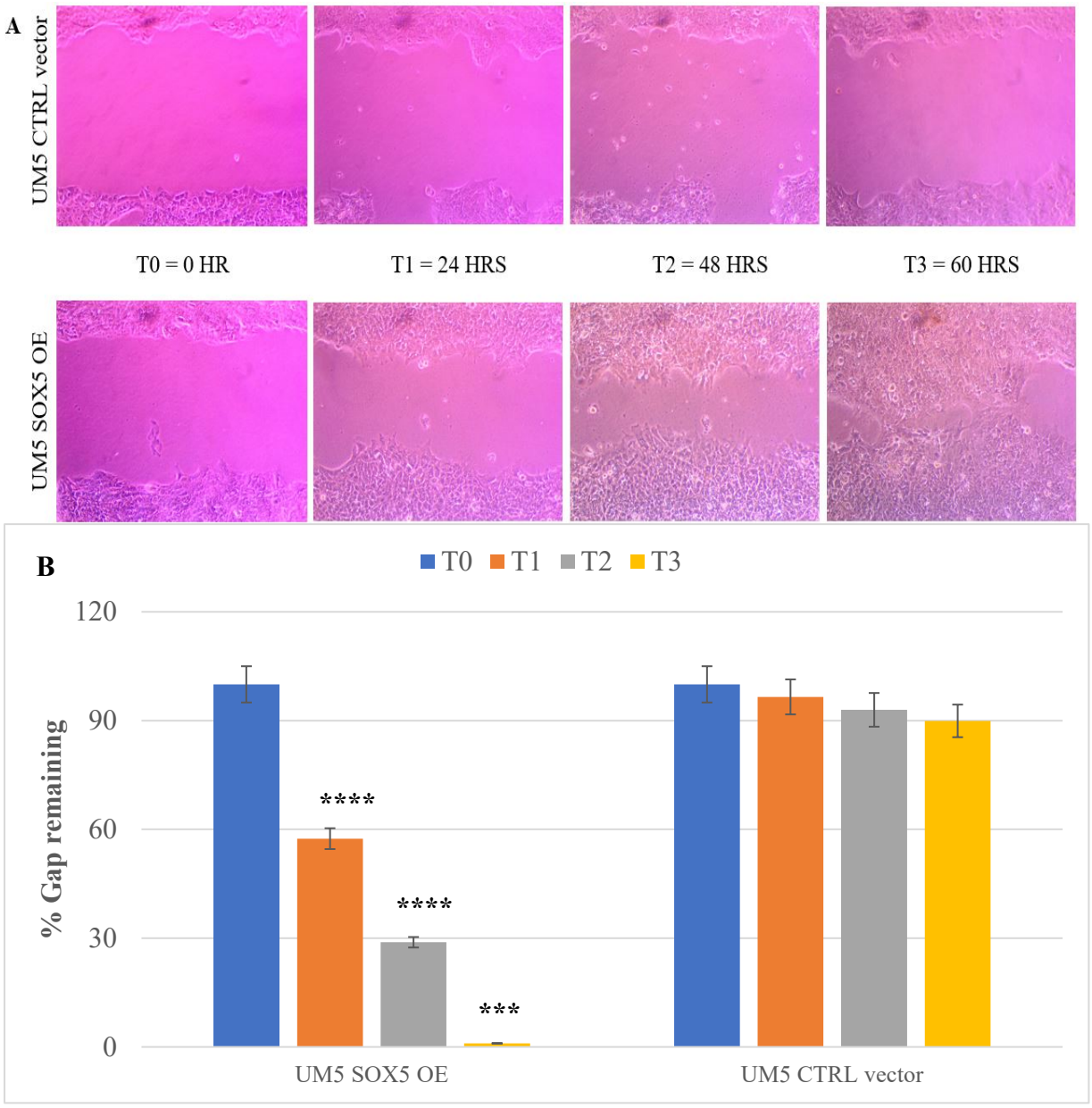
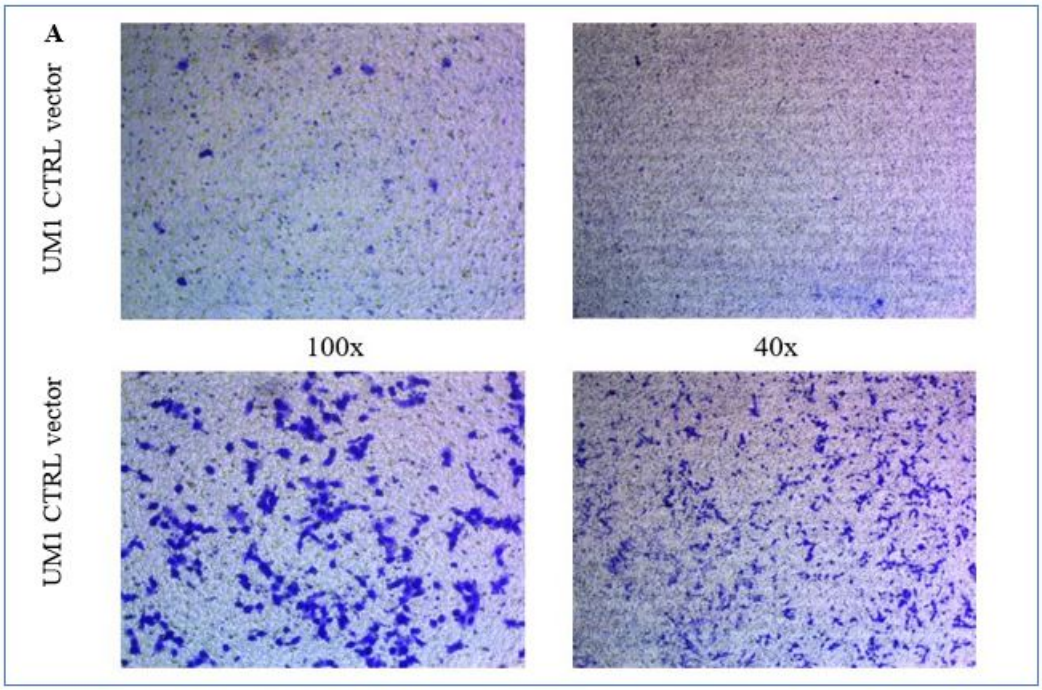


Figure 17. Migration assay for cell migration capability. **(A)** 100x visualization of gap closure at initial time of insert removal, 24 hours following, 48 hours following and 60 hours following. **(B)** Quantification of the area of the gap remaining at each time point in UM5, showing significant increased migration in *SOX5* overexpressing cells vs CTRL vector cells. (*** , $p < 0.001$, ****, $p < 0.0001$)



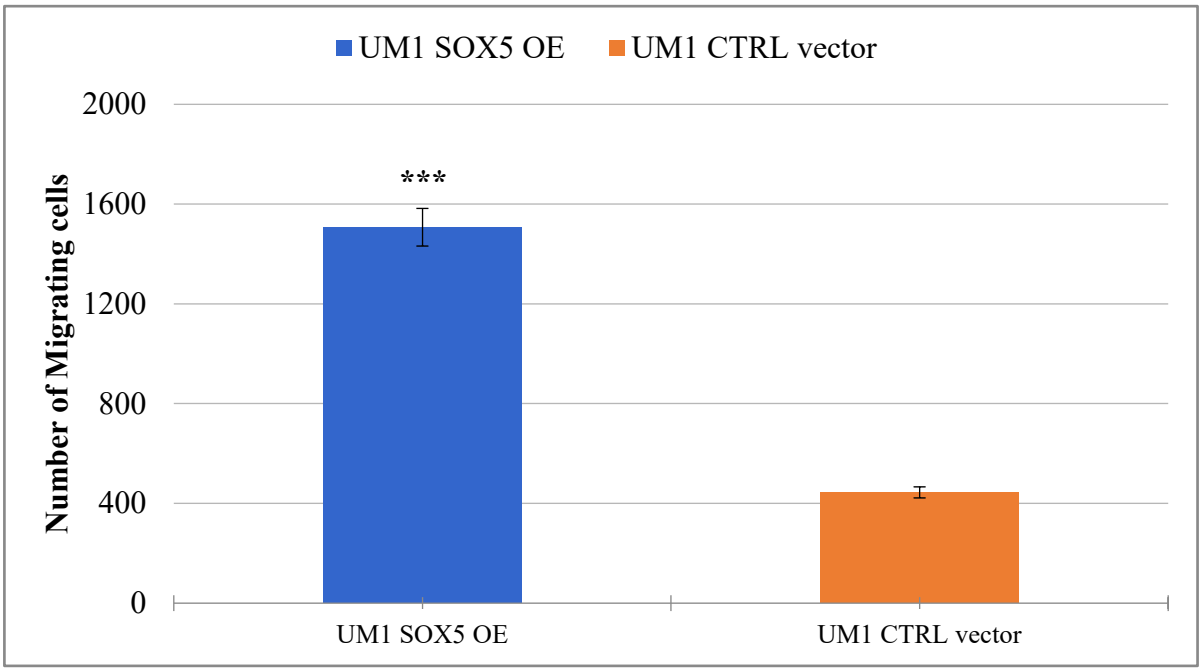


Figure 18. Migration Transwell assay for cell migration capability. **(A)** 100x and 40x visualization of migration of UM1 comparing *SOX5* OE plasmid treated cells vs control vector through migration transwell chambers. **(B)** Quantification of the number of migrating cells, showing increased migration in the UM1 *SOX5* OE plasmid treated group compared to control vector treated group. (***, $p < 0.001$)

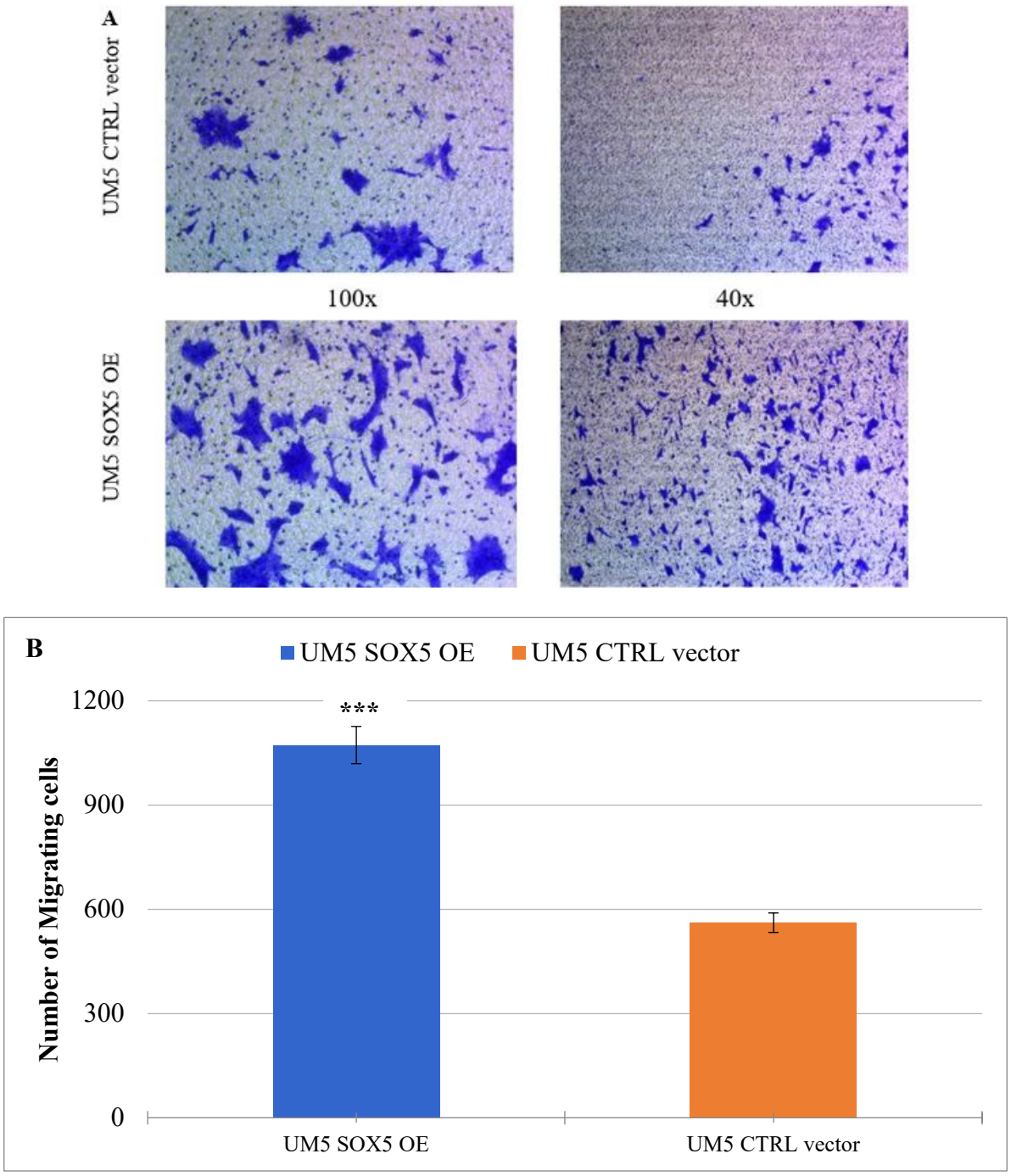


Figure 19. Migration Transwell assay for cell migration capability. **(A)** 100x and 40x visualization of migration of UM5 comparing *SOX5* OE plasmid treated cells vs control vector through migration transwell chambers. **(B)** Quantification of the number of migrating cells, showing increased migration in the UM5 *SOX5* OE plasmid treated group compared to control vector treated group. (***, $p < 0.001$)

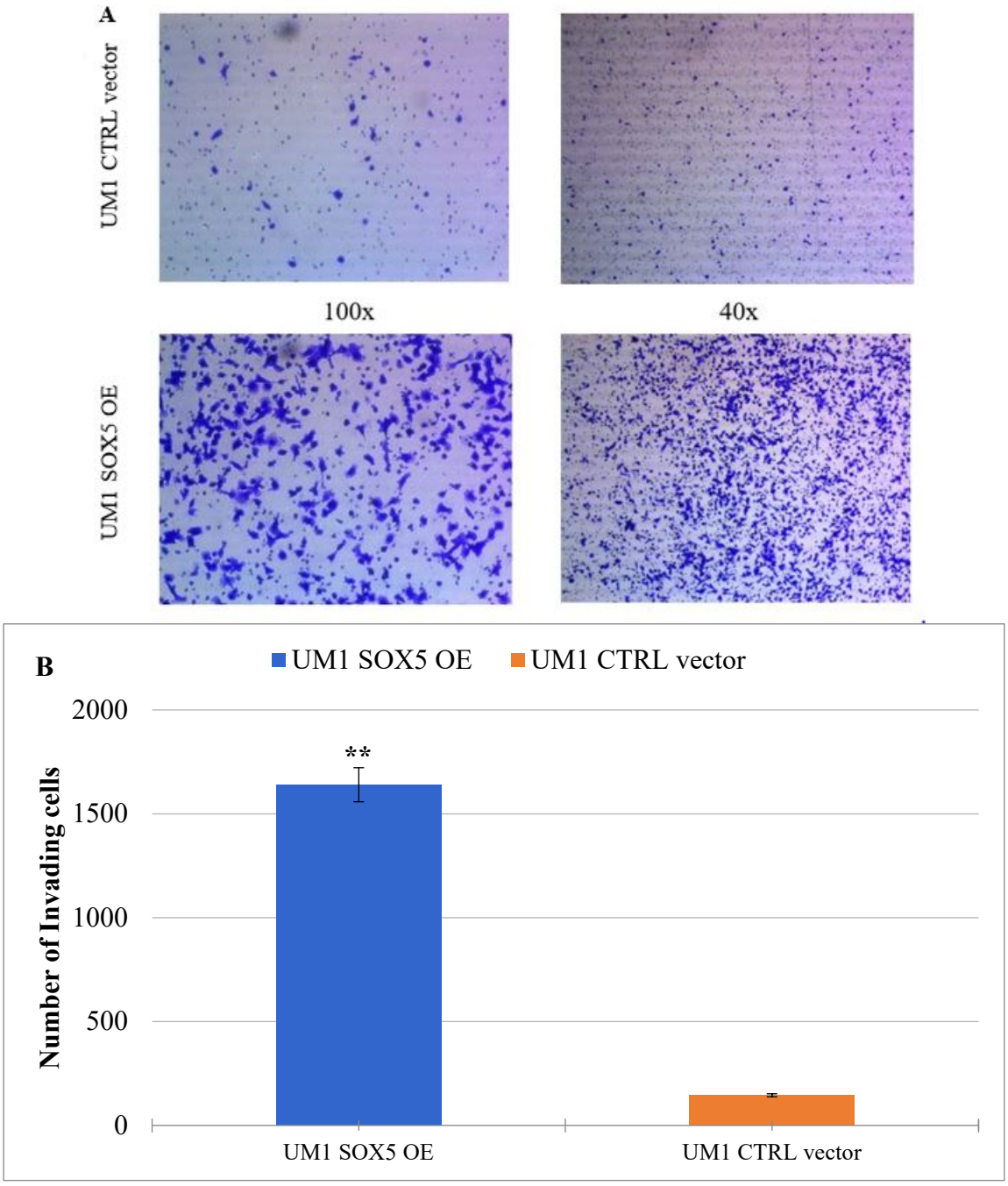


Figure 20. Invasion assay for of the invasion potential of UM1 cells with *SOX5* overexpression. **(A)** 100x and 40x visualization of migration of UM1 comparing *SOX5* OE plasmid transfected cells vs control vector treated through Matrigel invasion chambers. **(B)** Quantification of the number of invading cells, showing increased invasion in the UM1 *SOX5* OE group compared to control vector group. (***, $p<0.01$)

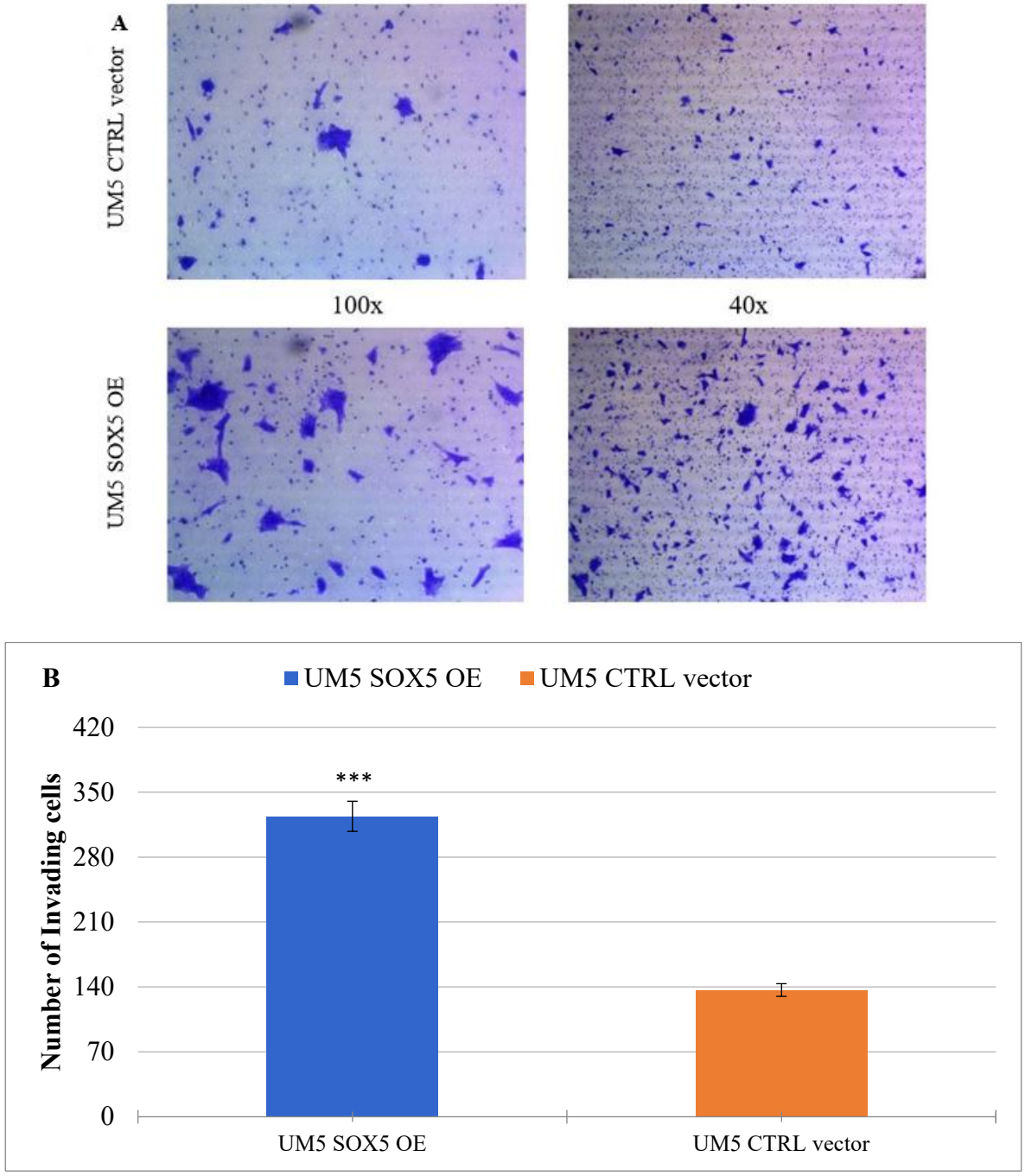


Figure 21. Invasion assay for the invasion potential of UM5 cells with *SOX5* overexpression. **(A)** 100x and 40x visualization of migration of UM5 comparing *SOX5* OE plasmid transfected cells vs control vector treated through Matrigel invasion chambers. **(B)** Quantification of the number of invading cells, showing increased invasion in the UM5 *SOX5* OE group compared to control vector group. (***, $p<0.01$)

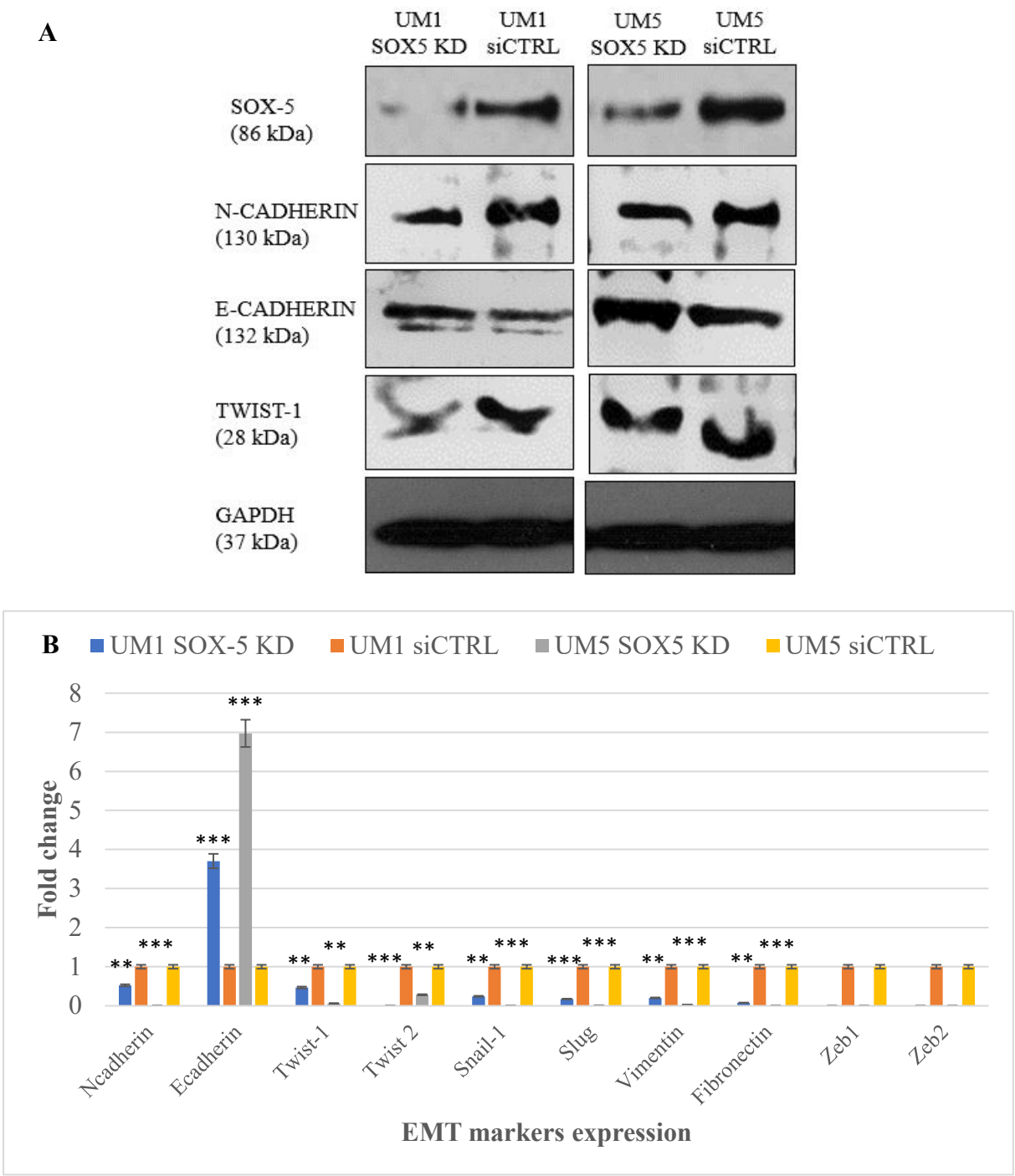


Figure 22. Analysis of EMT markers in UM1 and UM5 cells with *SOX5* knockdown vs control. **(A)** Western blot reveals N-Cadherin and TWIST-1 levels are reduced while E-Cadherin level is increased in *SOX5* knockdown group compared to control group with GAPDH as loading control. **(B)** qPCR analysis showing similar results with respect to these three EMT markers levels, with actin as loading control in UM1 and UM5. (* , $p<0.05$; ** , $p< 0.01$; ***, $p< 0.001$; ****, $p< 0.0001$)

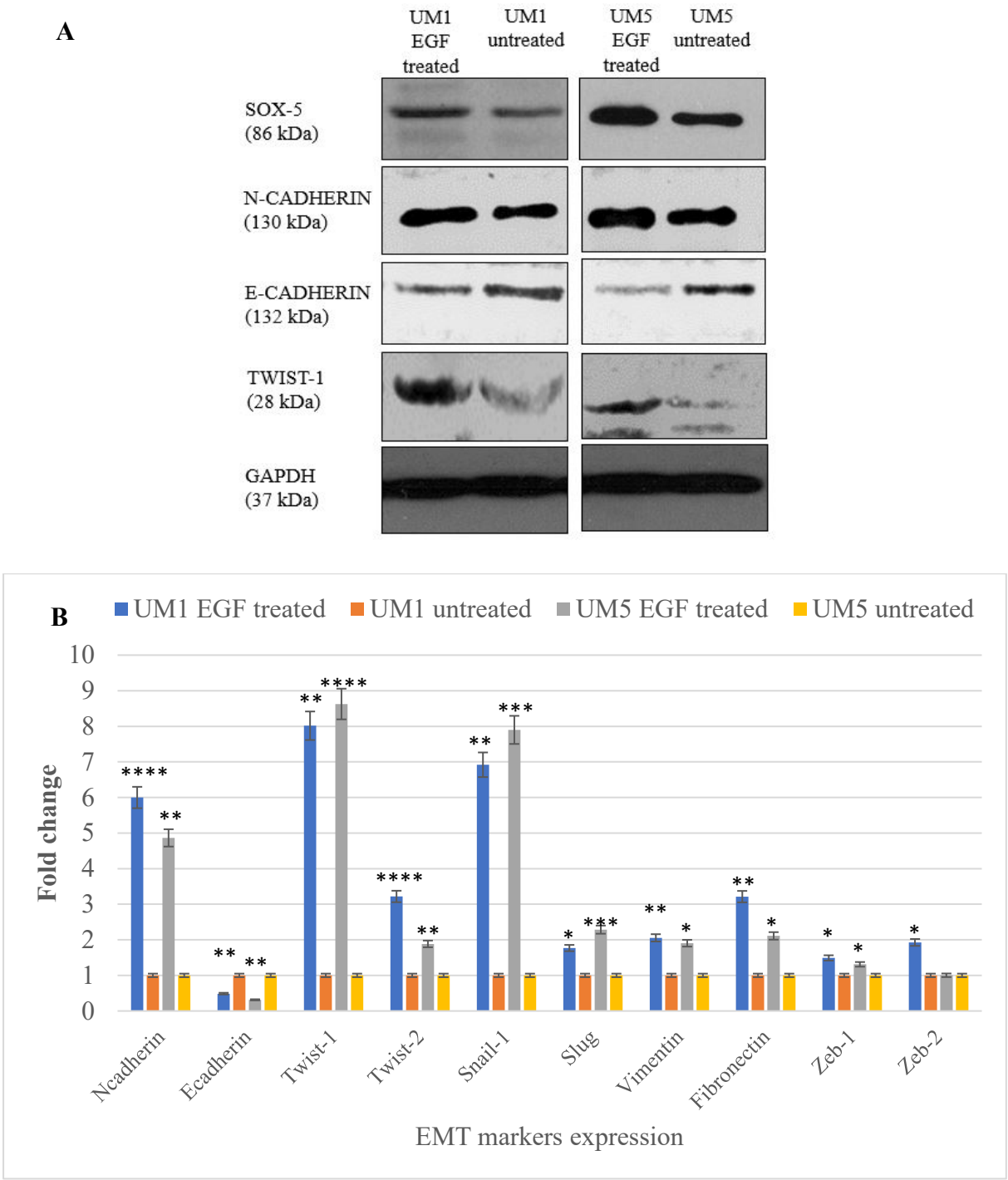


Figure 23. Analysis of EMT markers in EGF treated vs untreated UM1 and UM5 cells. **(A)** Western blot reveals N-Cadherin and TWIST-1 levels are higher while E-Cadherin level is reduced in EGF treated group compared to untreated group with GAPDH as loading control. **(B)** qPCR analysis showing similar results with respect to these three EMT markers levels, with actin as loading control in UM1 and UM5. (*, $p<0.05$; **, $p< 0.01$; ***, $p< 0.001$; **** ,$p< 0.0001$)

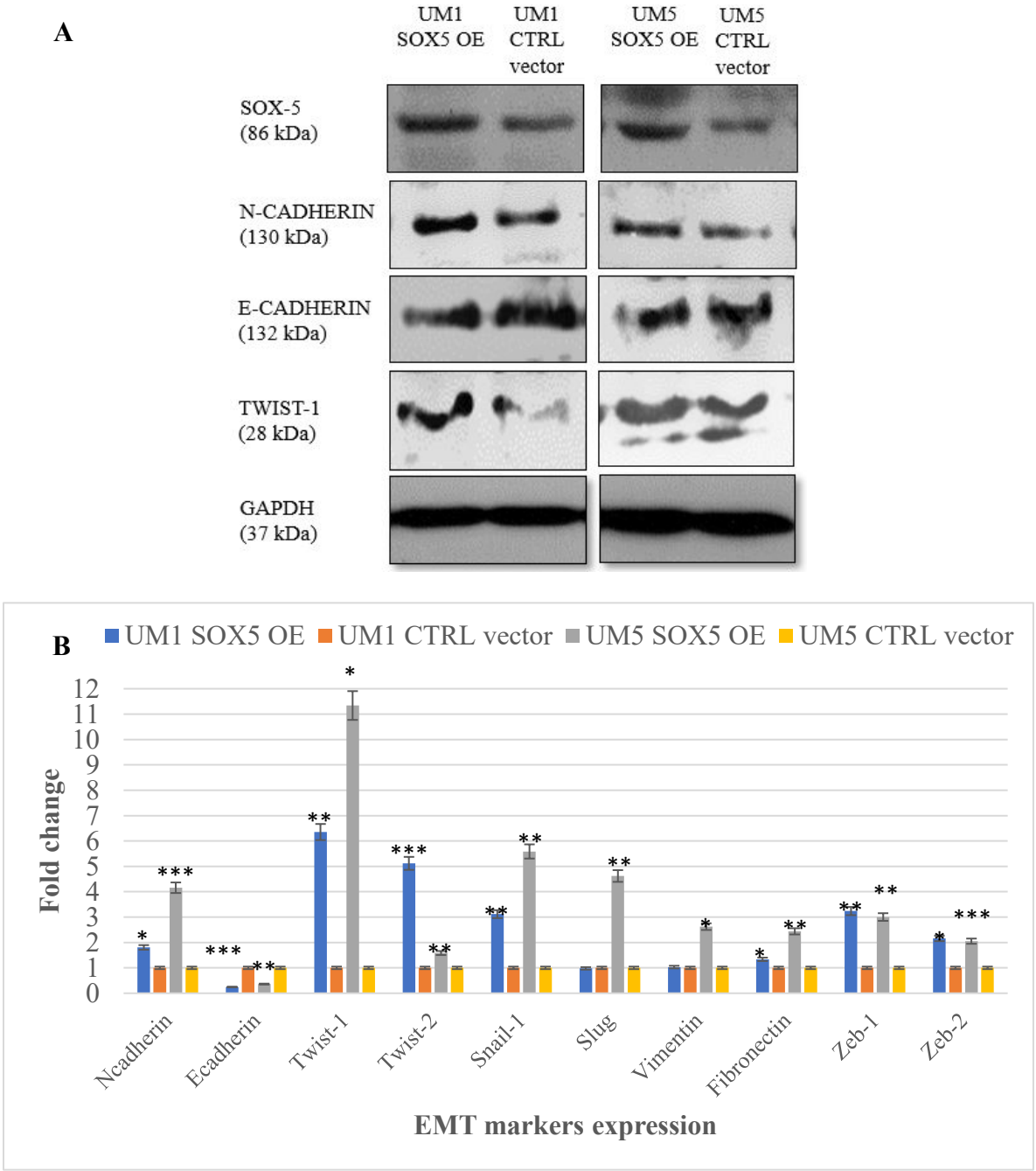
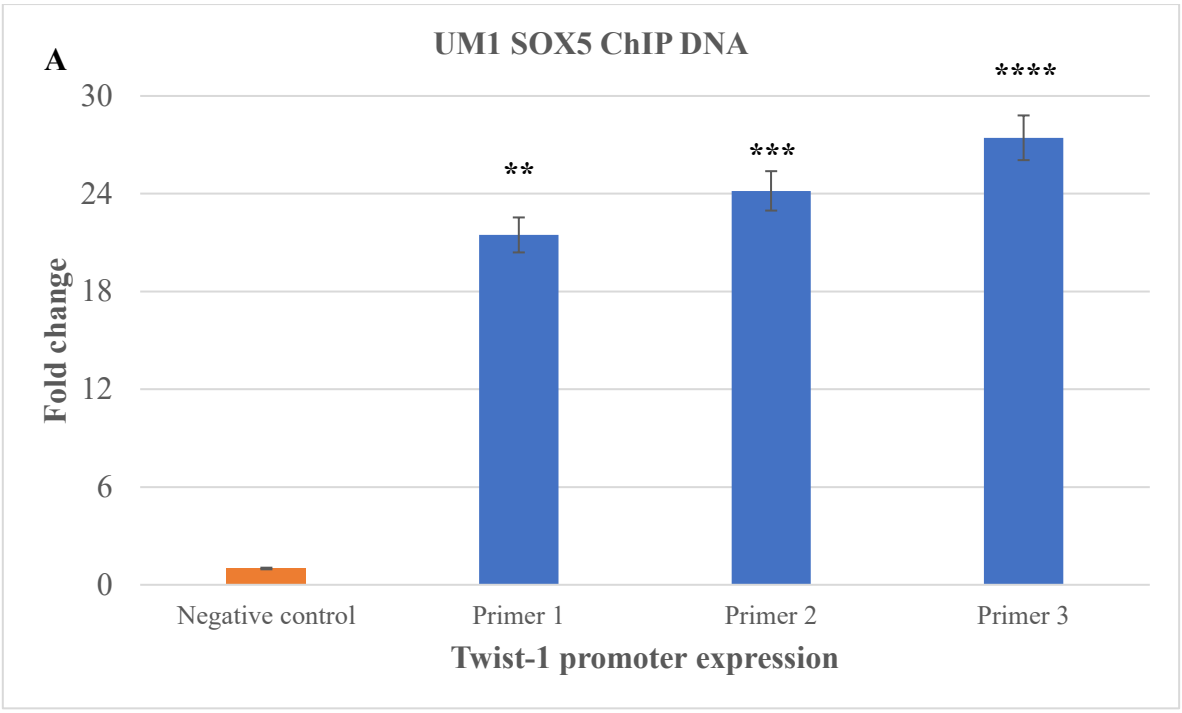


Figure 24. Analysis of EMT markers in *SOX5* overexpression by *SOX5* OE plasmid vs OE control in UM1 and UM5. **(A)** Western blot reveals N-Cadherin and TWIST-1 levels are higher while attenuated level of E-Cadherin is found in *SOX5* overexpressed group compared to the control group with GAPDH as loading control. **(B)** qPCR analysis showing similar results with respect to these three EMT markers levels, with actin as loading control in UM1 and UM5. (* , $p<0.05$; **, $p< 0.01$; ***, $p< 0.001$)



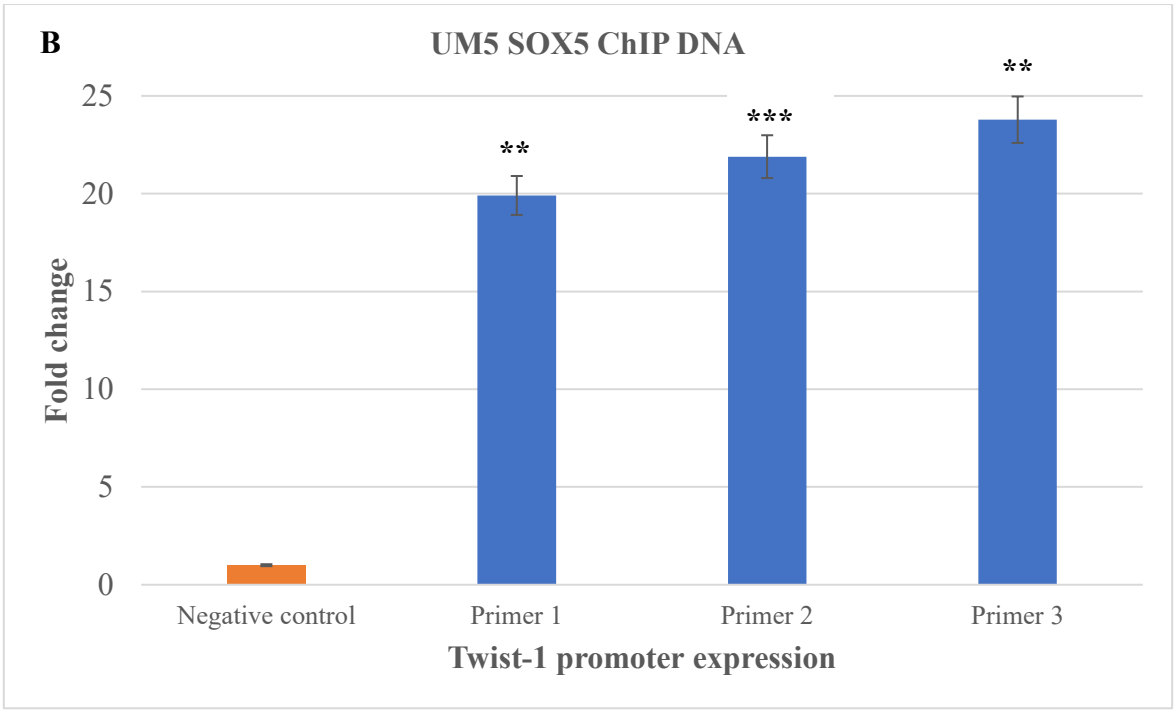


Figure 25. ChIP with qPCR analysis with SOX5 antibody to immunoprecipitated *TWIST-1* promoter. **(A)** in UM1 cells and **(B)** in UM5 cells, both showed significant fold increase in the levels of *TWIST-1* promoter across all three primer sequences compared to negative control as immunoprecipitation with normal rabbit IgG. (**, $p < 0.01$; ***, $p < 0.001$)

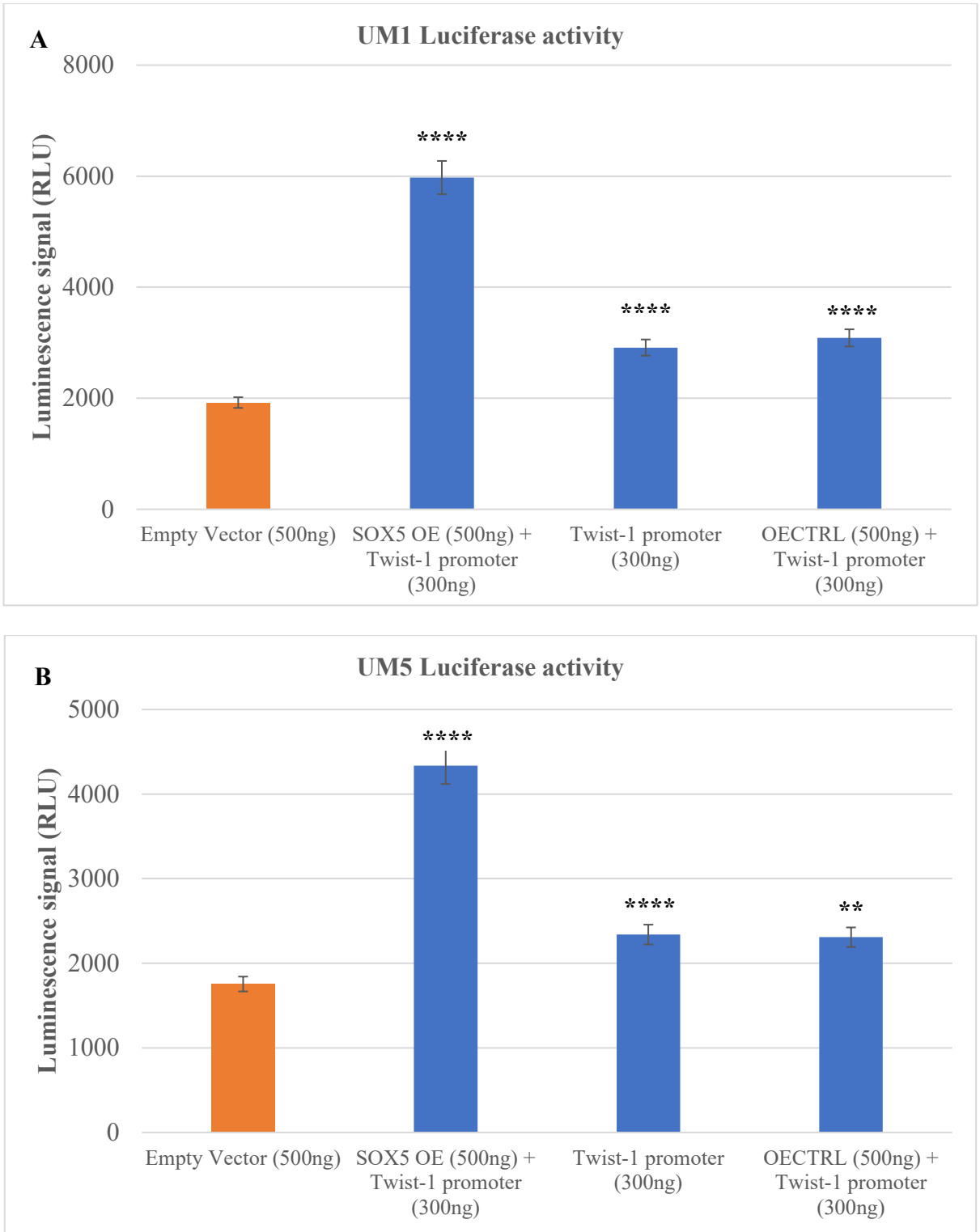


Figure 26. Luciferase reporter assay to analyze if SOX5 transcriptionally regulates *TWIST-1*. **(A)** in UM1 cells and **(B)** in UM5 cells, both showed most increased in measured luminescence signal activity when cells are treated with *SOX5* OE plasmid and *TWIST-1* promoter reporter construct compared to others three conditions. (**, $p < 0.01$; ***, $p < 0.001$)

REFERENCES

1. Lo Nigro, C., et al., Head and neck cancer: improving outcomes with a multidisciplinary approach. Cancer management and research, 2017. **9**: p. 363-371.
2. Leemans, C.R., B.J.M. Braakhuis, and R.H. Brakenhoff, The molecular biology of head and neck cancer. Nat Rev Cancer, 2011. **11**(1): p. 9-22.
3. Lefebvre, V., The SoxD transcription factors – Sox5, Sox6, and Sox13 – are key cell fate modulators. The International Journal of Biochemistry & Cell Biology, 2010. **42**(3): p. 429-432.
4. Pei, X.-H., X.-Q. Lv, and H.-X. Li, Sox5 induces epithelial to mesenchymal transition by transactivation of Twist1. Biochemical and biophysical research communications, 2014. **446**(1): p. 322-327.
5. Wang, D., et al., SOX5 promotes epithelial–mesenchymal transition and cell invasion via regulation of Twist1 in hepatocellular carcinoma. Medical Oncology, 2015. **32**(2): p. 10.
6. Hu, J., et al., Sox5 contributes to prostate cancer metastasis and is a master regulator of TGF-β-induced epithelial mesenchymal transition through controlling Twist1 expression. British journal of cancer, 2018. **118**(1): p. 88.
7. Chen, X., et al., SOX5 predicts poor prognosis in lung adenocarcinoma and promotes tumor metastasis through epithelial-mesenchymal transition. Oncotarget, 2018. **9**(13): p. 10891.
8. Zhang, D. and S. Liu, SOX5 promotes epithelial-mesenchymal transition in osteosarcoma via regulation of Snail. Journal of BU ON.: official journal of the Balkan Union of Oncology, 2017. **22**(1): p. 258.

9. Huang, D.Y., et al., Transcription factor SOX-5 enhances nasopharyngeal carcinoma progression by down-regulating SPARC gene expression. The Journal of Pathology, 2008. **214**(4): p. 445-455.

10. Kalyankrishna, S. and J.R. Grandis, Epidermal Growth Factor Receptor Biology in Head and Neck Cancer. Journal of Clinical Oncology, 2006. **24**(17): p. 2666-2672.

11. Reuter, C.W.M., M.A. Morgan, and A. Eckardt, Targeting EGF-receptor-signalling in squamous cell carcinomas of the head and neck. British Journal of Cancer, 2007. **96**(3): p. 408-416.

12. Wieduwilt, M. and M. Moasser, The epidermal growth factor receptor family: biology driving targeted therapeutics. Cellular and Molecular Life Sciences, 2008. **65**(10): p. 1566-1584.

13. Gkouveris, I., N. Nikitakis, and A. Sklavounou, p38 Expression and Modulation of STAT3 Signaling in Oral Cancer. Pathology & Oncology Research, 2018: p. 1-10.

14. Tanaka, S., et al., Sox5 and c-Maf cooperatively induce Th17 cell differentiation via RORγt induction as downstream targets of Stat3. Journal of Experimental Medicine, 2014. **211**(9): p. 1857-1874.

15. Suto, A., S. Tanaka, and H. Nakajima, Sox5 and Th17 cell differentiation. Oncotarget, 2015. **6**(24): p. 19952-19953.

16. Hardee, J., et al., STAT3 Targets Suggest Mechanisms of Aggressive Tumorigenesis in Diffuse Large B-Cell Lymphoma. G3: Genes|Genomes|Genetics, 2013. **3**(12): p. 2173-2185.

17. Yang, M.H., et al., Direct regulation of TWIST by HIF-1alpha promotes metastasis. Nat Cell Biol, 2008. **10**(3): p. 295-305.

18. Horikawa, T., et al., Twist and Epithelial-Mesenchymal Transition Are Induced by the EBV Oncoprotein Latent Membrane Protein 1 and Are Associated with Metastatic Nasopharyngeal Carcinoma. Cancer Research, 2007. **67**(5): p. 1970.

19. Hosono, S., et al., Expression of Twist increases the risk for recurrence and for poor survival in epithelial ovarian carcinoma patients. British Journal Of Cancer, 2007. **96**: p. 314.

20. Zhao, Z., et al., Multiple biological functions of Twist1 in various cancers. Oncotarget, 2017. **8**(12): p. 20380-20393.

21. Lovell-Badge, R., The early history of the Sox genes. The International Journal of Biochemistry & Cell Biology, 2010. **42**(3): p. 378-380.

22. Lefebvre, V., et al., Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. The International Journal of Biochemistry & Cell Biology, 2007. **39**(12): p. 2195-2214.

23. Ikeda, T., et al., The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. Arthritis & Rheumatism, 2004. **50**(11): p. 3561-3573.

24. Liu, C.-F., et al. Transcriptional control of chondrocyte specification and differentiation. in Seminars in cell & developmental biology. 2017. Elsevier.

25. Liu, C.-F. and V. Lefebvre, The transcription factors SOX9 and SOX5/SOX6 cooperate genome-wide through super-enhancers to drive chondrogenesis. Nucleic acids research, 2015. **43**(17): p. 8183-8203.

26. Wegner, M., All purpose Sox: The many roles of Sox proteins in gene expression. The International Journal of Biochemistry & Cell Biology, 2010. **42**(3): p. 381-390.

27. Nicholson, R., J. Gee, and M. Harper, EGFR and cancer prognosis. European journal of cancer, 2001. **37**: p. 9-15.

28. Yarden, Y., The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities. European journal of cancer, 2001. **37**: p. 3-8.

29. Kijima, T., et al., STAT3 activation abrogates growth factor dependence and contributes to head and neck squamous cell carcinoma tumor growth in vivo. Cell Growth and Differentiation-Publication American Association for Cancer Research, 2002. **13**(8): p. 355-362.

30. Shah, N.G., et al., Prognostic significance of molecular markers in oral squamous cell carcinoma: a multivariate analysis. Head & neck, 2009. **31**(12): p. 1544-1556.

31. Hanahan, D. and R.A. Weinberg, The Hallmarks of Cancer. Cell, 2000. **100**(1): p. 57-70.

32. Huang, B., et al., Bisphenol A represses dopaminergic neuron differentiation from human embryonic stem cells through downregulating the expression of insulin-like growth factor 1. Molecular neurobiology, 2017. **54**(5): p. 3798-3812.

33. Renard, E., et al., Sox9/Sox6 and Sp1 are involved in the insulin-like growth factor-I-mediated upregulation of human type II collagen gene expression in articular chondrocytes. Journal of molecular medicine, 2012. **90**(6): p. 649-666.

34. Brabletz, T., et al., EMT in cancer. Nature Reviews Cancer, 2018. **18**: p. 128.

35. Khan, M.A., et al., Twist: a molecular target in cancer therapeutics. Tumor Biology, 2013. **34**(5): p. 2497-2506.

36. Zeisberg, M. and E.G. Neilson, Biomarkers for epithelial-mesenchymal transitions. The Journal of clinical investigation, 2009. **119**(6): p. 1429-1437.

37. Nakayama, S., et al., Establishment of high and low metastasis cell lines derived from a human tongue squamous cell carcinoma. Invasion and Metastasis, 1998. **18**(5-6): p. 219-228.

38. Brenner, J.C., et al., Genotyping of 73 UM-SCC head and neck squamous cell carcinoma cell lines. Head & Neck, 2010. **32**(4): p. 417-426.

39. Lin, C.J., et al., Head and neck squamous cell carcinoma cell lines: Established models and rationale for selection. Head & Neck, 2007. **29**(2): p. 163-188.

40. Kordaß, T., et al., SOX5 is involved in balanced MITF regulation in human melanoma cells. BMC Medical Genomics, 2016. **9**(1): p. 10.

41. Liu, X., et al., Inhibition of TDP43-Mediated SNHG12-miR-195-SOX5 Feedback Loop Impeded Malignant Biological Behaviors of Glioma Cells. Molecular Therapy - Nucleic Acids, 2018. **10**: p. 142-158.

42. Wu, K., et al., Long noncoding RNA lnc-sox5 modulates CRC tumorigenesis by unbalancing tumor microenvironment. Cell Cycle, 2017. **16**(13): p. 1295-1301.

43. Wang, L., et al., HuR stabilizes lnc-Sox5 mRNA to promote tongue carcinogenesis. Biochemistry (Moscow), 2017. **82**(4): p. 438-445.

44. Momen-Heravi, F. and S. Bala, Emerging role of non-coding RNA in oral cancer. Cellular Signalling, 2018. **42**: p. 134-143.

45. Luo, X., et al., Long non-coding RNA implicated in the invasion and metastasis of head and neck cancer: possible function and mechanisms. Molecular Cancer, 2018. **17**(1): p. 14.

46. Renjie, W. and L. Haiqian, MiR-132, miR-15a and miR-16 synergistically inhibit pituitary tumor cell proliferation, invasion and migration by targeting Sox5. Cancer Letters, 2015. **356**(2): p. 568-578.

47. Si, C., Q. Yu, and Y. Yao, Effect of miR-146a-5p on proliferation and metastasis of triple-negative breast cancer via regulation of SOX5. Experimental and Therapeutic Medicine, 2018. **15**(5): p. 4515-4521.

48. Tian, R., et al., Differential expression of miR16 in glioblastoma and glioblastoma stem cells: their correlation with proliferation, differentiation, metastasis and prognosis. Oncogene, 2017. **36**(42): p. 5861.

49. Zhang, S., et al., Loss of SOX5 protein expression by RNAi in osteosarcoma cells suppresses cell proliferation and invasion. INTERNATIONAL JOURNAL OF CLINICAL AND EXPERIMENTAL PATHOLOGY, 2017. **10**(6): p. 6480-6487.

50. Yunhong, T., et al., Efficacy and safety of anti-EGFR agents administered concurrently with standard therapies for patients with head and neck squamous cell carcinoma: a systematic review and meta-analysis of randomized controlled trials. International Journal of Cancer, 2018. **142**(11): p. 2198-2206.

51. Akiyama, H., et al., The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes & Development, 2002. **16**(21): p. 2813-2828.

52. Nyati, M.K., et al., Integration of EGFR inhibitors with radiochemotherapy. Nature Reviews Cancer, 2006. **6**(11): p. 876.