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The Role of Integrin αV in Oral Cancer

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The Role of Integrin αV in Oral Cancer

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in Oral Biology

by

Jeremy Barrett

2015

Abstract

The Role of Integrin αV in Oral Cancer

By

Jeremy Barrett

Master of Science in Oral Biology

University of California, Los Angeles, 2015

Professor Dr. Shen Hu, Chair

Background: Oral cancer constitutes 2-3% of carcinomas worldwide and results in more than 128,000 deaths each year. The most common form of oral cancer, squamous cell carcinoma (OSCC), is derived from the oral epithelium. Invasive OSCC is extremely destructive with high metastatic potential due to the high vasculature within the oral cavity. SOX-11 is a transcription factor that has been shown to be up-regulated in cancer and recent studies in our lab have suggested an increase in SOX-11 expression in higher invasive oral cancer cells. By using

proteomic analysis, we have also demonstrated that knockdown of SOX-11 inhibited the expression of integrin αV (ITGAV) in oral cancer cells. However, the role and expression of ITGAV in oral cancer are not known.

Objectives: The objective of our research is to investigate if SOX-11 regulates the expression of ITGAV in oral cancer cells and to examine the role of ITGAV in oral cancer. We will also demonstrate the differential expression of ITGAV among normal human oral keratinocytes (NHOK), and high and low invasive cancer cells.

Methods: Both SOX-11 and ITGAV expression in high (UM1 and UM5) and low (UM2 and UM6) invasive cells were measured using western blotting and qPCR. Chromatin immunoprecipitation (ChIP) assay and siRNA knockdown were used to examine if SOX-11 regulates ITGAV expression in oral cancer cells. Invasion, migration, and proliferation assays were performed to evaluate if ITGAV plays an important role in oral cancer cell proliferation and invasion.

Results: *In silico* analysis through the Genematix software suite and *MatInspector* identified one potential binding site of SOX-11 to the promoter region of ITGAV at 90% confidence. However, twelve additional SoxC group binding sites were located and cross regulation within the SoxC group has been noted in other genes. ChIP assay showed SOX-11 binds to the promoter region of ITGAV gene. After SOX-11 knockdown with siRNA (90% efficiency), ITGAV showed a 40% decrease matching to our previous finding by LC-MS/MS. Next, both western blotting and qPCR showed a significant over-expression of ITGAV

($p < 0.01$) in highly invasive cancer cells (UM1 and UM5) when compared to low-invasive cancer cells (UM2 and UM6). ITGAV was knocked down in invasive UM1 (95% knockdown) and UM5 (63% knockdown) with siRNA and phenotypic studies were preformed. Wound healing assay showed a 33.1% (UM1) and 40.8% (UM5) decrease in migration capability ($p < 0.001$), invasion assay showed a 68% (UM1) and 65% (UM5) decrease in invasion potential ($p < 0.05$), and proliferation assay showed a decreasing proliferation trend in the knockdown group with significant decrease at day 2 and 3 (UM1) and day 2 and 4 (UM5) ($p < 0.05$).

Conclusions: SOX-11 is a transcription factor that binds to the promoter region of ITGAV gene and may regulate the expression of ITGAV in oral cancer cells. ITGAV is significantly over-expressed in highly invasive cancer cells compared to low invasive cancer cells and may play an important role in oral cancer cell migration and invasion.

The thesis of Jeremy Barrett is approved.

Robert Chiu

Carl Maida

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University of California, Los Angeles

2015

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INTRODUCTION

Cancer is a worldwide group of diseases that affect organ systems and tissue to cause an abnormal growth of cells that do not respond normally to external or internal cellular signaling[1]. In 2012, 14 million new cases were identified and 8.2 million cancer deaths were reported indicating that cancer is the leading cause of morbidity and mortality worldwide [1]. Cancers of the oral cavity and pharynx constitute 2% and 3% of cancers in women and men respectively with increased incidence at South-Central Asia and Melanesia[2-4](Figure 1). Worldwide this corresponds to 263,900 new cases and 128,000 deaths from oral cancer in 2008 with an average five year survival rate in the United states of 65%[2, 3]. This five year survival rate is variable with a decrease in the survival rate for African Americans due to decreased early detection.

The most common type of oral cancer is oral squamous cell carcinoma(OSCC)[5]. OSCC can be extremely damaging to surrounding tissue with erosive capabilities to regional soft tissue and osseous structures, including the maxilla and mandible[6]. OSCC often requires surgical removal which can leave patients with large debilitating oral and pharyngeal defects[7]. Oral cancers of the tongue and floor of the mouth have the lowest survival rate due to increased vascularity of the regions[4]. Tongue carcinoma has the lowest five year survival rate of oral cancer at 47% for white men and 27% for African American men[4]. Prevention of risk factors, including alcohol and smoking, and early diagnosis are critical factors in reducing the number of cases of oral cancer

and decreasing morbidity[4]. Throughout the next decade cancer incidence is predicted to increase and early diagnostic factors and treatment modalities will be needed to continue to attempt to lower the morbidly of oral cancer[1].

Regulation of extracellular and intracellular signaling along with extracellular matrix (ECM) interactions is crucial for differentiating between normal tissue and invasive cancers. The interaction with the ECM is critical for cellular survival signals and is involved in the initiation of cancer invasion and metastasis[8]. Therefore, transmembrane proteins involved in promoting survival, migration, proliferation, and differentiation are ideal targets for anticancer therapy. Integrins are a widely expressed family of cell surface membrane receptors which attach cells to the extracellular membrane, mediate cell-cell interactions, and promote outside-in and inside-out signaling in ligated and unligated forms[9-13]. The bidirectional signaling through integrins promote many different cellular responses including motility, differentiation, migration, adhesion, and proliferation and also play a role during embryogenesis including vascular formation[9, 10, 13]. Integrins are a αβ heterodimer which attach non-covalently upon ligand binding to transmit signaling, however, unligated forms also may transmit signals, including anti-apoptotic signals[9, 10]. In total, there are 18α and 8β which can configure to a total of 24 heterodimers[9].

One integrin in particular, Integrin αV (ITGAV), pairs with β1, β3, β5, β6, and β8[14]. Different ITGAV pairings bind different ligands and have multiple binding effects. For example ITGAV heterodimers binding factors can include

vitronectin, fibronectin, vWF, tenascin, osteopontin, fibrillin, fibrinogen, and thrombospondin which have multiple effects including involvement within the coagulation cascade (vWF). These proteins all contain the arginine-glycine-aspartic acid (RGD) which ITGAV has been shown to bind[15, 16]. Using synthetic RGD containing molecules ITGAV pathways have been disrupted showing ITGAV is susceptible to small molecular inhibitors[17]. In addition, expression of ITGAV may determine whether cells may survive in particular microenvironments based on extracellular binding[17].

Due to ITGAV affinity for binding vascular promoting ligands ITGAV has been shown to play a distinct role in multiple pathways of angiogenesis when formed as a dimer of αvβ3 and αvβ5. Integrin αvβ3 was the first integrin tied to angiogenesis and is particularly linked to angiogenesis as it is expressed in only angiogenetic endothelial cells but it is not expressed in normal endothelial cells[18, 19]. Interestingly, multiple pathways are used to initiate angiogenesis. Integrin αvβ3 requires TNF- α or FBF to activate angiogenesis and works through the activation of p21-activated kinase, however, integrin αvβ5 require VEGF or TGF- α and utilize the FAK or Src kinase [20]. This exhibits how different dimers utilizing ITGAV can influence downstream angiogenesis through alternate pathways.

Integrins, including αvβ3, αvβ5, αvβ6, have been shown to be directly associated with cancer growth and invasion[19, 21]. Physiologic responses of integrins during development include angiogenesis, cellular remodeling, and

vascular formation. These same processes turn pathologic with increased amount of integrins present. Increased integrin expression often occurs for tumor growth and to promote invasive characteristics[17]. Increased or upregulated integrins allow for cells to grow within a new environment after migration through outside-in and inside-out signaling causing intracellular remodeling to extracellular cues[17]. For example, in melanoma and pancreatic cancer, increased expression of αvβ3 is associated with increased disease progression and metastasis[22, 23]. Increased ITGAV dimerized as αvβ3 was shown as a key component for transendothelial migration for multiple invasive human cancers[24]. However, the role of integrin αV in oral cancer is unknown and no studies have been completed investigating the relationship of ITGAV in benign or invasive oral cancers.

Currently, anti-integrin αV therapy exists and involves antibodies directed toward ITGAV to prevent dimerization with a β-subunit, or small molecular inhibitors to block ITGAV when dimerized, however, no immunotherapy targets upstream transcription factors. SOX-11 is a transcription factor involved in embryonic development and the determination of cell fate during development. It functions during embryogenesis to promote nervous system development and assists with adult neurogenesis after growth is complete[25]. It is also observed in a wide range of tissues involved in epithelial-mesenchymal interactions[26]. Its role in cancer is unclear but in recent studies SOX-11 has been shown to be upregulated in gliomas, medulloblastomas, and non-B cell lymphomas[27, 28].

Our lab has recently shown through SOX-11 knockdown followed by tandem LC-MS/MS that ITGAV expression was decreased after the SOX-11 knockdown (Figure 2). This data, however, needs to be investigated further.

In this study, we investigate ITGAV regulation and its effect on oral cancers. First, we will look at upstream regulation of ITGAV to determine if the protein SOX-11 is a transcription factor for ITGAV through *in silico* analysis, chromatin immunoprecipitation, and silencing SOX-11 to evaluate ITGAV's expression through western blot. Secondly, we will explore multiple benign and invasive oral squamous cell cancers to analyze the differential expression of integrin αV in oral cancers through western blot and qPCR. Lastly, we will analyze the affect of ITGAV after knockdown in invasive oral cancers through proliferation, migration, and invasion assays.

MATERIALS AND METHODS

In Silico Analysis

Genomatix software suite was used to determine potential binding sites of the transcription factor SOX11 to the promoter of ITGAV. The accession number of ITGAV was uploaded to *Gene2Promoter* which allows the identification of promoter regions for the given gene. The promoter sequences that were obtained from *Gene2Promoter* were altered to be located at 500bp upstream or 100bp downstream of transcription initiation sites. This data was entered into *MatInspector* which searches for potential binding sites of transcription factors to the promoter regions of the input gene. Transcription factors were limited to V$SORY which limit the *MatInspector* search to SOX transcription factors. A common site analysis was preformed and the data was again limited to SOX-11. Finally the data was analyzed and filtered through *MatInspector.*

Cell culture

Four oral squamous cell cancer (OSCC) cell lines UM1, UM2, UMSCC-5 (UM5), and UMSCC-6 (UM6) were obtained from Dr. Yong Kim at the UCLA School of Dentistry. UM1 and UM5 are invasive cancers obtained from human tongue cancer. UM2 and UM6 are non-invasive cancers obtained from oral tongue cancer. UM1 and UM2 were obtained originally from the same patient. UM5 and UM6 are obtained from different patients. All OSCC cell lines were cultured in DMEM (Invitrogen Life Technologies, CA) with 10% fetal bovine

serum (FBS) (Gemini Bio-Products, CA), 100 units/mL penicillin G and 100 μg/mL streptomycin (1%) (Invitrogen Life Technologies, CA). Normal human oral keratinocytes (NHOK) were cultured in keratinocyte basal media containing keratinocyte growth factor. Cell culture medium was changed every three days and were maintained at 5% CO_2 with 95% air at 37 °C in a humidified incubator.

Western blotting

Cells were cultured until 80% confluence in 6cm plates, UM1, UM2, UM5, and UM6 cell lines were lysed in Rb buffer for 5-10 minutes. Remaining cells were scraped from the plate. The cell lysates were homogenenized for 30 seconds. The cell lysates were centrifuged at 15,000 rpm for 5 minutes at 4°C. The supernatants were removed and the protein was resuspended before the levels of proteins were quantified with micro BCA assay. 30ug of each cell line and 5ul of protein ladder (Santa Cruz Biotechnologies, CA) of cell lysate were loaded into NUPAGE Novex 4-12% Bis-Tris gel. The gel was ran with a buffer of MES, EDTA, SDS, and water at 35 mA for 4 hours. The gel was transferred to a nitrocellulose membrane in transfer buffer at 20 V for 30 minutes at room temperature.

After the transfer, the membrane was blocked with 5% milk in TBST for one hour at room temperature and milk discarded. The blocked nitrocellulose was incubated with primary antibodies at a dilution of 1:200 in 2% milk with TBST and hybridized on a shaker overnight at 4°C with a constant 60rpm. The following primary antibodies were used to perform western blot analysis: Integrin

αV (rabbit derived, Santa Cruz Biotechnolgy), SOX-11 (rabbit derived, Santa Cruz Biotechnology), GAPDH (mouse derived, Santa Cruz Biotechnology).

The membrane was washed 3 times for 5 minutes each on the shaker with TBST before adding either ECL goat anti-mouse IgG or ECL goat anti-rabbit IgG (Santa Cruz Biotechnology, CA) and incubated for one hour at room temperature on a shaker at 60rpm. The membrane was washed again with TBST 3 times for 5 minutes each.

Enhanced chemiluminescence (ECL) detection kit (GE healthcare) was used to detect the secondary antibody. The detection reagents were added to the membrane and excess flushed away so no excess remained. The membrane was incubated in the dark for 5 minutes at room temperature with the detection reagent. The membrane was transferred to a dark room cassette. Triplicate radiograph films(Thermo Scientific) were placed on the blot and exposed for 10-20 minutes before developing the films. The films were scanned and quantified with ImageJ (NIH).

Quantitative Polymerase Chain Reaction (qPCR)

UM1, UM2, UM5, and UM6 cell cultures were grown to 80% confluence in 6cm plate prior to mRNA collection. 400uL Rb buffer was added to each plate for 10 minutes and cell lysate was collected. The cell lysate was homogenized and centrifuged at 1500 rpm for 5 minutes. The supernatant was collected and RNAEasy kit (Genesee Scientific) was used to collect the mRNA. The mRNA

was then converted to cDNA using reverse transcription with the superscriptase III kit (Invitrogen) and the concentration was quantified using the Nanodrop machine. 1 uL (1:10cDNA dilution) of sample was pipetted into a 50uL microcentrifuge tube with 10 uL SYBR Green Supermix 2X, 0.2 uL of forward primer, and 0.2uL reverse primer (10 uM), and sterilized water until 20 uL final volume was achieved. The PCR was completed in qPCR with the following settings: denaturation at 94°C for 40 s, annealing at 55°C for 30s, extension at 68°C for 90 s for 40 cycles, and a final extension at 68°C for 8 min. The Cq values were obtained from the qPCR machine and analyzed for fold change using delta-delta Ct. The ITGAV primers that were used during qPCR and for CHiP can be found in Table 1.

Chromatin Immunoprecipitation (ChIP)

UM1 and UM5 cultures were grown to 80% confluency and treated with doxorubicin (0.4 μM; Sigma-Aldrich) or DRB (50 μM; Sigma-Aldrich) and washed with PBS. 1% formaldehyde/PBS solution was added to the cells for 15 min at room temperature to initiate DNA cross-linking. The cross-linking was stopped by addition of glycine until 125 mM final concentration was reached. The cells were washed twice with PBS and collected with RIPA buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl at pH 8, 5 mM EDTA, 20 mM NaF, 0.2 mM sodium orthovanadate, 5 μM trichostatin A, 5 mM sodium butyrate, and protease inhibitors). Sonication was used on the samples to generate DNA fragments <500 bp.

1 mg of protein extract was precleared for 2 hours with 30 µL of 50% G protein-Sepharose slurry before addition of Integrin αV (rabbit derived, Santa Cruz Biotechnolgy). Two micrograms of ITGAV antibody was added to the samples and incubated overnight at 4°C in the presence of 30 µL of protein G-beads that were preblocked with 1 mg/mL BSA and 0.3 mg/mL salmon sperm DNA. Antibodies against phosphorylated CTD were used in the sample. The immunocomplexes were recovered using anti-mouse IgM/protein G-Sepharose beads (Sigma-Aldrich). The beads were washed twice with RIPA buffer, four times with ChIP Wash Buffer (100 mM Tris-HCl at pH 8.5, 500 mM LiCl, 1% [v/v] Nonidet P-40, 1% [w/v] deoxycholic acid), two times with RIPA buffer, and two times with 1× TE. The immunocomplexes were eluted for 10 min at 65°C with 1% SDS, and cross-linking was reversed by adjusting to 200 mM NaCl and incubating 5 h at 65°C. The DNA was purified and the sample was quantified using Nanodrop and subject to qPCR as described previously.

siRNA Transfection

siRNA knockdown experiments were carried out in UM1 and UM5 cell lines for proteins SOX-11 and ITGAV.

Day One: Evenly plate cells

UM1 and UM5 cell cultures at 80% confluency in 6cm were washed with PBS three times, treated with 2ml trypsin for 5 minutes, quenched with 10ml of DMEM, and collected. The cells were centrifuged at 1500rpm for 5 minutes, and

resuspended in DMEM medium (Invitrogen) supplemented with 10% FBS (Gemini) and 1% Penicillin Streptomycin (Penstrep)(Invitrogen) then homogenized. The cell cultures were counted and transferred to a six well plate at 300,000 cells per well with even cell distribution throughout the plate. The cells were placed back into the cell culture incubator overnight.

Day Two: Preform Knockdown

Two solutions were made separately for this transfection with one complex being 0.1nmol of siRNA (either siITGAV, siSOX11, or siControl) (Santa Cruz Biotechnology) added to 100 uL of DMEM. The second solution consisted of 10 uL of Lipofectamine II transfecting reagent (Invitrogen) with 100 uL DMEM. The two solutions were mixed together and incubated at room temperature for 5 minutes. The culture medium from 6-well plates was removed the cell cells were washed once with PBS. 3 mL of DMEM and the transfecting solutions (siControl or siKnockdown) were added to the wells marked siControl or siKnockdown. The cells were then incubated in the CO_2 incubator over night.

Day Three: Stop Knockdown

After 16 hours the siControl or siKnockdown and transfection reagent was removed. The cells were washed with PBS three times and 3mL of fresh cell culture medium was added (DMEM medium (Invitrogen) supplemented with 10% FBS (Gemini) and 1% Penstrep). The cells were returned to the CO_2 incubator for 24 hours (mRNA collection) or 48 hours (protein collection).

Day Four: mRNA Collection

After 24 hours the mRNA was collected. The complete medium was removed and the cells were washed three times with PBS. The cells were lysed with RLT buffer for five minutes. The complete lysate was collected and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the mRNA was collected using RNAEasy extraction kit. It was immediately converted to cDNA using superscriptase III conversion kit (Invitrogen).

Day Five: Protein Collection

After 48 hours from transfection reagent removal the completed medium was removed and the cells were washed with PBS three times. The cells were lysed with RIPA buffer, and the protein was collected and quantified as described in the western blot section.

Invasion Assay

Invasion assay was conducted on siControl UM1, siControl UM5, siITGAV UM1, and siITGAV UM5. ITGAV was knocked down using siRNA as described above in UM1 and UM5 cell lines. Forty-eight hours after ITGAV knockdown was completed the complete medium was removed and the cells were washed with PBS one time. 300uL of trypsin was added to each well for 7 minutes then quenched with 3mL of complete media. The cells were centrifuged at 1500 rpm for 3 minutes and the supernatant was removed. The cells were resuspended in 5mL DMEM and homogenized. 500 uL of suspension was removed and the cell

number was analyzed using Vi-CELL XR (Cell Viability Analyzer) (Beckman Coulter). $5.0x10^4$ of each cell group were added into 24-well BD BioCoat Matrigel Invasion Chambers and DMEM was added up to 0.5mL. Complete medium was used as the chemoattractant and added to the wells of the BD Falcon TC Companion Plate. The invasion chambers were transferred to the wells containing the chemoattractant. The 24-well invasion plate was incubated for 22 hours in a humidified cell culture incubator.

After 22 hours the invasion wells were removed and the non-invading cells were removed with a cotton tipped swab two times. After the non-invading cells were removed the remaining invading cells were stained using the Diff-Quik staining kit. The cells were photographed using microscope with mounted camera and invading cells were counted in siITGAV and siControl wells and the results analyzed.

Migration Assay/Wound Healing Assay

The migration assay was conducted on siControl UM1, siControl UM5, siITGAV UM1, and siITGAV UM5. ITGAV was knocked down using siRNA as described above in UM1 and UM5 cell lines. Forty-eight hours after ITGAV knockdown was completed the complete medium was removed and the cells were washed with PBS one time. Using a sterile 200uM pipette 3 scratches were made vertically at thirds across the 6 well plate. The wells were washed with PBS once. 3mL of complete media was added to each well. An initial 0hr picture was obtained of each scratch in control and knockdown wounds. Photos were

obtained of each wound at 6hr, 24hr, and 48hr intervals. At each measurement the thickness of the wound was recorded, the cells were washed with PBS, and fresh complete medium was added.

Proliferation Assay

Proliferation assay was conducted on siControl UM1, siControl UM5, siITGAV UM1, and siITGAV UM5. ITGAV was knocked down using siRNA as described above in UM1 and UM5 cell lines. Forty-eight hours after ITGAV knockdown was completed the proliferation assay was started and will be considered Day 1 of proliferation assay. On Day 1, the complete medium was removed and the cells were washed once with PBS. The cells were lysed with 300uL 0.25% trypsin (Invitrogen) for 7 minutes and quenched with 1.5mL complete media. The cells were carefully checked under a microscrope to make sure 100% of the cells were free from the plate. The cells were removed from the wells and counted using Vi-CELL XR (Cell Viability Analyzer) (Beckman Coulter). The process was followed at 24hr intervals for four days. The data was collected from Vi-CELL XR.

Statistical Analysis

The statistical analysis conducted throughout this project consisted of using Student's t-test to obtain statistically significance difference between two sets of data. A value of $P<0.05$ was considered as statistically significant.

RESULTS

In Silico Analysis

The *In Silico* Analysis using the Genomatic Software Suite was preformed as a model to test, through computer simulation, if the transcription factor SOX-11 has the potential to bind to the promoter region of ITGAV. Only known promoter regions of the gene ITGAV along with promoters identified according to *Gene2Promoter* were used in *MatInspector* to identify potential binding sites of SOX-11 to the promoter region of ITGAV. We identified 1 potential binding site with 89% confidence at position 252-274 upstream from transcription initiation site (Figure 2 & 3). 12 additional binding sites were located for other members of the SoxC group (SOX-4 and SOX-9).

Chromatin immunoprecipitation

There has been no study showing the binding of SOX-11 to the promoterof ITGAV. To test whether SOX-11 can bind to ITGAV's promoter region, a ChIP assay was preformed on invasive cancer UM5 cell line, which has been shown to have an upregulated expression of SOX-11. The ChIP was preformed using a SOX-11 specific polyclonal antibody followed by a qPCR using primers for the regulatory region of ITGAV. We positively identified binding of SOX-11 upstream of the ITGAV transcription initiation site. The DNA that was obtained from the ChIP was amplified using primers upstream to ITGAV. There was a significant

amplication after qPCR analysis showing a 7 fold change against the control with no SOX-11 anitbody (*p = 0.005*) (Figure 4).

siRNA knockdown of SOX-11

To further analyze the effect that SOX-11 has on the expression of ITGAV we preformed a knockdown of SOX-11 using siRNA in UM1 cell line. After SOX-11 was confirmed to be knocked-down the expression of SOX-11 and ITGAV was analyzed by western blot using ITGAV and SOX-11 specific polyclonal antibodies. We concluded that after the knockdown of SOX-11 there was a reduced expression of ITGAV and quantification of the western using ImageJ resulted in a 39.4% decrease in ITGAV expression(Figure 5).

Differential expression of ITGAV between high and low invasive cancer cells

Invasive cancer cells lines, UM1 and UM5, and non-invasive cancer cell lines, UM2 and UM6, were selected to analyze for ITGAV expression through western blot and quantitative polymerase chain reaction. Whole cell lysate of all cell lines was subject to western blotting with primary polyclonal antibody to ITGAV (Figure 6). The results show that the expression of ITGAV has increased expression in invasive cancer cell lines (UM1 and UM5) compared with non-invasive cancer cell lines (UM2 and UM6). Quanitification of the western blot in ImageJ showed a significant increase of ITGAV in UM1 cells than in UM2 and UM6 cells (*p<0.01)* and a significant increase of ITGAV in UM5 cells than in UM2

and UM6 cells (*p<0.01).* The normal human oral keratinocyte (NHOK) expression of ITGAV remains high as it is subject to different culturing media containing growth factors that promote increased cell surface receptors.

Next, we used qPCR to quantify the gene expression of ITGAV in NHOK, UM1, UM2, UM5, and UM6 cells. The mRNA was extracted from all cell lines and quantified using PCR in triplicates (Figure 7). The results match the protein expression levels indicating an increased expression of ITGAV in invasive oral cancer cells versus low-invasive cancer cells. UM1 had a 91-fold and 104-fold increase over UM2 and UM6 respectively (*p<0.01),* while UM5 showed a 44-fold increase and 58-fold increase over UM2 and UM6 (*p<0.01).*

Knockdown of ITGAV suppresses the invasion of UM1 and UM5 cells

The invasion assay was used to test the change in invasion capability of malignant oral cancers (UM1 and UM5) after ITGAV was knocked-down. To begin the invasion assay a knockdown of ITGAV in UM1 and UM5 was initially completed, techniques were optimized, and final results were verified with western blot (Figure 8). After the knockdown of ITGAV was idealized, the invasion assay was preformed with knockdown ITGAV against a si-scramble control in UM1 and UM5. The results show that a significantly decreased number of UM1 (68% reduction) and UM5 (65% reduction) cells invaded through the membrane towards the chemoattractant after knockdown ($p < 0.05$) (Figure 9).

Knockdown of ITGAV inhibits the migration of UM1 and UM5 cells

Wound healing assay was preformed to test the change in UM1 and UM5 migration capability after ITGAV knockdown. After ITGAV knockdown was idealized the migration assay was preformed with a 200uM wound and photos were obtained at multiple time points (Figure 10). The results indicate that there is significant decrease in migration capability after ITGAV was knocked-down. In UM1 a there was an average of 33.1% (UM1) and 40.8% (UM5) reduction in migration ($p < 0.001$).

Knockdown of ITGAV suppresses the proliferation of UM1 and UM5 cells

ITGAV was knocked down in UM1 and UM5 cells and the effect on UM1 and UM5 proliferation rates was measured over four days. Overall trend shows that ITGAV knockdown has a decreased proliferation in UM1 and UM5 cell lines. UM1 has significant decrease on day 2 and day 3 ($p<0.05$) and UM5 shows significant decrease on day 2 and day 4 ($p<0.05$). (Figure 11).

DISCUSSION

Alteration of ITGAV regulation is a common abnormality that contributes to cancer pathogenesis. The ability of ITGAV in dimer formation to deliver outside-in and inside-out signaling leading to increased angiogenesis, anti-apoptotic signals, and survival signals in new environments make up regulated ITGAV a powerful pro-invasive and pro-metastatic protein. However, before this study the understanding of upstream regulatory mechanisms of ITGAV is limited, the discrepancies in regulation of ITGAV between benign and invasive cancer is not fully understood, and the differential expression of ITGAV in oral cancer was unknown. Our goal was to begin data collection in each of these categories to help express ITGAV's role in leading to a more aggressive cancer and at the same time describing ITGAVs role in oral cancer.

Our lab has previously shown that the transcription factor SOX-11 is up regulated in the invasive OSCC cell lines UM1 and UM5 compared with benign OSCC UM5 and UM6. This data led to an increased interest in SOX-11 to determine which possible downstream targets may contribute to increased cancer pathogenesis. To begin investigating downstream proteins a LC-MS/MS was preformed between the highly invasive OSCC UM1 and altered UM1 with SOX-11 K/D which showed 80% of ITGAV after knockdown. Due to the high regulation of ITGAV we determined ITGAV as a good potential target.

Using the MS data as a base we first wanted to determine if ITGAV was indeed potentially regulated by SOX-11. We started with an *In silico* study using

Genomatix Software Suite and *MatInspector* to verify that a potential binding between SOX-11 and ITGAV. Even thought only 1 potential binding site for SOX-11 occurred at 89.6% confidence there were 12 potential binding sites for other SoxC group members SOX-4 and SOX-12. The SoxC group has shown to have overlapping expression patterns and molecular patterns possibly due to conserved domains[29]. SOX-11 may bind at other SoxC regions in ITGAV's promoter to increase regulation, however, this will need to be evaluated further in future studies. Next, we confirmed a potential binding of SOX-11 to ITGAV promoter using ChIP assay and we obtained a positive result showing a eight fold increase in qPCR analysis versus the control indicating that SOX-11 does bind to ITGAV's promoter region (Figure 4). Finally, a knockdown of SOX-11 in UM1 was preformed followed by Western Blot of ITGAV and SOX-11 proteins. After the SOX-11 knockdown with siRNA was optimized to a 95% knockdown ITGAV was analyzed by western showed and decrease of expression by 39.4% as quantified by ImageJ (Figure 5). Through, *in silico* analysis, ChIP assay, and siRNA knockdown of SOX-11 and evaluation through LC-MS/MS and western blot we determined that SOX-11 is a potential transcription factor in UM1 and UM5 cell lines.

Our next step was to determine the expression of ITGAV in OSCC and establish phenotypic traits that ITGAV promotes in oral cancer. To initiate investigation our cell lines were decided based on invasive and benign traits. Interestingly, UM1 and UM2 are cancers harvested from the same patient,

however, UM1 is malignant and had spread to distant sites within the patient and UM2 is premalignant without current metastatic potential[30]. UM5 is an invasive tongue cancer and UM6 is a premalignant cancer each from separate patients. After idealizing the western blot the ITGAV protein levels from each cell line were measured and quantified with ImageJ and qPCR. The results indicate that there is a significant difference in the increase of ITGAV in UM1 and UM5 invasive cell lines against the less invasive UM2 and UM6. This data matches the same trend in SOX-11 expression that our lab previously concluded. Which indicates that SOX-11 is highly expressed in invasive oral cancers and ITGAV follows the same pattern.

Finally, the phenotypic trend of ITGAV was evaluated to understand ITGAV's role in invasion, proliferation, and migration in invasive OSCC. To evaluate the difference of ITGAV we decided to conducted an siRNA knockdown of ITGAV in UM1 and UM5 and study the resultant phenotypic changes. The siRNA knockdown of ITGAV was idealized in UM1 and UM5 with a 94.8% knockdown in UM1 and 63.9% knockdown in UM5 (Figure 8). The invasion assay showed a significant decrease in the amount of UM1 and UM5 invasion after ITGAV was knocked down (Figure 9). After performing the invasion assay it appears UM1 has a higher invasion rate than UM5, and UM5 prefers to migrate in clumps of cells unlike UM1 which moves more independently of one another. However even with UM5's decreased invasion capability it remains significantly decreased after ITGAV was knocked down. Next the migration was preformed

after ITGAV was knocked down in UM1 and UM5. In both UM1 and UM5 siITGAV groups there was a significant decrease in the amount of wound closure at each time point. Similar to the invasion assay, the migration assay showed a decreased amount of wound closure at each time point in UM5 than UM1, and this extended the UM5 to 30 hours before first closure of the control was seen as opposed to 18 hours in UM1. Regardless, in both groups the wound of the knockdown group remained open after the control groups wound already fused completely. Finally the proliferation assay was completed in the experimental ITGAV knockdown group against the control, si-scramble, group. The results indicate that after the first day the proliferation of the control group was greater than that of the knockdown group. These three studies show that decreasing ITGAV leads to reduced malignant cancer traits and further the relationship that ITGAV has on increasing cancer pathogenesis.

CONCLUSION

In conjunction with our previous LC-MS/MS data showing decrease in ITGAV expression when SOX-11 was knocked down, we have now demonstrated that SOX-11 is a potential transcription factor for ITGAV using *In Silico* data analysis, ChIP assay, and siRNA knock down of SOX-11. The role of ITGAV in oral cancer was previously not understood. However, we have found that ITGAV is significantly up-regulated in invasive oral cancer cells compared to low-invasive oral cancer cells and ITGAV promotes the invasion, migration, and proliferation of oral cancer cells.

The limitations of the study are bound by the effects of using transient silencing to knockdown our proteins of interest. While siRNA allowed us to decrease the protein concentration of ITGAV and SOX11 in our experiments, it does not guarantee a complete knockdown. Therefore, a more ideal complete knockout technique would eliminate the protein of interest and allow for a study with no interference of the knockout protein. Additionally, future studies will be needed to verify SOX11 is a transcription factor for ITGAV and additional studies will be needed to verify ITGAV's true role within oral cancer. To complete the link that SOX11 is a transcriptional regulator of ITGAV additional functional studies will be needed to verify that SOX11 controls the rate of ITGAV transcription. In addition, upregulation of SOX11 should be completed and resultant downstream effects of ITGAV quantified. The verification of ITGAV's role in oral cancer would ideally require a complete knockout cell line. A

knockout cell line of ITGAV would allow true evaluation of ITGAV's function role in OSCC with additional phenotypic studies. A stable knockout cell line would also allow for an animal model study to verify our findings and additional metastatic studies *in vivo.*

While ITGAV is known to increase the aggressive potential in specific types of cancer it has not been evaluated in oral cancer. The results that we found in this study suggest that ITGAV increases the invasive ability of OSCC as well. Sub-types of cancer can be widely variable, but to find a protein, ITGAV, that increases invasive potential across multiple strains of highly invasive OSCC from multiple patients translates to earlier identification of a more aggressive carcinoma on diagnosis. After further testing of ITGAV's role in oral cancer, targeting ITGAV may allow new cancer treatment modalities and chemotherapy to be utilized against aggressive OSCC with a goal to increase the currently low five-year survival rate.

FIGURES AND TABLES

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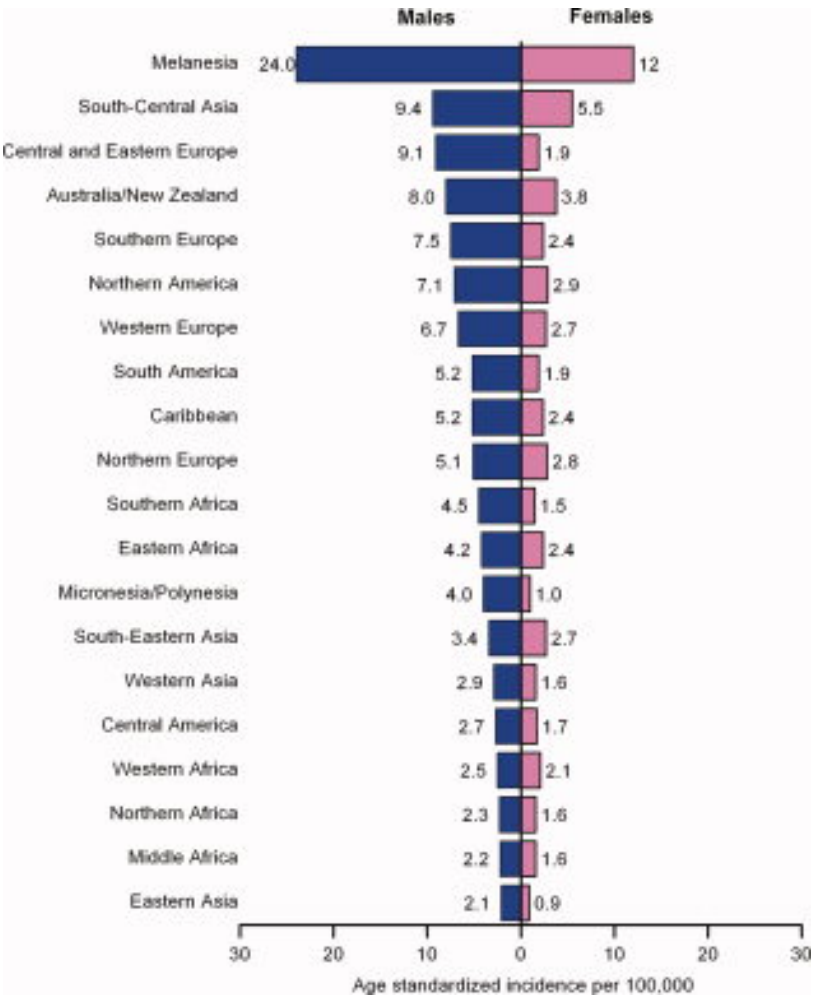


Figure 1. Age-Standardized Oral Cavity Cancer Incidence Rates by Sex and World Area[2].

Check transcription factor <-> matrix family assignment				Position					
Matrix Family	Detailed Family Information	Matrix	Detailed Matrix Information	from	to	anchor	Strand	Matrix sim.	Sequence (red: cl-value > 60 CAPITALS: core sequence)
V$SORY	SOX/SRY-sex/testis determinig and related HMG box factors	V$SOX11.01	SRY-related HMG-box gene 11	252	274	263	(-)	0.896	atggaACAAggaaagcaaaggac

Figure 2. *In Silico* analysis of SOX-11 transcription factor binding to promoter region of ITGAV.

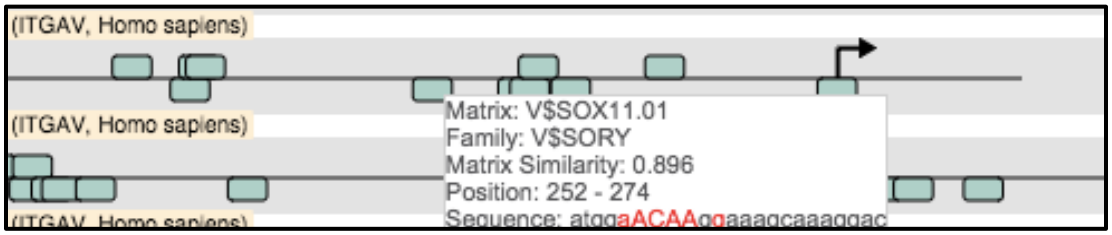
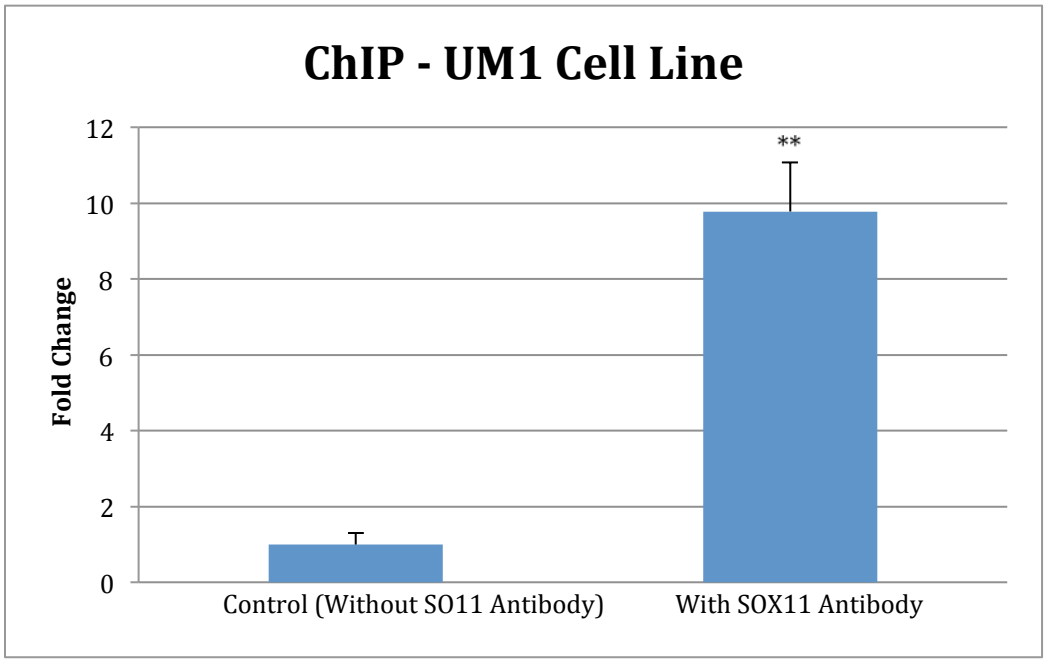


Figure 3. Location of SOX-11 transcription factor binding site relative to transcription initiation.



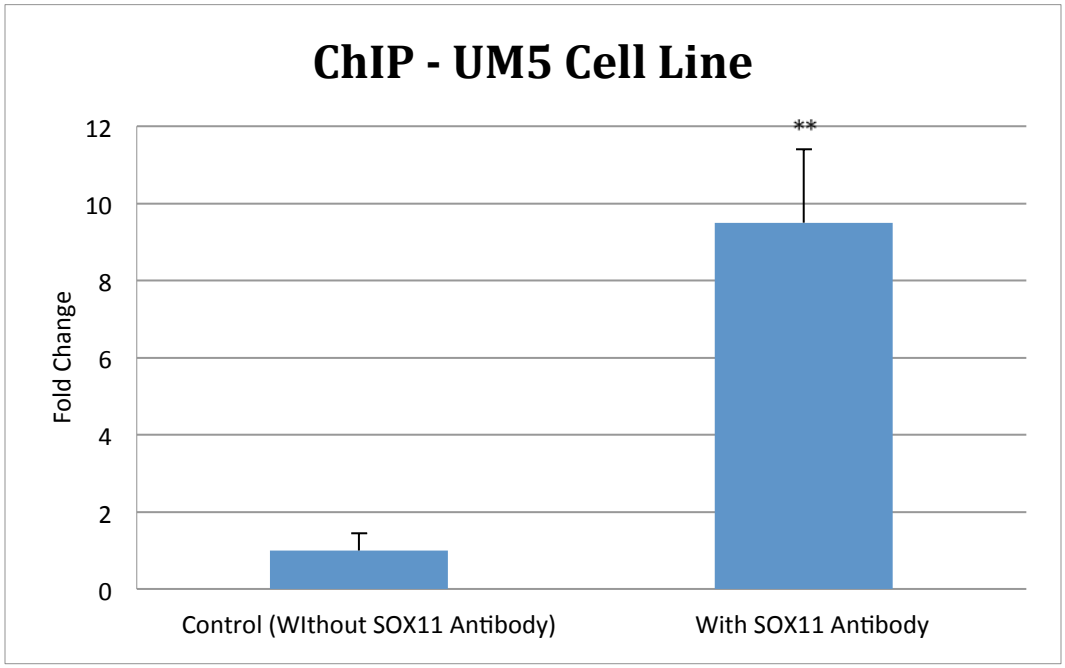
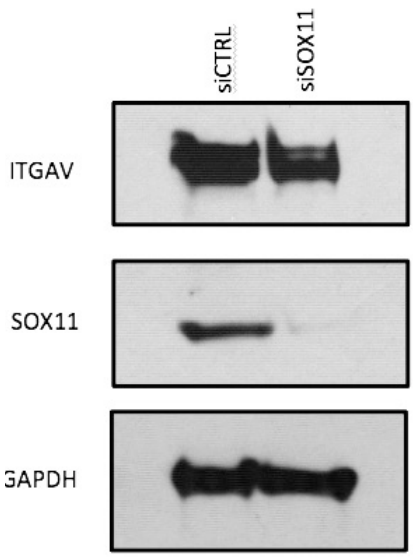


Figure 4. qPCR of DNA obtained from ChIP assay with and without SOX-11 polyclonal antibody in UM1 and UM5 cell lines (*p<0.001)*. (* indicates *p < 0.05*, ** indicates *p < 0.001*)

5A.)



5B.)

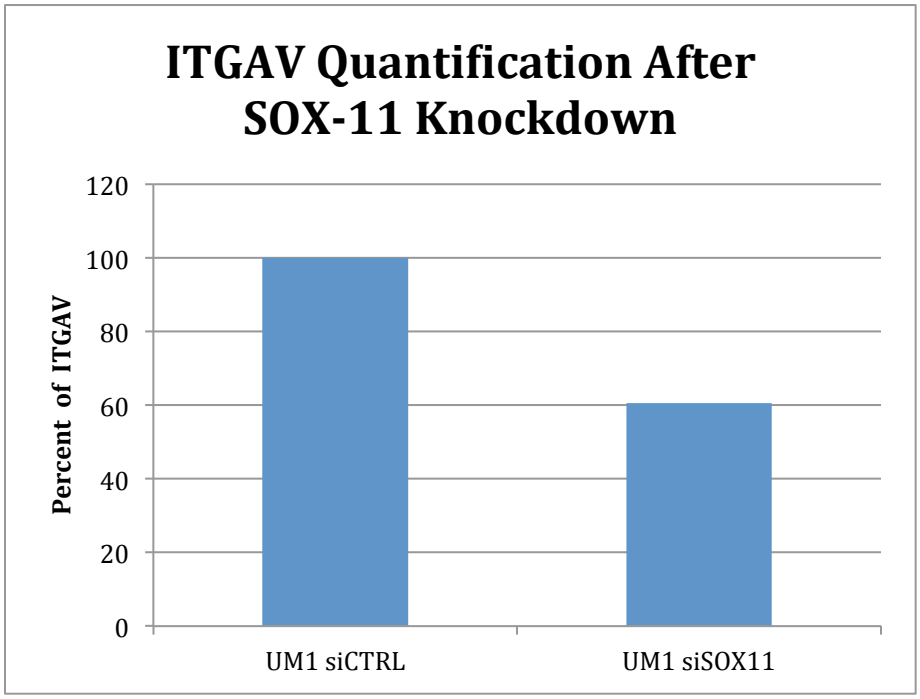
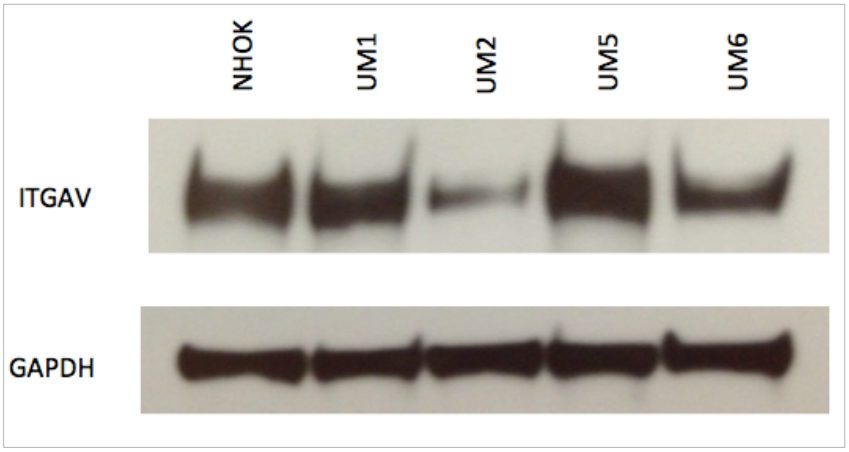


Figure 5. Knockdown of SOX-11 inhibits the expression of ITGAV. A.) Western blot analysis showing SOX11 knockdown and resultant decrease in ITGAV expression; B.) Quantification of ITGAV expression change after SOX11 knockdown using ImageJ.

6A.)



6B.)

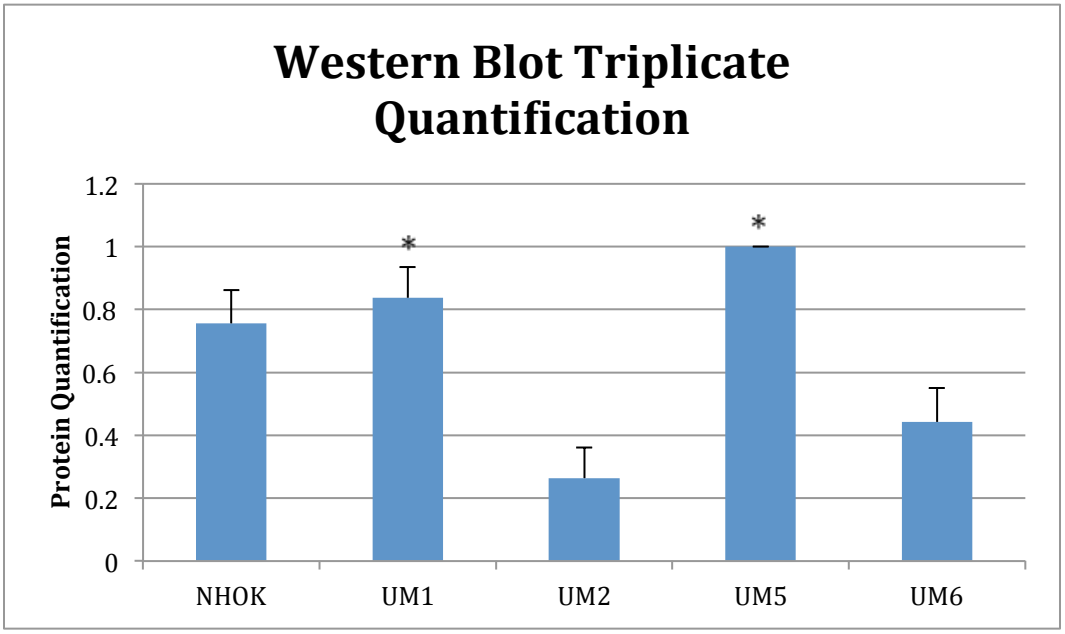


Figure 6. A.) Western blotting analysis showing the expression of ITGAV between highly invasive cancer cells(UM1 and UM5) and low invasive cancer cell lines (UM2 and UM6); B.) Quantification of western blotting results using ImageJ. The expression levels of ITGAV in UM1 and UM5 cells are significantly higher than UM2 and UM6 cells ($p < 0.05$). (* indicates $p < 0.05$, ** indicates $p < 0.001$)

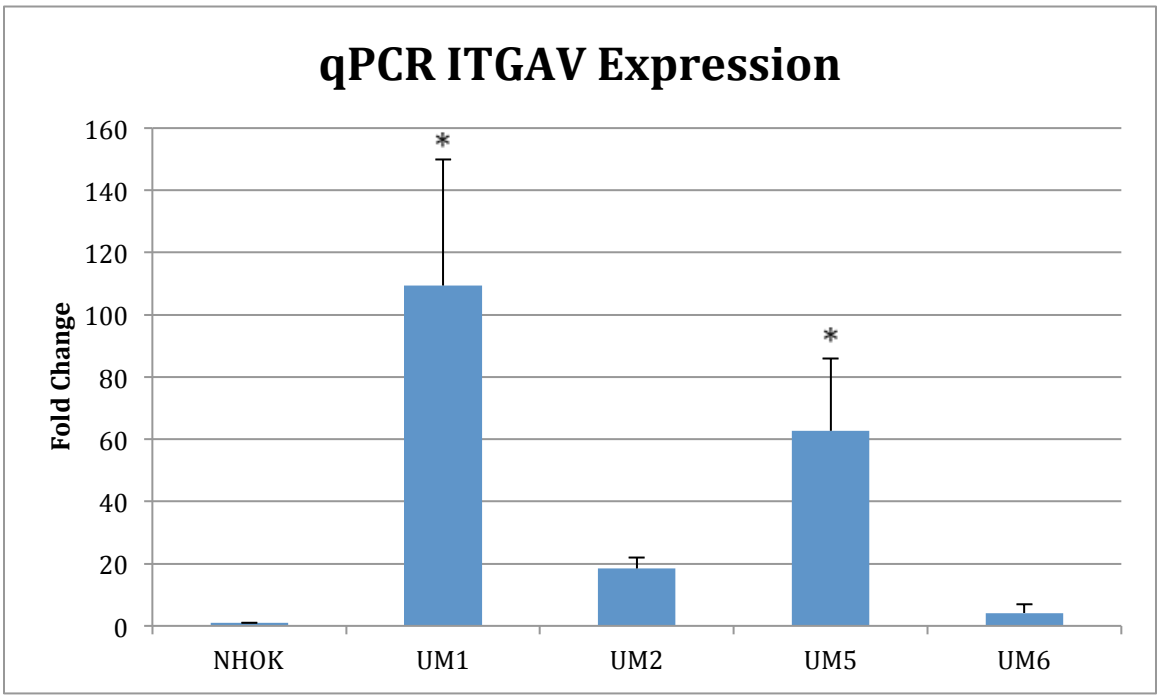
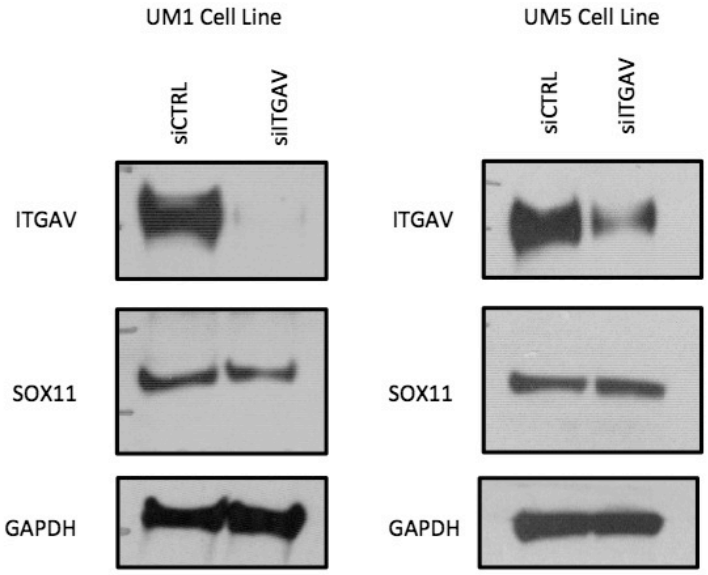
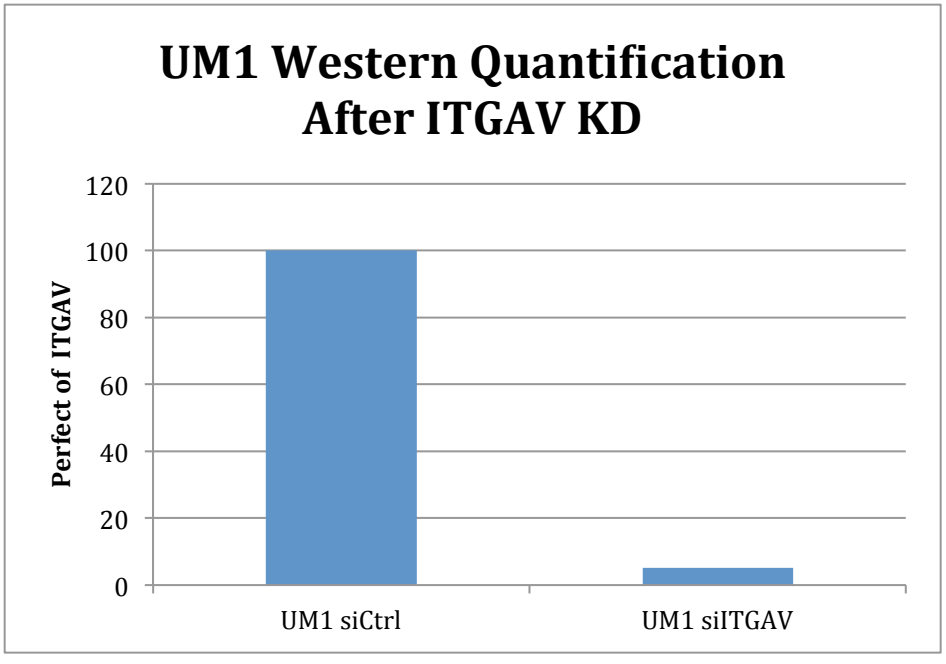


Figure 7. qPCR analysis showing the increased expression of ITGAV in highly invasive cancer cell lines (UM1 and UM5) compared to low-invasive cancer cell lines (UM2 and UM6). The gene expression of ITGAV in UM1 and UM5 cells are significantly higher than UM2 and UM6 cells ($p < 0.05$).
(* indicates $p < 0.05$, ** indicates $p < 0.001$)

8A.)


UM1 Cell Line
UM5 Cell Line
siCTRL
siITGAV
siCTRL
siITGAV
ITGAV
ITGAV
SOX11
SOX11
GAPDH
GAPDH

8B.)


UM1 Western Quantification
After ITGAV KD
Perfect of ITGAV
120
100
80
60
40
20
0
UM1 siCtrl
UM1 siITGAV

8C.)

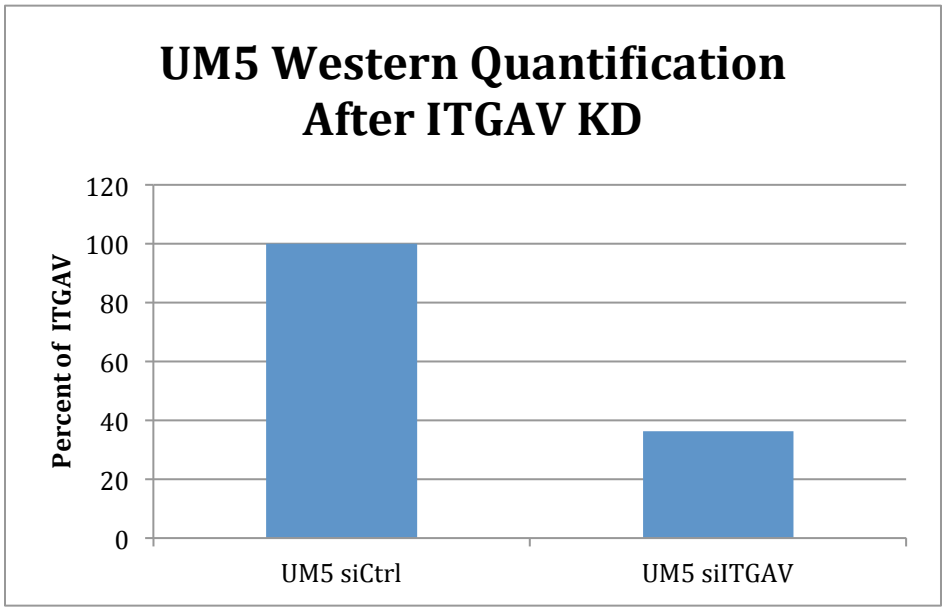
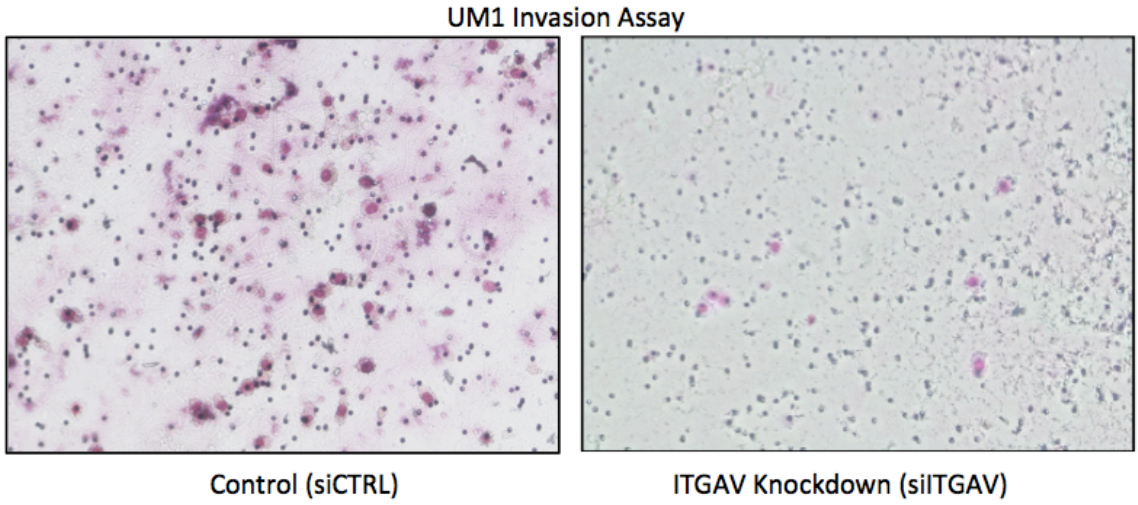
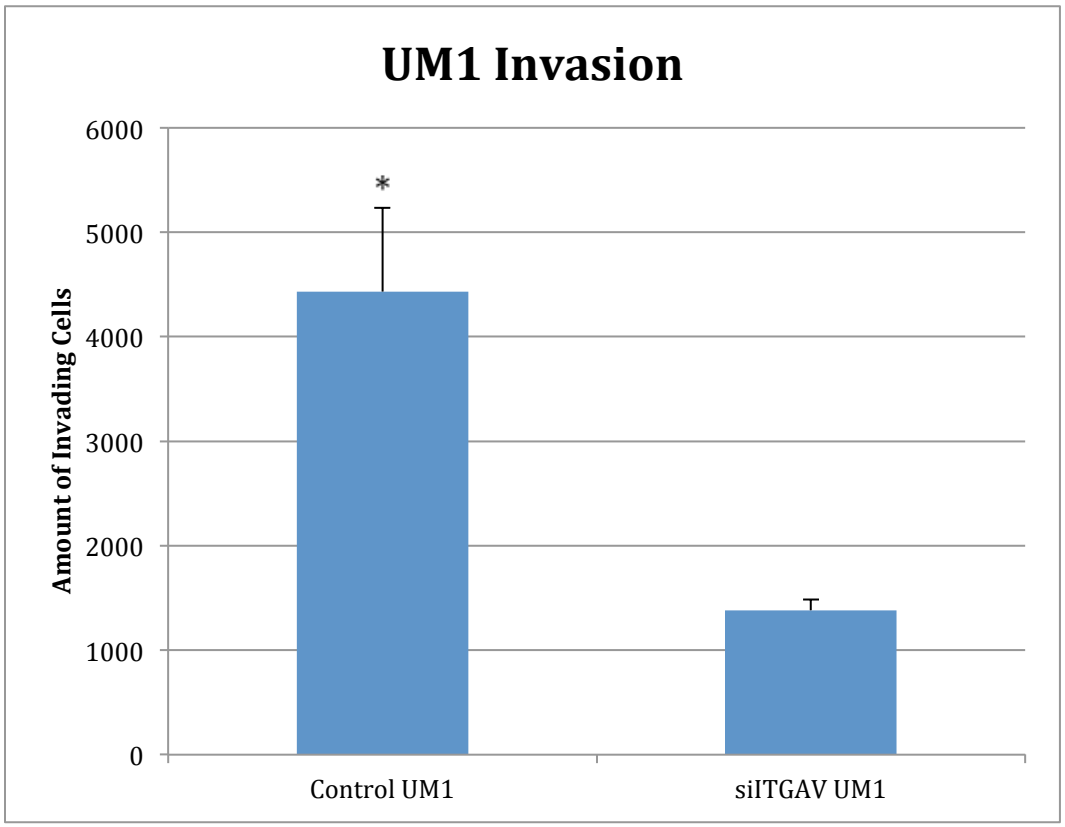


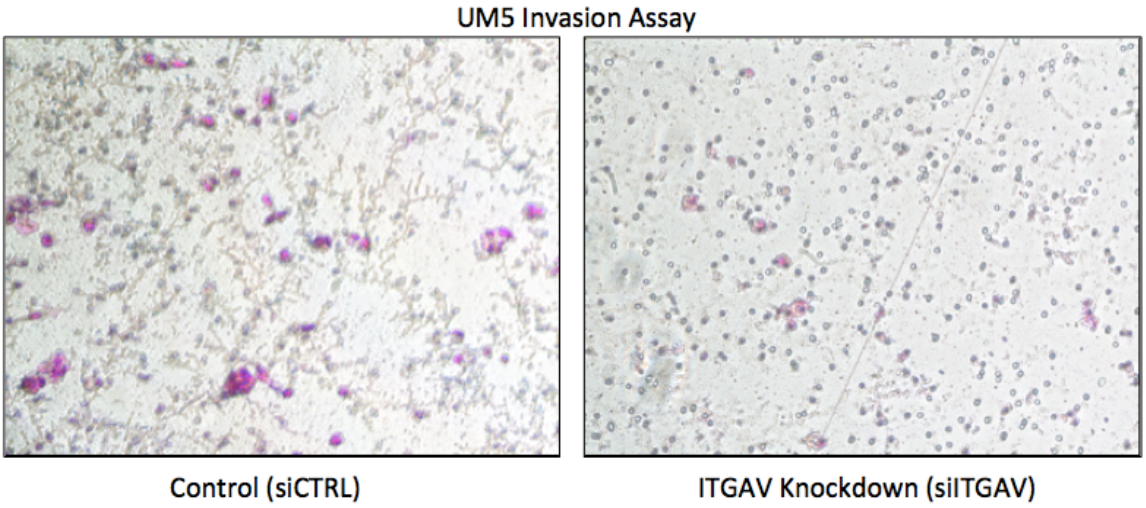
Figure 8. ITGAV knockdown was confirmed by Western blotting. A.) Western blot of ITGAV in UM1 and UM5 cell lines after ITGAV knockdown. B.) Quantification of ITGAV knockdown in UM1 cells using ImageJ. C.) Quantification of ITGAV knockdonw in UM5 cells using ImageJ.

9A.)


UM1 Invasion Assay
Control (siCTRL)
ITGAV Knockdown (siITGAV)


UM1 Invasion
Amount of Invading Cells
6000
5000
4000
3000
2000
1000
0
*
Control UM1
siITGAV UM1

9B.)



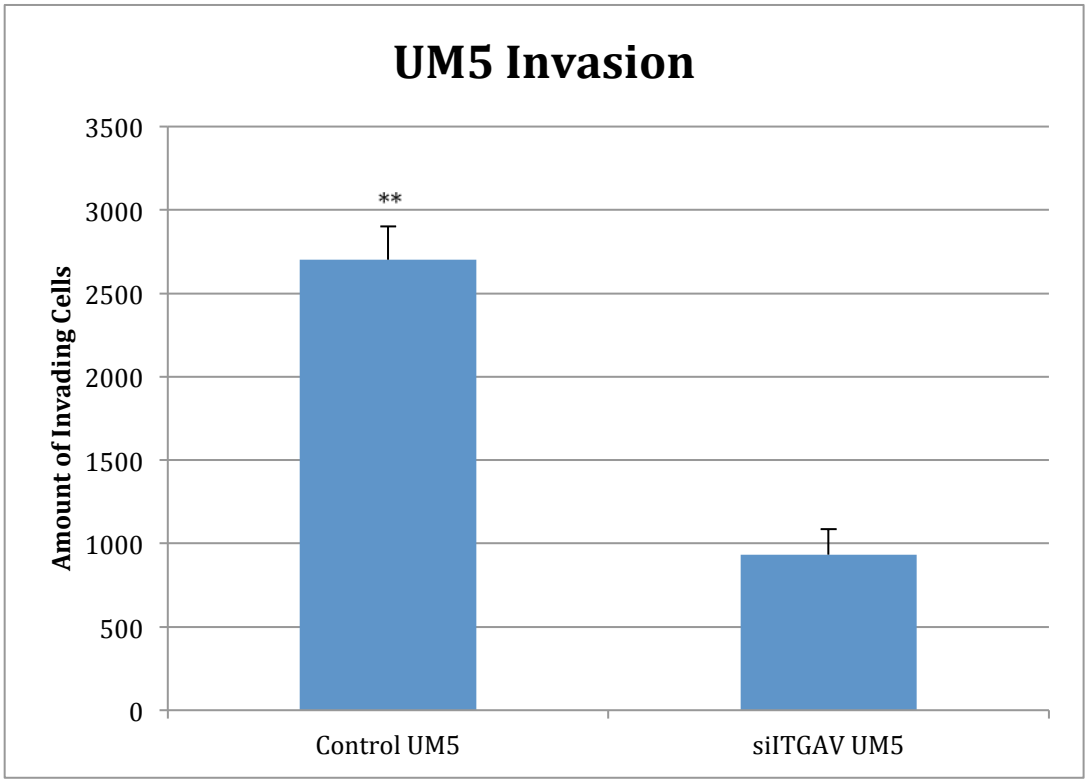
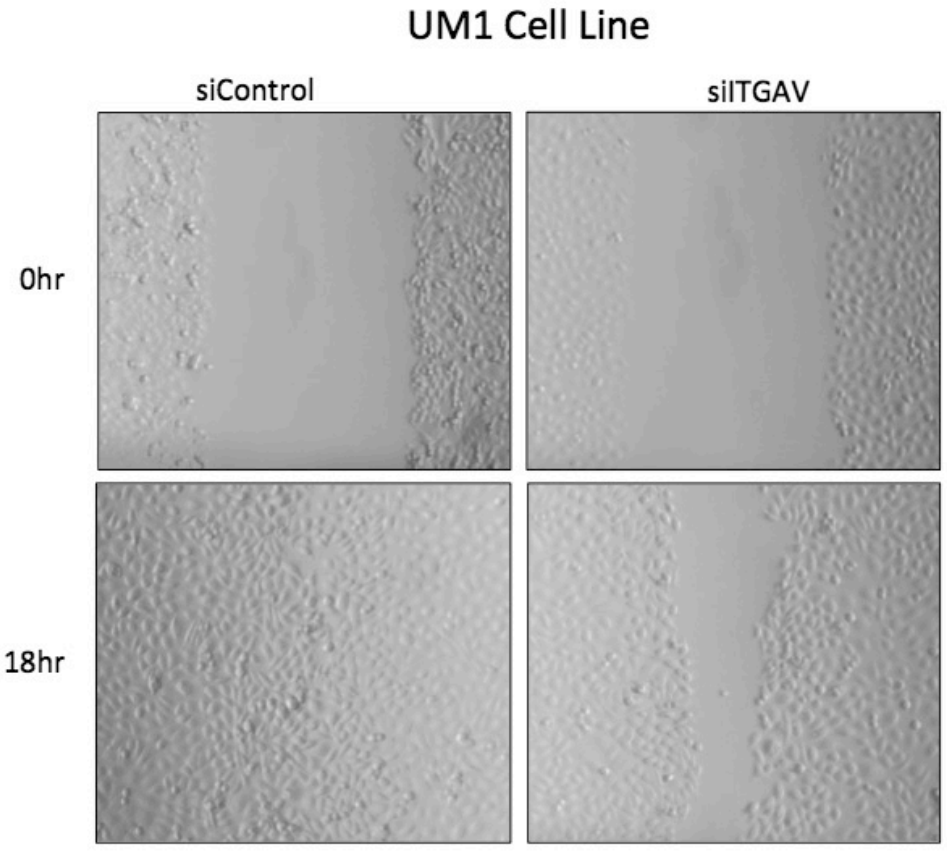
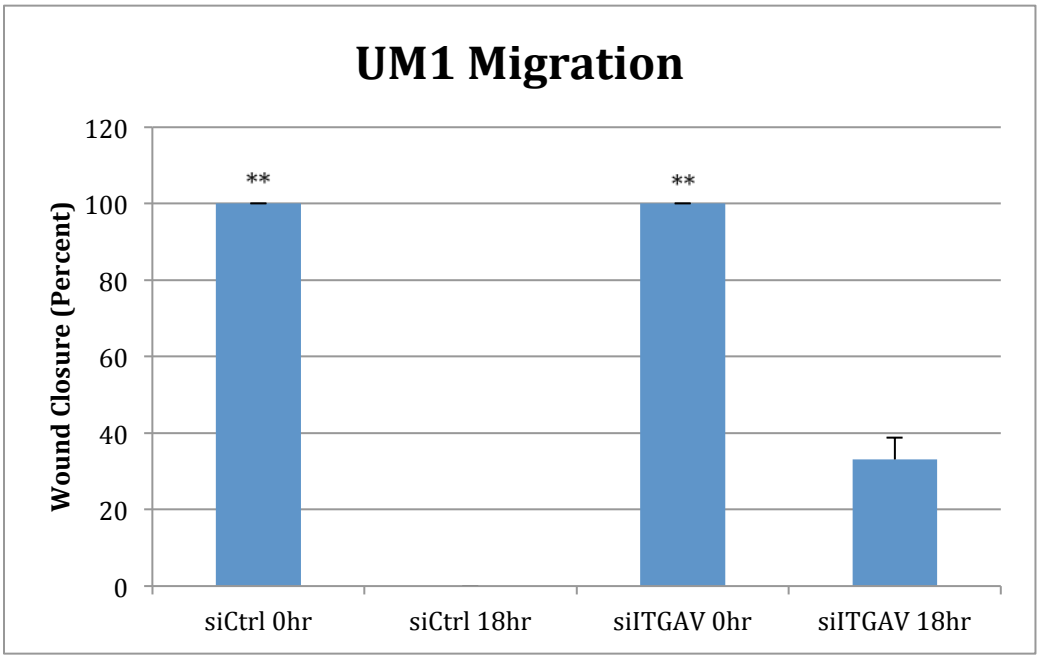


Figure 9. UM1 (A) and UM5 (B) invasion assays comparing control and knockdown ITGAV. After siRNA knockdown, UM1 showed a 68.8% (*p<0.05*) decrease in invasion potential and UM5 showed a 65.4% (*p<0.001*) reduction in invasion potential. (* indicates $p < 0.05$, ** indicates $p < 0.001$)

10A.)


UM1 Cell Line
siControl
siITGAV
0hr
18hr


UM1 Migration
Wound Closure (Percent)
120
100
80
60
40
20
0
**
**
siCtrl 0hr
siCtrl 18hr
siITGAV 0hr
siITGAV 18hr

10B.)

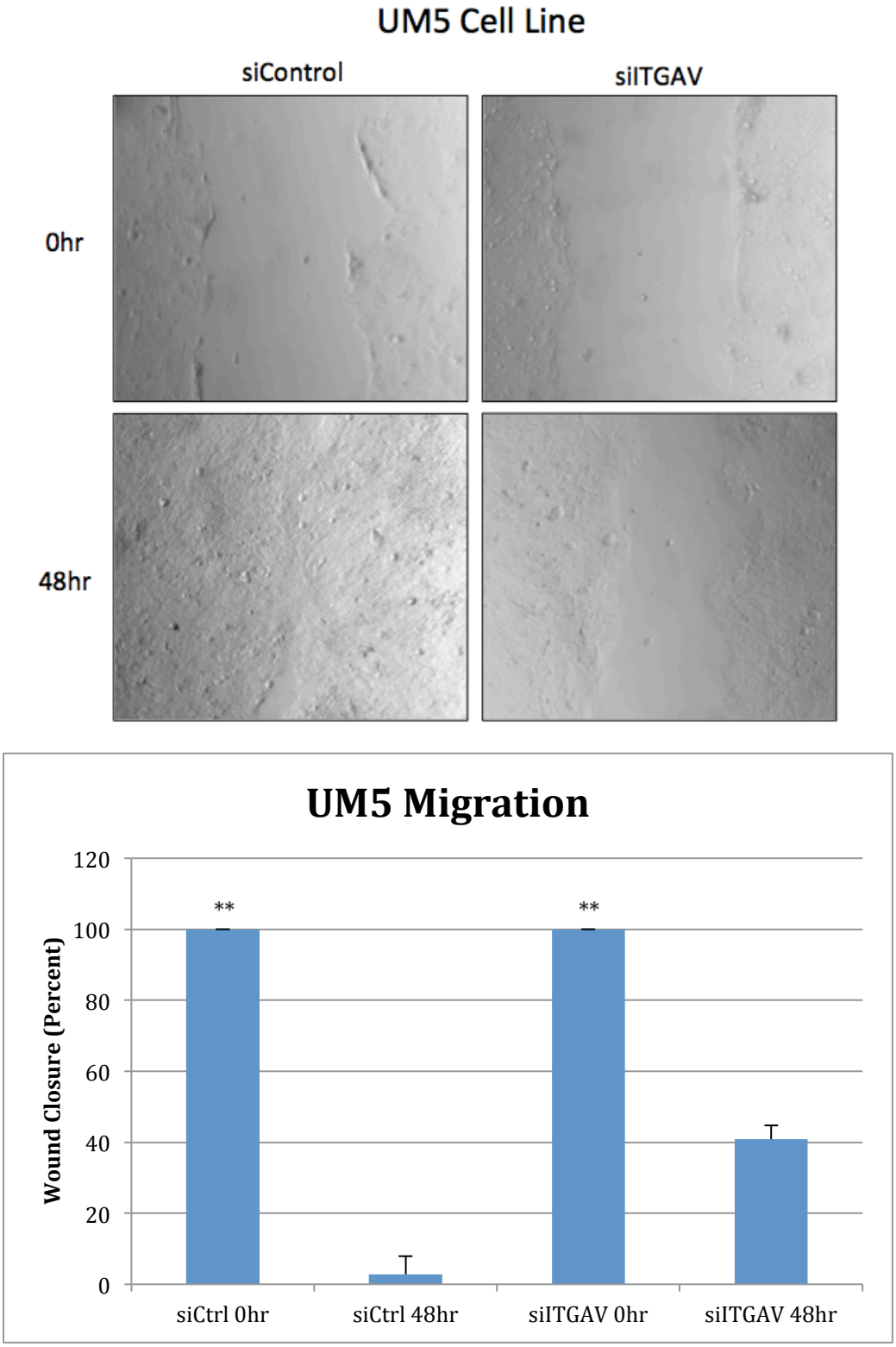
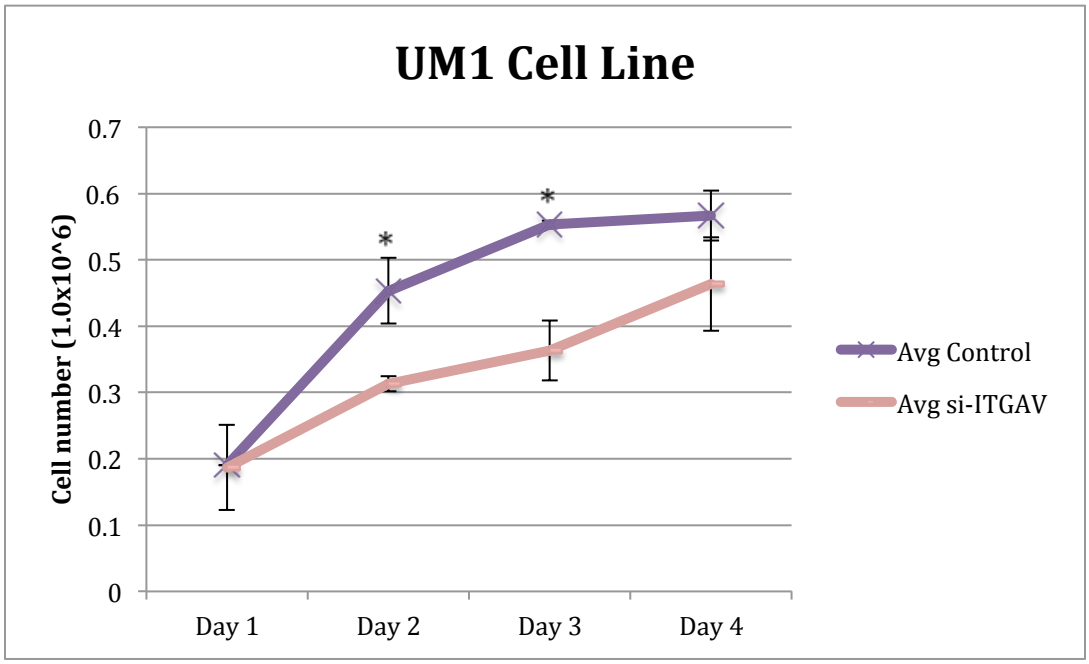


Figure 10. UM1 (A) and UM5 (B) migration assay comparing control and knockdown ITGAV. In UM1 and UM5, ITGAV knockdown resulted in a 33.1% and 40.8% residual wound after control wound closure (*p<0.001)*.
(* indicates $p < 0.05$, ** indicates $p < 0.001$)

11A.)



11B.)

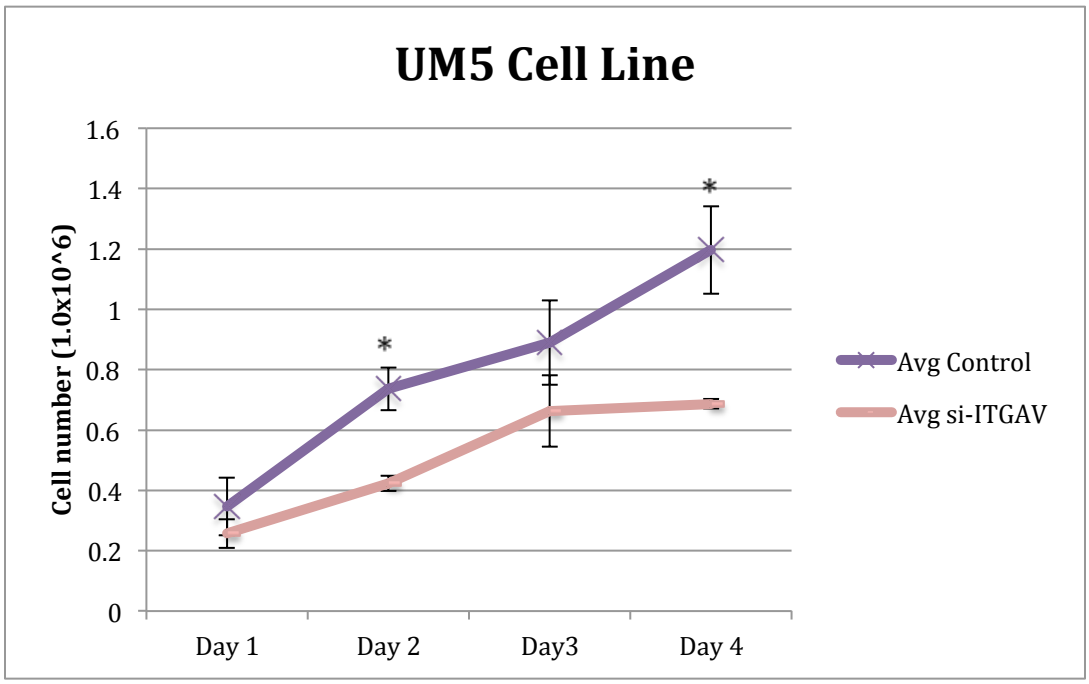


Figure 11. UM1 (A) and UM5 (B) proliferation assay comparing control and knockdown ITGAV. Overall trend shows that ITGAV knockdown has a decreased proliferation in UM1 and UM5 cell lines. UM1 has significant decrease on day 2 and day 3 (*p<0.05)* and UM5 shows significant decrease on day 2 and day 4 (*p<0.05).* (* indicates $p < 0.05$, ** indicates $p < 0.001$)

Table 1. List of primers of ITGAV used during qPCR.

Gene	Forward Primer	Reverse Primer
ITGAV Promoter 1	TGCCCTGCGAATCCTTTCTT	CGTGTTTCTGCTGCTTAGCC
ITGAV Promoter 2	GCCTTATTTCACCGGTGTGC	AAGGATTCGCAGGGCAAAGA

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