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Transforming Growth Factor- β 1 activates Np63/c-Myc to promote Oral Squamous cell carcinoma

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Abstract

Objective—During the development of oral squamous cell carcinoma (OSCC), the transformed epithelial cells undergo increased proliferation resulting in tumor growth and invasion. Interestingly, throughout all phases of differentiation and progression of OSCC, TGF β 1 induces cell cycle arrest/apoptosis, however; the role of TGF β 1 in promoting cancer cell proliferation has not been explored in detail. The purpose of this study was to identify the effect of TGF β 1 on OSCC cell proliferation.

Methods—Using both human OSCC samples and cell lines (UMSCC38 and UMSCC 11B), we employed biochemical experiments to show protein, mRNA, gene expression and protein-DNA interactions during OSCC progression.

Results—Our results showed that TGF β 1 increased OSCC cell proliferation by up-regulating the expression of Np63 and c-Myc oncogenes. While the basal OSCC cell proliferation is sustained by activating Np63, increased induction of c-Myc causes unregulated OSCC cell proliferation. Following induction of the cell cycle by Np63 and c-Myc, cancer cells that halt c-Myc activity undergo EMT/invasion while those that continue to express Np63/c-Myc undergo unlimited progression through the cell cycle.

Conclusion—We conclude that OSCC proliferation is manifested by the induction of c-Myc in response to TGF β 1 signaling, which is essential for OSCC growth. Our data highlights the potential role of TGF β 1 in the induction of cancer progression and invasion of OSCC.

Keywords

OSCC; TGF β ; Np63; c-Myc; cell cycle

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Introduction

The incidence of malignancies of the oral cavity is estimated at around 42,000 cases in the United States annually, most of which are oral squamous cell carcinomas (OSCC).¹ An increasing body of evidence demonstrates that Transforming Growth Factor β 1 (TGF β 1), acting in concert with its downstream effectors, plays a pivotal role in regulating the initiation and progression of OSCC. To prevent tumorigenesis, during the early stage of tumor development TGF β 1 acts as a tumor suppressor, relying on its ability to inhibit cell proliferation by inducing cell cycle G₁ arrest.² However, when compared to normal cells, most of the squamous carcinoma cells are either completely or partially defective in their physiologic response to inhibiting the TGF β 1 signaling mechanism. This is one of the key mechanisms that promotes the oncogenic development of epithelial-derived tumors.³

TGF β 1 functions in a variety of cellular processes including cell proliferation, differentiation, apoptosis, epithelial-mesenchyme transition (EMT)/invasion, matrix synthesis and immune responses.⁴ TGF β 1 initiates these processes by activating Smad-dependent and/or Smad-independent pathways, such as the phosphoinositide-3 kinase (PIK3) and MAPK signaling pathways.^{5–7} Paradoxically, TGF β 1 also functions as a key oncogenic promoter during the later stages of epithelial-derived tumors.^{8, 9} Its pro-survival function can be attributed to Smad-independent pathway effectors, such as PI3K and AKT pathways.^{10, 11}

One of the mechanisms by which TGF β 1 can stimulate differentiation is by inducing p63, located on chromosome 3q27–29. In fact, p63 was shown to be elevated in up to 80% of OSCC cases.¹² The p63 gene encodes two major protein isoforms, TAp63 and Np63, both of which have transactivating and transcriptional repressing activities regulating a wide range of target genes.¹³ In most basal epithelial cells, the predominant isoforms expressed at the protein level are of the Np63 type, and are believed to be master regulators of epithelial differentiation.¹⁴ Surprisingly, the most documented oncogene, c-Myc, can also be activated to induce cell proliferation which is mediated via the TGF β /Smad pathway in several malignancies^{15, 16} including OSCC.⁹

Characterization of the response to TGF β 1 in OSCC should be of clinical significance, as elucidating its role may contribute to our understanding with regards to how signaling pathways are switched, ultimately altering the course of OSCC differentiation. Based on our results, we conclude that while TGF β 1 has distinct cellular functions, including stimulation of OSCC cell survival/basal cell proliferation and epithelial mesenchymal transition (EMT) by Np63, the switch to upregulating the cell cycle, therefore supporting cancer growth, is mediated via the induction of c-Myc in UMSSC38 cells.

Material and Methods

Reagents and antibodies

All the antibodies and reagents were used according to the conditions recommended by the manufacturers/providers and confirmed by us previously.^{16–19} Sources are listed in Supplementary Table 3.

Tissues and Cell lines

Human normal oral gingival mucosa (control) and oral squamous cell carcinoma (OSCC) tissue samples were kindly provided by Dr. Peter J. Giannini (University of Nebraska Medical Center). The protocol for the use of human samples was reviewed and approved by the IRB (#113–15-NH) and conducted in full compliance with federal regulations and UNMC Institutional Review Board policies. 8µm sections of tumor tissue were hematoxylin and eosin (H&E) stained according to our established protocols.^{16–19} We utilized 10 (ten) samples of normal oral gingival mucosa (as control) as well each category of tumor differentiation stage (well, moderately and poorly-differentiated OSCC).

The human head and neck squamous cell carcinoma cell lines, we used 6 (six) oral squamous cell carcinoma cell lines established at the University of Michigan, UMSCC10B, UMSCC11B, UMSCC17B, UMSCC23, UMSCC38 and UMSCC74B (generously provided by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI). But only 2 cell lines (UMSCC38 and UMSCC11B) responded to TGFβ1, others did not demonstrate any cellular changes (either morphological, phenotypically or biochemical). Hence, we chose to focus strictly to the cell lines that responded to dissect the signaling pathway and limit our study to explore how TGFβ1 activates downstream signaling pathways to induce cellular changes (proliferation and EMT). We felt that it would be much more constructive to focus on these two cell lines and investigate its mechanisms in OSCC progression. UMSCC38 was obtained from the primary OSCC from the primary site of origin, whereas UMSCC11B was obtained from a secondary OSCC resulted from relapse and recurrence at the primary site. The nature and characteristics of these cell lines are described in Lin et al.²⁰ Cells were propagated in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO₂. Human oral/laryngeal SCC cell lines were obtained from Dr. Thomas Carey (University of Michigan, Ann Arbor, MI) in 2010 and 2011. These cell lines were previously characterized genetically and morphologically^{21–28} (15, 16), and have been retested and authenticated in the present study. Table 1 from this paper shows genotyping data obtained with 10 common tetranucleotide repeat sequences on 73 of the most commonly used UM-SCC head and neck cell lines. Representative photographs from each of the genetically characterized cell lines is illustrated in the paper as well as on the University of Michigan Head and Neck Cancer SPORE web page (<http://www.med.umich.edu/cancer/hn-spore/>) for easy access for other investigators who have these lines in their laboratory.

Immunofluorescence Analysis

8µm normal oral gingival mucosa and OSCC sections were immunofluorescence stained according to our established protocols.^{16–19} Sections were mounted with DAPI contained Vectashield mounting medium (Vector Laboratories), and imaged with the Nikon A1 confocal system (Nikon Instruments Inc., Melville, NY) on a Nikon 90i upright fluorescence microscope.

2.5×10^4 UMSCC 38 and 11B cells/well were cultured in 10% FBS/DMEM on glass coverslips until they reached 40~60% confluence. Cells were washed with sterilized PBS twice prior to being applied to a 48 h serum-free starvation (the synchronization) in DMEM, and then treated with TGFβ1 in 0.2% FBS DMEM. The subsequent fixation and staining of

cells were carried out as previously described by us.^{16–19} Stained cells were mounted with Vectashield mounting medium containing DAPI. All images were obtained on an inverted motorized microscope (Axiovert 200M, Zeiss) using SlideBook 5.0 image acquisition software (Intelligent Imaging Innovations Inc, Denver). Five microscopic fields were selected randomly (40× magnification), in which, both immunofluorescence and DAPI stained cells were counted manually as the number of positive stained cells and total cells respectively; the ratio of the positively stained cells was then calculated.

Cell proliferations and viability assays

Trypan Blue exclusion assay was undertaken to evaluate cell number using Trypan Blue Solution (Life Technologies, Grand Island, NY) manufacturer protocol. Briefly, UMSCC 38 and 11B cells were plated onto six-well cell culture plates at 2.5×10^4 UMSCC cells/well in 2mL of culture medium with FBS/DMEM (0.2% and/or 10%) with or without 2, 5 and 10ng/mL of recombinant TGFβ1 treatment for 24, 48 and 72 hours at 37°C. The cells were harvested by suspension in 0.4% solution of trypan blue in buffered isotonic salt solution, pH 7.2 to 7.3. Cell counts were performed in triplicates using hemocytometer, with Trypan blue exclusion to identify.

We also undertook both *MTT* and *Neutral Red (NR)* assays using manufacturer protocol as detailed in Vybrant® MTT cell proliferation assay kit and Neutral Red stain (Life Technologies, Grand Island, NY), both assays measure metabolic function/activity in mitochondria (MTT) and lysosomes (NR). Briefly, 2.5×10^4 UMSCC 38 and 11B cells underwent treatment with TGFβ1 (2, 5 and 10ng/mL) for 24, 48 and 72 h (with 0.2% and/or 10% FBS/DMEM). Treatments were terminated with the addition of NR stock solutions to the cells in 96-well microtitre plates for 4 h at 37°C. For NT, added solution was aspirated and wells were rinsed with 4% formaldehyde containing 1% calcium chloride, followed by 1ml of glacial acetic acid in 100ml of 50% ethanol for 15 min. For *MTT*, cell treatments were terminated with 10μL of MTT solution for 2 h at 37°C until the purple formazan crystal developed. For both, the comparative analysis of the absorbances were plotted at 550nm optical density, and for each treatment three replicates were examined.

Western Blot Analysis

UMSCC cells were cultured in 10% FBS/DMEM until they reached 50% confluence. Then, medium was replaced with FBS-free DMEM for a 48 h serum-starvation before TGFβ1 treatments. For protein expression of UMSCC cells by western blot, total protein was extracted. For nuclear and cytoplasmic proteins, we used the nuclear extraction kit from Chemicon Nuclear Extraction Kit (Millipore, MA) as performed previously.^{16–19} The concentrations of the nuclear, cytoplasmic and total proteins were obtained using NanoDrop 2000c spectrophotometer (Thermo Scientific, Rockford, IL). 30μg protein samples were electrophoresized in SDS-PAGE and probed by various antibodies according to the standard Western blot protocol.

Intensity of the band was measured using the Carestream Molecular Imaging Software version 5.3.1 (Rochester, NY). To perform a t-test analysis of mean intensity measurements, a ROI analysis was conducted from data converted to Microsoft Excel from the exported

“.txt” files. Data points for all samples are paired by spatial arrangement on gel and compared pairwise to minimize the impact of subtle background artifacts on image analysis.

Flow Cytometry Assay

2.5×10^4 UMSCC cells/well were cultured in 10% FBS medium until they reached 40~60% confluence in 6-well plates. After serum starvation, cells were cultured in 0.2% FBS/DMEM with recombinant TGF β 1. Cells without TGF β 1 treatment served as a negative control, while those grown in 10% FBS represented a positive control. Cells were then harvested and prepared for subsequent cell cycle analysis, including EdU incorporation and PI staining, which were detailed in our published protocols.^{16–18} Flow cytometry was conducted on a BD FACSArray Bioanalyzer (BD Biosciences, CA) and analyzed with FlowJo_V10 software.

Transwell cell migration assay

The cell migration assay was performed three times as described in Nawshad et al.²⁹ using Innocyte™ Cell Migration Assay (EMD Biosciences, CA). For the cell migration assay, UMSCC38 and 11B cell lines were treated with TGF β 1 (5ng/mL) for 24 and 48 h. The endogenous c-Myc and Smad4 were blocked 24 h prior to TGF β 1 and continued for an additional 24 and 48 h in the presence of TGF β 1, with media changed every 24 h. PI3 Kinase was blocked 60 min prior to TGF β 1 treatment. Briefly, 8 μ m pore size transwell migration chambers in 96-well plates were used for migration analyses. Both treated and untreated cells were allowed to migrate across the membrane insert toward media in the presence of serum for 48 h at 37°C (chemotactic migration). Cells that migrated through the membrane and attached to the lower side of the cell culture insert (each/per field) were subsequently detached using a cell detachment buffer containing Calcein-AM fluorescent dye (excitation max.: 485nm; emission max.: 520nm). The data were obtained using a BD Bioanalyzer™ fluorescent plate reader (BD Biosciences, CA). The mean of 10 areas was determined and represented in Fig. 5D.

Cell Treatment

UMSCC38 and 11B cells were treated with, *a*) TGF β 1 (2, 5 and 10 ng/mL) for 6, 12, 24, 36 and 48 h; *b*) 0.2% FBS/DMEM, negative ‘untreated’ (UnTr) control and *c*) 10% FBS/DMEM, ‘positive’ control. *d*) Inhibition (with short hairpin RNA, and small synthetic chemical inhibitor) and activation (with full-length cDNA) as confirmed by us previously.^{16–19}

To inhibit Np63, c-Myc and Smad4 (Smad dependent pathway), cells were both transiently transfected with small hairpin RNA (shRNA) for Np63 (pRetrosuper-sh- Np63), c-Myc (pRetrosuper-sh-c-Myc) and Smad4 (pRetrosuper-sh-Smad4) (Addgene, MA), for 24 h by themselves followed by an additional 24 h in the presence of TGF β 1 treatment, which specifically targets the coding region of murine Np63, c-Myc and Smad4. To activate, we transfected cells with Np63 and c-Myc, full-length Np63 (pCMV-Entry-p63; Origene, MD) and c-Myc cDNAs (pcDNA3.3 c-Myc; Addgene, MA) for 24 h by themselves followed by an additional 24 h with TGF β 1. The pRetrosuper-sh- Np63, pRetrosuper-sh-c-Myc, pRetrosuper-shSmad4 and pCMV-Entry-p63, pcDNA3.3 c-Myc were transfected into both

cells by gently adding solution containing 10 μ l of Lipofectamine 2000 in 300 μ l of Opti-MEM medium for 24 h, as described previously.^{16, 18} The Smad-independent pathway via PI3 kinase was blocked using commercially available small synthetic chemical inhibitors, LY294002 (20 μ M) (Cell Signaling, MA) for 60 min. Following all inhibitions (pRetrosuper-shSmad4 and chemical inhibitors), TGF β 1 (5 ng/mL) was added in the presence of these molecules for another 24 h prior to mRNA extraction. All inhibitors/activators were used at the concentration and time points recommended by the respective manufacturer/provider as well as confirmed by us previously.^{16–19} Media changed every 24 h. Details of these plasmids/constructs are described in Supplementary Table 4.

qRT-PCR

As described previously,^{16, 18} UMSCC38 and 11B cells were serum starved for 48 h, followed by treatment with TGF β 1 (5 ng/mL) for additional 24 h. RNA was harvested using the RNeasy Mini Kit (Qiagen, CA) according to the manufacturer's instructions. RNA integrity was assessed using formaldehyde gels in 1 $\times$ TAE buffer, and RNA purity and concentration were determined by 260/280 ratio on a Nanodrop 2000C (Thermoscientific, MA). The sequences of primers for cyclins, c-Myc and GAPDH (detailed in Supplementary Table 1,2) were obtained from the Invitrogen online PCR primer design site, and were synthesized at the Molecular Biology Core Facility, UNMC. Gene expression was determined by normalization with the control gene, GAPDH. Each RT-PCR experiment was performed in triplicate.

Transfection and luciferase assay

The TGF β -mediated effect on Np63 and c-Myc promoter activity was measured through firefly luciferase assays in both cell lines as previously shown by us.^{16, 18} UMSCC38 and 11B cells were transiently transfected with Np63 promoter: Np63 promoter region (pGL3-2k Np63; -1848 to +152) generously provided by Dr. Iyoto Katoh (University of Yamanashi, Japan). We also employed several deletions/mutations of Smad binding sites on the c-Myc promoter as detailed in Supplementary Table 4. UMSCC38 and 11B cells were transiently transfected with pBV-Luc plasmid harboring the c-Myc promoter (2500bp; pBV-Luc-Myc-WT plasmid) or derivative plasmids containing mutations in TBE1 (pBV-Luc-TBE1mut) or TBE2 (pBV-Luc-TBE2mut) or both sites (pBV-Luc-TBE1/2mut) [obtained from Addgene (Cambridge, MA)]. Additionally, we used c-Myc promoter region deletion constructs: pBV-Luc-DEL1 (WT, with intact TBE1, TBE2 and TIE sites), pBV-Luc-DEL3 (without TBE1, but with intact TBE2 and TIE), pBV-Luc-DEL4 (without TBE1 and TBE2 but with intact TIE) also obtained from Addgene. All these plasmids have been used by us previously and details of c-Myc luciferase protocol have been reported previously.^{16–18} For the pGL3-2k Np63 luciferase assay we followed the protocol provided by Dr. Katoh.³⁰ Lipofectamine 2000 transfection reagent (Invitrogen, CA) was used according to the manufacturer's protocol. Transfection of UMSCC38 and UMSCC11B cells with pGL3-empty vector and pBV-Luc vector without the c-Myc promoter region (empty vectors) were performed as negative controls. Recombinant TGF β 1 (5ng/mL) was added to the transfected cells for 24 h, and luciferase activity was detected using the Sirius luminometer (Berthold Detection Systems, NC). Results are reported as mean $\pm$ SD of at least three independent experiments.

Chromatin immunoprecipitation (ChIP) assay

As described previously,^{16, 18} equal quantities (4×10^7 cells) of the untreated control (0.2% FBS), TGF β 1 (5 ng/mL) stimulated cells in 80%–90% monolayers were fixed with 1% formaldehyde for 10 min at RT for chromatin cross-linking. The reaction was stopped by adding glycine to a final concentration of 0.125M and the cells were immediately washed twice with ice-cold PBS and harvested by scraping in ice-cold 1×PBS+PMSF. The Simple ChIP™ Enzymatic Chromatin IP kit (Magnetic beads) (Cell Signaling, MA) was used according to the method successfully used by us previously.^{16, 18} Briefly, cross-linked chromatin was isolated from the lysates by sonication 3 times for 6 seconds (s) each, using the Microson XL 2000 sonicator. Immune precipitation of the cross-linked chromatin with rabbit anti-Smad4 antibody (Cell Signaling, MA) was subsequently performed. For negative control, rabbit IgG (Cell Signaling, MA) and for positive control, Histone H3 antibody were used. The 100 μ l chromatin samples in binding buffer from the Chromatin IP kit (Cell Signaling, MA), containing 15 μ g chromatin DNA were incubated overnight with antibodies at 4°C. The subsequent binding with magnetic beads, immunocomplex washing and DNA extraction were carried out following the manufacturer's protocol. Purified DNA was analyzed by PCR with primers specific for Smad4-binding elements in the c-Myc promoter. The primer design is detailed in Supplementary Table 2.

Resulting DNA fragments were detected by electrophoresis in 1.5% agarose gel. To quantitatively analyze Smad4 binding with three potential Smad binding sites in c-Myc promoter following TGF β 1 treatment, real-time PCR analysis of DNA, purified from the immunocomplexes with anti-Smad4 antibody, was performed. DNA samples were subjected to quantitation by real-time PCR using RealMasterMix Sybr Rox (5Prime, Hamburg, Germany). Reactions were performed in a 20 μ l volume according to the manufacturer's recommendations. The mean $\pm$ SD obtained from three independent experiments were compared.

Statistical Analysis

Data from at least three replicates for each parameter were evaluated and analyzed for significance by SPSS 14.0 and one-way ANOVA was conducted. Quantitative RT-PCR data were analyzed by two-way ANOVA (OriginPro v. 8.0) and Student's *t* test (Microsoft Excel). The significance level was set as 0.05. A p-value of ≤ 0.05 was considered significant.

Results

Characterization of proliferation status of various grades of OSCCs

To assess the actively cycling status of OSCC; PCNA, Ki67, cyclin A, p63 and TGF β 1 were immunofluorescence stained and compared against E-Cadherin (Fig 1, green) to demonstrate the epithelial architecture, in addition to cytokeratin 14 (Supplementary Figure S2, green) to reveal that the malignant epithelial tissue in the ECM is of basal epithelial origin.

In gingival mucosa, PCNA expression was confined to cell nuclei within the basal, parabasal and suprabasal layers (Fig 1, red, a), Ki67 was limited to mostly basal and few parabasal epithelial nuclei (Fig 1, red, e), cyclin A expressed in epithelial cells and extra cellular matrix (ECM) (Fig 1, red, i), while Np63 revealed nuclear positivity in basal and parabasal keratinocytes (Fig 1, red, m). Their expression showed a very similar pattern in well- and moderately-differentiated OSCC; demonstrating positivity in basal, parabasal and/or suprabasal layers of the tumor nests. However, their expression decreased in cells toward the center of the tumor nest (Fig 1, red, b, c, f, g, j, k, n, o). Nevertheless, in the tumor nest of poorly-differentiated OSCC, where the cancer cells lost their epithelial features, the markers were expressed in only a small portion of cancer cells (Fig 1, red, d, h, l, p), showing a significantly decreased expression profile. TGFβ1 revealed both nuclear and cytoplasmic expression in the epithelium as well as in the ECM (Fig 1, red, q–t).

In well- and moderately-differentiated OSCC, E-cadherin (green) expression was strictly confined within the epithelial membrane of the squamous layers. However, in poorly-differentiated OSCC, its expression was noticeably reduced, demonstrating a discontinuous pattern (Fig 1, green, d, h, l, p and t), while cancer cells dispersed throughout the full thickness of the tumor nest revealed loss of characteristic epithelial features. Our results suggest that in early stage OSCC there is preservation of normal epithelial architecture and morphology, which however, were lost as oral carcinomas progressed to the later stages (Fig. 1 a, e, i, m and q). The expression pattern of these proteins was similar, indicating a generalized high-proliferative activity in the epithelia. Cytokeratin14 showed strong cytoplasmic expression in all epithelial cells, further confirming that the epithelial cells invading in the ECM originate from the squamous epithelial lining (Fig S2).

TGFβ1 effects on the population of UMSCC cells expressing proliferation proteins

UMSCC cells were assessed for their cell proliferation status by evaluating PCNA, Ki67, cyclin E2 and Np63 in response to TGFβ1 treatment. Our results demonstrate that TGFβ1 exerts both a dose- and time-dependent inductive effect on the numbers of cells positively stained with these key proliferation effectors in both cell lines up until 24 h (Fig 2A, B). However, unlike the increased cell proliferation at 48 h in UMSCC38 (Fig 2A, f–h, 2C), UMSCC11B resulted in two distinctive observations: (1) the numbers of UMSCC11B cells positively stained decreased sharply; (2) most UMSCC11B cells lost their cobblestone morphology, developing an elongated, fibroblast-like morphology, implying that they were undergoing EMT (Fig 2B, f–h). These data indicate that TGFβ1 is a facilitator of cell proliferation in UMSCC38, but to a lesser degree in UMSCC11B, inducing EMT-like changes instead.

The number of cells expressing those proteins in both cell lines was plotted in Fig 2C, confirming the protein expression findings and pattern in Fig 2A and B. The actual numerical value is shown in Supplementary Table 5.

TGFβ1 effects on DNA synthesis rate of UMSCC cell lines

In order to investigate the role of TGFβ1 in UMSCC cell cycle and proliferation, we used the EdU incorporation assay to assess the ability of TGFβ1 to promote DNA synthesis in those cell lines. Only EdU positive cells in S phase were shown in Fig 3A–B.

For UMSCC38 cells, uptake of EdU, indicative of new DNA synthesis and cell proliferation increased in a time- and dose-dependent fashion by TGFβ1 treatment when compared with untreated cells. Only 4.43% of untreated UMSCC38 cells were incorporated with EdU, but for positive control groups of 6, 12, 24, 36 and 48 h, the percentages were 19.0, 30.2, 43.7, 53.4 and 75.1, respectively. All TGFβ1 treated groups differed significantly from control groups, while between the positive controls at different time points, the difference was also significant. For 2 ng/mL TGFβ1 treated cells, percentages of cells incorporated with EdU were 8.76, 13.23, 13.7, 15.5 and 23.2 for 6, 12, 24, 36 and 48 h. And the percentages were 12.2, 17.9, 21.1, 23.7 and 31.8 for 5 ng/mL TGFβ1 treated cells, while for 10 ng/mL TGFβ1 groups, 9.39, 13.7, 18.7, 20.6 and 24.3, respectively.

Our data demonstrate that the percentage of EdU incorporation increased upon TGFβ1 treatment in a dose- and time-dependent manner. It is obvious that the percentage of S phase cells increase chronologically. And among TGFβ1 treatment groups, 5 ng/mL TGFβ1 had the greatest effect on DNA synthesis in UMSCC38 cells (Fig 3A, ii). Similarly, our data also confirmed that UMSCC 38 cells are progressively proliferating as detected by Trypan blue assay, clearly demonstrating an increase in cell counts by the TGFβ1 treatment groups (Fig 3A, iii). The treated cells were viable as confirmed by MTT/NT ratio (Fig. 3A, iv).

However, for UMSCC11B cells, TGFβ1 treatment did not stimulate DNA synthesis progression in a similar manner as that for UMSCC38 cells. 3.33% of untreated cells were in S phase. Whereas, 10.4%, 17.4%, 30.6%, 38% and 42% of positive control UMSCC11B cells were EdU positive for 6, 12, 24, 36 and 48 h, separately. As to cells treated with TGFβ1 for 6, 12 and 24 h, there were 7.42%, 6.44%, 5.0% of cells positive for EdU in 2 ng/mL TGFβ1 treatment group; 8.37%, 6.41% and 5.87% of EdU positive cells in 5 ng/mL groups; and 8.21%, 8.54% and 4.63% of cells were in S phase in 10 ng/mL group. However, when cells were treated for a longer time, the portion of S phase cells decreased sharply. The percentages of EdU positive cells in 2, 5 and 10 ng/mL TGFβ1 treatment groups were 4.79, 2.39 and 4.05 at 36 h; and 3.74, 3.34, 9.25 at 48 h, similar to what was observed with untreated cells. All TGFβ1 treatment groups differed significantly from positive control groups, while amongst them, the difference was also significant at different time point. Similarly, UMSCC38 cells treated with 5 ng/mL TGFβ1 showed the greatest effect when compared with the other groups. In agreement with EdU incorporation results, our data confirmed that unlike UMSCC 38 cells, UMSCC 11B cells were not progressively proliferating beyond 24 h as detected by the Trypan blue assay, clearly demonstrating an absence of persistent growth as indicated by cell counts obtained from different TGFβ1 treatment groups (Fig 3B, iii) following 24 h. However, these treated cells were viable as confirmed by MTT/NT ratio (Fig. 3B, iv) after 24 h.

The seemingly unintuitive results can be attributed to the dichotomous nature of the TGFβ1 cytokine, which is known to exert multiple cellular outcomes depending upon dose and time.

Our results suggest that TGF β 1 promotes DNA synthesis in UMSCC38 cells and acts as a potent oncogenic factor (Fig 3A). In UMSCC11B cells, TGF β 1 treatment resulted in a negligible increase in EdU incorporation when compared to controls. TGF β 1 seemed to moderately promote DNA synthesis in the initial 24 h only, after which, it lost its pro-proliferative effect for prolonged treatments beyond 24 h (Fig 3B). These data suggest that UMSCC11B cells demonstrate significantly less proliferative activity and are largely refractory to the pro-proliferative TGF β 1 signaling when compared to their counterpart UMSCC38 cell.

The effect of TGF β 1 on cell cycle progression in UMSCC cell lines

To demonstrate the effect of TGF β 1 on distribution of cell cycle phases, synchronized UMSCC38 and 11B cells were measured for DNA content with propidium iodide (PI).

TGF β 1 treatment induced progression through the cell cycle of UMSCC38 cells, and the induction was time-dependent. The percentages of untreated cells in G₀/G₁, S and G₂/M phase were 86.3, 5.41 and 5.81, respectively. However, for positive control cells, there were 46.5%, 43.7% and 8.01% of cells in three phases at 24 h, while at 48 h, 17%, 75.1% and 2.35% of cells were in these phases. The distribution of cell cycle stages of TGF β 1 treatment groups differed significantly from control groups. Also, there was a significant difference in the cell cycle stages among all treated groups. Cells in G₀/G₁ phase decreased obviously when treated with TGF β 1; 78.3%, 73.7%, 66.8%, 65.7% and 62.5%, respectively, for 5 ng/mL TGF β 1 treated for 6, 12, 24, 36 and 48 h. Whereas, cells in S phase increased significantly chronologically, supported by 12.2%, 17.9%, 21.1%, 23.7% and 31.8% at 6, 12, 24, 36 and 48 h; and those in G₂/M phase were 6.33%, 6.81%, 8.79%, 6.95% and 7.61%, separately.

For UMSCC11B cells, TGF β 1 treatment did not induce progression through the cell cycle. The percentages of untreated UMSCC11B cells in G₀/G₁, S and G₂/M phase were 79.2, 3.33 and 3.0, respectively. All cell cycle stages of treated groups differed significantly from positive controls. For positive control cells, there were 51.1%, 30.6% and 15.5% of cells in these phase at 24 h, while at 48 h, 39%, 42% and 13.4% of cells were in G₀/G₁, S and G₂/M phase. However, there was no significant difference on the percentage of cells in G₀/G₁ and G₂/M phase among different TGF β 1 treatment groups. As to cells treated with 5 ng/mL TGF β 1 for 6, 12, 24, 36 and 48 h, there were 77.5%, 78.8%, 81.5%, 86.1% and 81.8% of cells in G₀/G₁ phase; 8.37%, 6.41%, 5.87%, 2.39% and 3.34% of cells in S phase; and 11.5%, 12.3%, 10.0%, 8.65% and 9.67% of cells in G₂/M phase. After TGF β 1 treated for 6, 12 and 24 h, the amount of cells in S and G₂/M phase increased significantly as compared with untreated cells. Whereas, for cells treated for 36 and 48 h, the ratio of S phase cells decreased obviously, showing no significant difference with that of untreated groups.

Our results suggest that TGF β 1 induces progression of successive cell cycles and is a fundamental cytokine that propagates UMSCC38 cell proliferation (Fig 3C). Unlike UMSCC38, UMSCC11B cell cycle progression was not enhanced by TGF β 1 treatment and no significant change was observed when compared to the untreated cells (Fig 3D, ii). These results suggest that while TGF β 1 is a potent inducer of the cell cycle in UMSCC38 cells as late as 48h, it cannot stimulate progression of successive cell cycles in UMSCC11B cells.

This may be due to the fact that at this stage UMSCC11B cells are programmed for cellular differentiation, such as EMT, during which persistence of the cell cycle will negatively affect such phenotypical changes. These UMSCC cell lines revealed a different degree of proliferation, and FACSArray data revealed no cell death by PI in the sub-G₁ population segment where it shows dead cell/debris.

TGFβ1 activate both Smad-dependent and Smad-independent pathways in UMSCC cells

To evaluate the pathways TGFβ1 uses to induce cell cycle progression, protein expression by western blot analysis of phosphor-Smad, phospho-AKT, and Np63 were undertaken. Cells were treated with TGFβ1 (2, 5 and 10 ng/mL) for 30 min for phospho-Smad2, AKT proteins and 24 h for Np63 detection.

Phosphorylation of Smad2 is a clear indication of an active and functional Smad pathway in the UMSCC38 but in limited fashion in the UMSCC11B cells. Our results indicate that unlike UMSCC11B, TGFβ1 activates the Smad-dependent signaling pathway in UMSCC38 cells. However, the results (Fig. 4A) also showed increased phosphorylation level of pAKT, by TGFβ1 treatments in the UMSCC11B, unlike UMSCC38 cells, indicating that PI3 kinase was activated by TGFβ1 in UMSCC11B. Intriguingly, the level of Np63 protein increased in response to TGFβ1 treatments equally in both cell lines. These data suggest that activation of the Smad-independent pathway, PI3 kinase (AKT), is regulated by TGFβ1 in the UMSCC11B cells while UMSCC38 cells predominately use the Smad pathway. The current results support the notion that TGFβ1 stimulates both Smad-dependent and independent pathways in UMSCC38 and 11B autonomously (Fig. 4A).

Induction of cyclins by TGFβ1 in the UMSCC cells

Complete assessment of cyclin mRNA levels show that the mRNA levels of cyclin A and B were higher than cyclin D and E in both cell lines. The data support the hypothesis that TGFβ1 induces the cell cycle by increasing mRNA levels of cyclins, with cyclin mRNA levels falling sharply after 24 h in UMSCC 11B cells, unlike in the UMSCC38 cells, which continue to escalate all cyclin mRNA levels (Fig 4C). These results indicate that cell cycle status of UMSCC38 and UMSCC11B are uniquely different and UMSCC11B cease to proliferate after 24 h of TGFβ1 treatment. Since cyclin D is the only member of the cyclin protein family involved in regulating cell cycle progression in all phases of the cell cycle, we investigated whether Np63 and c-Myc are interdependent in activating cyclin D, therefore, cell cycle. We showed that induction of c-Myc and Np63 (by cDNA) resulted in an increase in cyclin D mRNA levels in UMSCC38 cells, but with very limited effect on UMSCC11B cells. However, such effect on cyclin D was significantly increased with c-Myc induction, when compared to Np63. Similarly, absence of Np63 and c-Myc (by shRNA) reduced mRNA levels of cyclin D with pronounced effect by c-Myc inhibition compared to Np63 (Fig 4D). These outcomes of inhibition and activation of Np63 and c-Myc had a more noticeable effect on UMSCC38 cells while having a restricted influence on UMSCC11B cell lines. These data suggest that enhanced induction of the cell cycle in UMSCC38 cells is regulated by both Np63 and c-Myc, but the basal level cell cycle seen in UMSCC11B cells is due to its failure to induce c-Myc significantly.

TGFβ1 activates Np63 gene activity

Interestingly, the effect on Np63 gene activity as measured by luciferase 2K Np63 promoter region was more balanced in both UMSCC38 and 11B cell lines. Although gene activity was lower in the UMSCC11B cell line, the trend of increased Np63 gene activity was symmetrical (Fig 4E). However, blocking both Smad4 and PI3K significantly reduced the Np63 gene activity, indicating that both UMSCC38 and 11B utilize Smad dependent and Smad independent pathways respectively to stimulate Np63, which seems to be required for inducing basal cell cycle and survival.

c-Myc promoter activity and Smad binding elements

From earlier studies by us¹⁶ and others,¹⁵ it has been shown that c-Myc has several potential Smad Binding Elements (SBEs); namely TBE1, TBE2 and TIE. A schematic diagram of the 2.5kb c-Myc promoter showing the three potential SBEs is shown in Fig 5A. The transfected cells were treated with TGFβ1 (5 ng/mL) for 24 h, and luciferase assays demonstrated increased transactivation of the c-Myc promoter in response to TGFβ1 (pBV-Luc-DEL1-WT). Since the SBEs are functional and important in the activation of c-Myc promoter, as shown in Fig 5B, we wanted to identify if these SBEs harbor Smad proteins on the c-Myc promoter. We performed a chromatin immunoprecipitation (ChIP) assay to determine if Smad4 was bound to the SBEs (TBE1, TBE2 and TIE). Our data suggests that while TGFβ1 induces increased c-Myc promoter activity in the UMSCC38 cell lines, UMSCC11B had limited c-Myc promoter activity (Fig 5C). Both functional SBEs (TBE1 and 2) are necessary to induce optimum c-Myc gene activity since mutations of these (TBE 1 and 2) regions drastically reduced the effect of TGFβ1 on c-Myc promoter activity (Fig 5B). These data, combined with the observation that mutation of TBE1 and 2 decreased luciferase expression by the greatest amount (Fig 5B), suggest that TBE1 and 2 are the most important regions in the c-Myc promoter for Smad4 mediated transactivation induced by TGFβ1 signaling in UMSCC38 cells, but not in UMSCC11B cells.

TGFβ1 facilitates cell migration in UMSCC11B but not in UMSCC38 cells

Based on the data showing a dramatic inhibition of the cell cycle in UMSCC11B cells after 24 h of TGFβ1 (Fig. 4C), as well as limited c-Myc promoter activity and no Smad binding to c-Myc promoter (Fig. 5B and C), unlike in UMSCC38 cells, we explored the role of TGFβ1 on cell migration in both cell lines. We undertook a transwell cell migration assay (Fig 5D) at 24 and 48 h of TGFβ1 treatment using a combination of treatment conditions (blocking c-Myc, Smad4 and PI3K). The results demonstrate that TGFβ1 causes increased cell migration in UMSCC11B when compared to UMSCC38 cell lines. Furthermore, the addition of TGFβ1 for up to 48 h results in a dramatic increase in UMSCC11B cell migration. In addition, UMSCC11B cell migration can be halted significantly when PI3 kinase is blocked.

Discussion

Oral squamous cell carcinoma (OSCC) is the most common intraoral malignancy often with a dismal outcome.³¹³² Defining the exact signal cascades that may be implicated in various epithelial-derived carcinomas cells is essential for developing effective targeted therapeutic

regimens. In this study, using different grades of OSCC and primary (UMSCC38) and secondary tumor (UMSCC11B) cell lines, we investigated the role of TGF β 1 in squamous cell proliferation and differentiation. We demonstrated that TGF β 1 had a more pronounced effect in UMSCC38 cell proliferation than UMSCC11B. We additionally demonstrated that TGF β 1 activated both Smad-dependent and Smad-independent pathways in UMSCC38 and 11B cells lines, respectively, to induce cell survival/basal cell proliferation via Np63. Our results demonstrate that TGF β 1 is capable of facilitating OSCC cell proliferation via

Np63/c-Myc working in tandem in the initial phase, while in the later phase, TGF β 1 induces EMT in the absence of c-Myc. This dual role is not unique, as TGF β 1 is known to stimulate multiple cellular functions and phenotypes based on dose, time and the microenvironment. It is important to point out that, these UMSCC cell lines are extremely heterogeneous (origin, etiology, genetic alterations, location, degree/phase of cancer progression and secondary metastasis varies from one cell line to the other) like most cancer cell lines and they are destined to act differently by TGF β 1 treatments. And genetic drift over time in cultured cell lines are bound to show diverse cellular changes using established cell lines. Because of the critical diversity in native and in vitro nature of UMSCC cell lines as model systems, it is more than likely that they ought to behave different under the same treatment condition. Therefore, here, we emphasize more into each measurement/ test accurately determines important variation in our techniques for two UMSCC cell lines in response to TGF β 1 in a time and dose dependent manner establishing validity, reliability, and replicability achieving appropriate levels of statistical power and sensitivity. And demonstrate that it is quite plausible for TGF β 1 to cause EMT in one cancer cell line and proliferation in another and the purpose of this paper is to the mechanism of TGF β 1 signaling that causes these two separate cellular phenomenon, which not rare but a typical response to those two cell lines.

The nuclear localization of PCNA and Ki67 is an indication of cell proliferation during the late G₁ and S phases of the cell cycle.³³ First, we established that the degree of differentiation and progression of OSCC and UMSCC cell lines were directly related to the chronological progression of the cell cycle, in addition to increased expression of well-established cell proliferation proteins including PCNA, Ki67, cyclins and Np63. Interestingly, TGF β 1 was co-expressed in the same cells that also harbor the different cell proliferation proteins present at different stages of cancer progression and differentiation, indicating the decisive role that TGF β 1 plays in regulating cancer cell cycle and proliferation involving Np63. The uniform expression of E-cadherin in early stages and significant decreased expression in the later stages attests to the loss of cell-cell adhesion, therefore, promoting EMT in poorly-differentiated squamous cell carcinomas. Our data suggest when comparing UMSCC38 and 11B cells, UMSCC38 cells tend to maintain increased cell proliferation in response to TGF β 1. Conversely, UMSCC11B cells cease their upregulation of the cell cycle beyond 24 h, ultimately undergoing EMT at 48 h.

We demonstrated that while TGF β 1 stimulated the cell cycle via Np63 and c-Myc in varying degrees in UMSCC38 and 11B cell lines, UMSCC38 cells were affected to a greater extent. Our results demonstrate that TGF β 1 has an effect on the UMSCC38 cell cycle that is in accordance with the chronological progression through different phases of the cell cycle, however, such induction is limited in UMSCC11B cells. Moreover, termination of the cell

cycle in the UMSCC11B beyond 24 h of TGF β 1 induction implies that UMSCC11B cells might undergo cellular cytoskeletal rearrangement for migratory phenotype during which cessation of the cell cycle is very common.³⁴

It is well established that TGF β 1 can induce signals using either Smad-dependent or Smad-independent pathways, or both.⁴ However, while Smad2/Smad4 is a functional regulator of the Smad pathway in UMSCC38 cells lines, UMSCC11B cell lines independently utilize the PI3K/AKT pathway. Given the uniqueness of UMSCC38 and UMSCC11B cells in terms of their cell cycle response to TGF β 1, utilization of separate pathways by UMSCC38 and 11B would appear to be expected. This divergence of signaling mechanisms may be the deciding factor for continuation of the cell cycle in UMSCC38 as opposed to cessation in UMSCC11B cells. These combined results indicate that TGF β 1 initiates signals via both Smad-dependent and Smad-independent pathways in UMSCC38 and 11B cells respectively.

Np63 is preferentially expressed in proliferative keratinocytes.³⁵ Its expression is restricted to the nuclei of cells in the basal and parabasal layers of the stratified squamous epithelium, suggesting its involvement in the development and maintenance of epithelial structures;³⁶ and a regulatory role in epithelial proliferation and differentiation.³⁷ Accordingly, our

Np63 luciferase assay data demonstrates that TGF β 1 signals via both Smad-dependent and Smad-independent pathways in UMSCC38 and 11B cell lines, respectively, induces Np63 gene activity. An increased in Np63 expression was detected in up to 80% of primary OSCC tumors.¹²³⁸ However, since Np63 expression falls in UMSCC11B cells at 48 h in response to TGF β 1, this may be indicative of that they are undergoing EMT. This switch, as seen in UMSCC11B cellular behavior and phenotype, facilitates invasion at this stage of cancer progression. This is in contrast to the Np63 dependent pro-proliferation, a pro-survival pathway that seems to exist in invasive SCCs. The signaling mechanisms that trigger the shift in UMSCC11B but not in UMSCC38 cells may be the result of different mechanisms in Smad-dependent and Smad-independent pathways. Moreover, as shown in the UMSCC38 cells, enhanced Np63 expression appeared to be a prerequisite for well-differentiated OSCCs to maintain their noninvasive phenotype.³⁰ The above findings suggest that loss of Np63 confers the invasive behavior that is observed in poorly differentiated OSCCs, as Np63 is a well-known anti-metastatic protein.³⁹

It has been well established that c-Myc is an oncogene whose role in cell proliferation has been demonstrated in various systems.^{40–43} While we demonstrated that TGF β 1 is capable of inducing cell cycle progression, we also showed that UMSCC38 cell proliferation is achieved by the induction of c-Myc gene activity, which requires functional Smad signaling. However, such signaling is limited in UMSCC11B cells, therefore, downregulating the cell cycle as well. Our data also indicate that TGF β 1 mediates its effect on c-Myc via binding of Smad to the TBE1 and 2 elements, particularly in UMSCC38 cells, but not in the UMSCC11B cells. In addition, we demonstrated that cessation of a robust cell cycle (via cyclins/c-Myc in UMSCC11B), resulted after 24 h of TGF β 1 treatment, and that only UMSCC11B cells undergo EMT, but not the UMSCC38 cells. Interestingly, the signaling pathway that TGF β 1 utilizes to induce EMT in the UMSCC11B cells is via non-Smad, PI3K/AKT pathways, while UMSCC38 cells maintain Smad pathways to activate c-Myc-dependent cell proliferation. TGF β 1 promotes the growth, progression and migration of

established tumors.⁴⁴ Furthermore, TGFβ1 is known to promote the transcription of Snail and SIP-1, two repressors of E-cadherin expression, thereby diminishing E-cadherin and inducing EMT, which is critical for cancer cell motility, invasion and metastasis.⁴⁵

To correlate the degree of differentiation *in vitro* (consistent with most studies and generally accepted protocol), we demonstrated that oral cancer cell lines undergo cell proliferation and transformation (which are phases of differentiation *in vivo*) in relation to changes in cell proliferation and EMT proteins in response to TGFβ1. Overall our results demonstrate that induction of the cell cycle is a key phase that takes place during OSCC progression and tumor growth, and is dependent on c-Myc induction. Based on our solid data, we postulated that (Fig 6): (a) TGFβ/Smads/ Np63/c-Myc pathway remains active in the noninvasive primary but higher proliferative attributes of OSCCs, particularly UMSCC38 and (b) TGFβ/PI3K/AKT/ Np63 pathway is functional (in the secondary tumor that resulted from relapse and recurrence at the primary site) at the inception stage of invasive OSCC from the secondary, as seen in the UMSCC11B cell lines. We further conclude that while TGFβ1 has distinct functions in cancer progression, its downstream signaling partners and transcription factors regulate ultimate cancer cell fate and promote the transition from dysplasia to invasive squamous cell carcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations and acronyms

TGFβ	Transforming Growth Factor β
Np63	Truncated-p63
EdU	5-Ethynyl-2'-deoxyuridine
PCNA	Proliferating Cell Nuclear Antigen
PI3K	Phosphoinositide-3 Kinase
SBE	Smad Binding Element
OSCC	Oral Squamous Cell Carcinoma
EMT	epithelial mesenchymal transition

TBE	TCF-binding element
TIE	TGFβ1 inhibitory element
TβRI/II	TGFβ receptor type I/II

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Statement of Clinical Relevance

Our results imply that OSCC progression depends on active TGF β 1 signaling and we propose that restricting TGF β 1 signaling and/or its down stream partner c-myc may have important clinical and pharmaceutical significance.

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Fig. 1. Different grades of OSCC express PCNA, Ki67, cyclin A, Np63 and TGFβ1 in comparison to E-cadherin

(a,e,i,m) Proteins expression in normal oral gingival mucosa (red): PCNA, Ki67 and Np63 revealed nuclear positivity staining in epithelia cells, while cyclin A expressed in both the epithelial cells and ECM. **(b,c,f,g,j,k,n,o) Proteins expression in well- and moderately-differentiated OSCC (red):** All of them showed a very similar pattern as they were in normal oral gingival mucosa. But their expression decreased in the cells toward the center of the tumor nest. **(d,h,l,p) Proteins expression in poorly-differentiated OSCC (red):** In the tumor nest, these proteins expressed only in a small portion of cancer cells (red), showing a significantly decreased expression fashion except for Ki67. **(q-t) TGFβ1 expression in OSCC (red):** TGFβ1 showed both nuclear and cytoplasmic staining in the epithelium as well as in the ECM (red). **(at) E-cadherin expression in OSCC (green):** E-cadherin expression was strictly within the epithelial membrane of the squamous layers, except in poorly-differentiated OSCC, it was reduced and discontinuous (green, d, h, l, p and

t). In all slides, nuclei were stained with DAPI (blue). Scale Bar at 400µm (p) for low magnification; 100µm (p, inset) for higher magnification.

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Fig 2A

Fig 2B

Fig 2C

Fig. 2.

(A) TGFβ1 regulates PCNA, Ki67, cyclin E2, Np63 and E-cadherin expression in UMSSC38 cells

Column a). PCNA, Ki67, cyclin E2 and Np63 revealed negative staining in untreated UMSSC38 cells. **Column b).** All the cells grown in 10% FBS/DMEM (positive control) were nuclear positive stained by PCNA, Ki67, cyclin E2 or Np63. **Column c–h).** PCNA, Ki67, cyclin E2 and Np63 expression in TGFβ1 treatment cells. The number of nuclei positively stained cells increased in a time and dose dependent manner, compared with untreated groups. Furthermore, as indicated by E-cadherin staining along the cell membrane (red), the cobblestone morphology was preserved in all treated UMSSC38 cells.

(B) TGFβ1 regulates PCNA, Ki67, cyclin E2, Np63 and E-cadherin expression in UMSSC11B cells.

Column a–b). PCNA, Ki67, cyclin E2 and Np63 expressed very similarly as that in UMSSC38 untreated and positive control cells. **Column c–e).** 24 h TGFβ1 treatment

increased the number and intensity of PCNA, Ki67, cyclin E2 and Np63 (green) expression in UMSCC11B cells compared to untreated control groups. Also the expression pattern of the epithelial marker, E-cadherin (red), was concentrated on the cell membrane, forming a continuous membranous, similar as that in the untreated cells. **Column f–h**). When treated with TGFβ1 for 48 h, the number of positive cells decreased significantly, and the membranous expression of E-cadherin (red) was reduced and interrupted thereafter. (C). Percentage of immunofluorescence positively stained UMSCC38 and UMSCC11B cells.

For both UMSCC38 and 11B cells, only very limited untreated cells express PCNA, Ki67, cyclin E2 or Np63. **(a–d) Percentage of positively stained UMSCC38 cells for PCNA (a), Np63 (b), Ki67 (c) and cyclin E2 (d)**. Over 90.0 percent of UMSCC38 cells grown in 10% FBS were positively stained for these proteins. When cells were treated with TGFβ1 for 24 h, the percentages increased as the dilutions of TGFβ1 were raised. At 48 h, the percentages raised to very similar level with that of positive control (10% FBS) groups. **(e–h) Percentage of positively stained UMSCC11B cells for PCNA (e), Np63 (f), Ki67 (g) and cyclin E2 (h)**. Almost 95.0 percent of cells grown in 10% FBS were positive stained for PCNA, Np63, Ki67 and cyclin E2. When cells were treated with TGFβ1 for 24 h, the percentages increased obviously as the dilutions of TGFβ1 were raised. But at 48 h, the percentages of positively stained cells reduced obviously, very similar as that of untreated cells.

Fig 3A

Fig 3B

Fig 3C

(i)

(ii)

Fig 3D

(i)

(ii)

Fig. 3. (A) The effect of TGFβ1 on DNA synthesis in UMSSC38 cells

(i) Using BD Biosciences FACSArray, a significant proportion of cells were found to occupy distinct cell cycle phases including G₀/G₁, S, and G₂/M within the selected boxed region. Larger boxed region identifies all proliferating cells as determined by their levels of DNA synthesis as assessed by PI within the G₀/G₁, S and G₂/M phases of the cycle. The smaller inset box includes the cells that are incorporated with EdU or S phase cells. Only results of 5 ng/mL TGFβ1 treatment groups and control groups were shown in this figure.

(ii) In the untreated (0.2% FBS) control group, few untreated UMSCC38 cells were incorporated with EdU. The percentages were raised in a time-dependent manner when cells were grown in 10% FBS (positive control). All the TGFβ1 treatment groups differed significantly from the control groups, while between the treated cells at different time point, the difference was also significant. And among TGFβ1 treatment groups, 5 ng/mL TGFβ1 had the greatest effect on DNA synthesis in UMSCC38 cells. A p-value of 0.05 was considered significant (as indicated by *). The one-way ANOVA indicated that the values differ significantly across the treatment groups. Here, we only show the EdU positive cells in S phase.

(iii) In response to TGFβ1, UMSCC38 cells showed progressive increased in the number of cells in the span of 0–72h when compared to negative control, untreated cells as determined by healthy log phase culture with 0.4% Trypan blue stain. All the TGFβ1 treatment groups differed significantly from the control groups, while between the treated cells at different time point, the difference was also significant. And among TGFβ1 treatment groups, 5 ng/mL TGFβ1 had the greatest effect on cell proliferation as determined by cell number in UMSCC38 cells. A p-value 0.05 and 0.005 were considered significant (as indicated by * and ** respectively). The one-way ANOVA indicated that the values differ significantly across the treatment groups.

(iv) Similarly, both MTT (mitochondria) and NT (lysosome) metabolic function that determines cell viability and proliferation shown to be increased progressively in UMSCC38 cells in 0–72h when compared to negative control, untreated cells, in response to TGFβ1. And all the TGFβ1 treatment groups differed significantly from the control groups, while between the treated cells at different time point, the difference was also significant. A p-value 0.05 and 0.005 were considered significant (as indicated by * and ** respectively). The one-way ANOVA indicated that the values differ significantly across the treatment groups.

(B) The effect of TGFβ1 on DNA synthesis in UMSCC11B cells.

(i) In UMSCC11B cells, TGFβ1 treated for 6, 12 and 24 h increased EdU incorporation compared to untreated group, but thereafter, EdU incorporation was reduced and there was no significant difference between them and untreated control groups. Only results of 5 ng/mL TGFβ1 treatment groups and control groups were shown in this figure.

(ii) For untreated cells (0.2% FBS), few were in S phase. Whereas, 10.4%–42.0% UMSCC11B cells were EdU positive when cultured in 10% FBS (positive control) for 6–48 h. However, when cells were treated with TGFβ1, at the very beginning, the portion of S phase cells increased to some extent, but when treated for longer time, this portion decreased sharply, showing no significant difference from untreated cells. All the TGFβ1 treatment groups differed significantly from positive controls, while between the positive controls, the difference was also significant. Also, cells treated with 5 ng/mL of TGFβ1 showed the greatest effect, compared with 2 and 10 ng/mL TGFβ1 treatment groups. A p-value of 0.05 was considered significant (as indicated by *), and p-value 0.005 was indicated by **.

(iii) In response to TGFβ1, UMSCC 11B cells showed increased in the number of cells in the first 24 h followed by cessation of cell proliferation, when compared to negative control, untreated cells as determined by opacity density of MTT/NT. But the progression ceased following 24 h of TGFβ1 treatment. The treatment groups differed significantly from the control groups, while between the treated cells at different time point, the difference was

also significant. A p-value 0.05 and 0.005 were considered significant (as indicated by * and ** respectively). The one-way ANOVA indicated that the values differ significantly across the treatment groups.

(iv) MTT/NT assays show increased in cell viability by all the TGFβ1 treatment groups that differed significantly from the control groups, while between the treated cells at different time point, the difference was also significant. A p-value 0.05 and 0.005 were considered significant (as indicated by * and ** respectively). The one-way ANOVA indicated that the values differ significantly across the treatment groups.

(C) Induction of cell cycle progression by TGFβ1 in UMSCC38 cells.

(i). DNA content and cell cycle distribution of UMSCC38 cells treated with TGFβ1. Only results of 5 ng/mL TGFβ1 treated cells and control groups were shown. Cells cultured in 10% FBS for 24 and 48 h were set as positive control. TGFβ1 treatment for 6, 12, 24, 36 and 48 h enhanced UMSCC38 cell cycle progression, as shown by the reduction in the number of G₀/G₁ phase cells, with a subsequent increase in the number of cells in S and G₂/M compared with the untreated control group (0.2% FBS).

(ii). The percentage of UMSCC38 cells in each phase of cell cycle. Only data from 5 ng/mL TGFβ1 treatment groups and control groups were shown in this Figure. TGFβ1 treatment induced progression through the cell cycle, and the induction was time-dependent. Also, there was significant difference on the cell cycle stages among all the TGFβ1 treatment groups. The one-way ANOVA indicated that the values differed significantly across the treatment groups. A p-value of 0.05 was considered significant (as indicated by *), while p-value 0.005 was indicated by **.

(D) Induction of cell cycle progression by TGFβ1 in UMSCC11B cells.

(i) DNA content and cell cycle distribution of UMSCC11B cells treated with TGFβ1 (5 ng/mL) for 0, 6, 12, 24, 36 and 48 h were assessed by PI staining. Only the data of cells treated with 5 ng/mL TGFβ1 and the control groups were shown in this figure. TGFβ1 treatment did not enhance UMSCC11B cell cycle progression, as shown by no obvious changes in the number of cells in different phases, compared with the untreated groups (0.2% FBS).

(ii) The percentage of UMSCC11B cells in each phase of the cell cycle. TGFβ1 treatment did not induce progression through the cell cycle. The percentages of UMSCC11B cells in each cell cycle stage for TGFβ1 treatment groups differed significantly from the positive control (10% FBS for 24 and 48 h) groups. However, there was no significant difference on the percentage of cells in G₀/G₁ and G₂/M phase among different TGFβ1 treatment groups. After TGFβ1 treated for 6, 12 and 24 h, the amount of cells in S and G₂/M phase increased significantly as compared with those in untreated groups. Whereas, for cells treated for 36 and 48 h, the ratio of S phase cells decreased obviously, similar as that of untreated groups. The one-way ANOVA indicated that the values had no significant difference among the treatment groups. A p-value of 0.05 was considered significant (as indicated by *).

Fig 4

Fig 4

(D)

(E)

Fig. 4. Pathways used by TGFβ1 during UMSCC cell proliferation

(A) TGFβ1 uses both Smad dependent and Smad independent pathways during UMSCC cell proliferation. pSmad2 was expressed in UMSCC38 cells but the expression

was indifferent with TGFβ1 treatment of different dosages, however, its expression in the UMSCC11B was negligible in 2 and 5 ng/mL treatments and very low in 10 ng/mL treatment. Subsequently, western blot analysis of phosphorylated proteins was performed to determine the effects of TGFβ1 on Smad-independent pathways. pAKT showed no expression in UMSCC38 cells with any doses of TGFβ1 treatments. UMSCC11B showed similarly increased expression of pAKT with no difference in the TGFβ1 treatment

conditions. Np63 expression in both UMSCC38 and 11B were comparable and showed increased expression in a dose dependent manner in comparison to control (actin).

(B) All three doses of TGF β 1 treatment groups differed significantly amongst themselves and in between two cell lines ($p < 0.05$, as indicated by *) from the untreated control cells (0.2% FBS) ($p < 0.005$, as indicated by **). Results from the blots (Fig 4A) and the intensity of the bands were measured using the Carestream Molecular Imaging Software version 5.3.1 (Rochester, NY). To perform a t-test analysis of mean intensity measurements, a ROI analysis was done from the data to Microsoft Excel software. Data points for all samples are paired by spatial arrangement on gel and compared pairwise to minimize the impact of subtle background artifacts on image analysis.

(C) Regulation of cyclin mRNA by TGF β 1. Cyclin D, E, A and B mRNA expressions were determined by real-time PCR in TGF β 1 (5 ng/mL) treated UMSCC38 and 11B cells every 12 h for 48 h. TGF β 1 treated cells had higher cyclins (D, E, A and B) mRNA expression levels compared to the untreated control (0.2% FBS). When compared to UMSCC11B, TGF β 1 had chronologically higher cyclins (D, E, A and B) mRNA levels in UMSCC38 cells in a time dependent fashion till 48 h. However, the levels sharply dropped after 24 h in the UMSCC11B cell lines and continued to decline until 48 h.

(D) Regulation of cyclin D mRNA by TGF β 1 in UMSCC cells: The levels of cyclin D mRNAs in response to TGF β 1, when Np63 and c-Myc are either activated (by full-length cDNA) or repressed (by pRetrosuper-shRNA), was measured. To induce cyclins, TGF β 1 functions on both Np63 and c-Myc, as activation and repression of Np63 and c-Myc significantly affects cyclin D mRNA levels. The change in mRNA levels was determined by comparison to untreated control (UnTr, 0.2% FBS) and plotted as fold change/s (mean $\pm$ s.d.; n=3; * $P < 0.05$ compared with controls; ** $P < 0.005$ compared with TGF β 1 treatments).

(E) TGF β 1 stimulates Np63 gene activity in UMSCC cells: TGF β 1 increased Np63 promoter activity chronologically starting at 6 h (5 and 6 fold in UMSCC38 and 11B respectively) and reaching the peak at 24 h (103 and 98 fold in UMSCC38 and 11B respectively). However, when both Smad and Smad independent pathways were blocked,

Np63 promoter activity was repressed in both UMSCC38 and 11B cell lines. The results are shown as a mean $\pm$ SD obtained from three independent chromatin preparations ($p < 0.05$ as indicated by * and $p < 0.005$ as indicated by **).

Fig 5
(A)

(B)

Fig 5

(C)

(D)

Fig. 5. TGFβ1 induces c-Myc gene activity by stimulating Smad promoter binding

(A) Schematic diagram of the human c-Myc promoter: ~2.5 kb, based on previous studies^{15,16}, showing three potential Smad Binding Elements (SBE): TBE1 (between -359 and -329 bp), TBE2 (between -95 and 46bps) and TIE. Based on these findings, we predicted that Smad-dependent transcriptional regulation is mediated by these SBEs, which might suggest that c-Myc is a direct down-stream TGFβ1 target.

(B) Smad binding elements are essential for c-Myc gene activity: TGFβ1 treatment resulted in an approximate 130-fold increase of luciferase activity in cells transfected by pBV-Luc-DEL1-WT compared with an empty vector (control) in UMSCC38 cells, whereas c-Myc gene activity increased by approximately 25 folds in UMSSCC11B cells, attesting that TGFβ1 is more potent in activating the c-Myc promoter in UMSCC38 cells compared to 11B cells. However, in response to TGFβ1 transactivation, deletions of the SBE (TBE1) in the c-Myc promoter (pBV-Luc-DEL3) decreased the promoter activity proportionately in both UMSCC38 and 11B but the levels were dramatically reduced when both TBE1 and TBE2 were deleted (pBV-Luc-DEL4). When the TBE1 region was mutated (pBV-Luc-TBE1 mut) and with TBE2 (pBV-Luc-TBE1/2 mut), c-Myc gene activity was reduced significantly

in both UMSCC38 and 11B cell lines. Background luciferase activity (empty vector) was subtracted from all data. Error bars indicate standard deviation of three independent preparations. The results are shown as a mean $\pm$ SD obtained from three luciferase values (p 0.05 as indicated by * and p 0.005 as indicated by **).

(C) Smad, activated by TGF β 1, binds to the c-Myc promoter: The RT-PCR analysis displayed that Smad4 is strongly bound to TBE 1 and 2 sites in UMSCC38 cells, instead of TIE site. On the contrary, UMSCC11B did not show any significant binding of Smad4 to any of the SBEs of c-Myc promoter in response to TGF β 1. Moreover, in UMSCC38 cells, amongst the three binding sites, TBE2 showed highest binding to Smad4. The specificity of the bindings was confirmed by the positive (Histone H3) and negative (Rabbit IgG) controls (data not shown). The results are shown as a mean $\pm$ SD obtained from three independent chromatin preparations (p 0.05 as indicated by * and p 0.005 as indicated by **). Statistically significant differences between TGF β 1 treated or untreated (0.5% FBS) samples (p 0.005 as indicated by **) were observed. For Input control for quantitative real-time of immunoprecipitated chromatin, chromatin was isolated and analyzed by quantitative PCR with primers recognizing TBE1, TBE2 and TIE Smad4 binding elements in c-Myc promoter with DNA standards described in B.

(D) TGF β 1 induces UMSCC11B migration via PI3 Kinase: Results show that only a few UMSCC38 cells migrated at 24 and 48 h compared to untreated control. But UMSCC11B cells, at the same time points, showed a significant number of cells migrated to the lower side of the upper chambers following TGF β 1 treatment, with the most migration observed at 48 h. TGF β 1 treatments (24 and 48 h) in both groups differed significantly from their respective untreated control (UnTr) groups. The results are shown as a mean $\pm$ SD obtained from three independent values (p 0.05 as indicated by * and p 0.005 as indicated by **).

Fig. 6. Schematic diagram of the proposed mechanism of OSCC proliferation and invasion
 Based on our data, we hypothesized that in the initial stage, UMSCC cell proliferation (both in the primary, UMSCC38 and secondary tumor, UMSCC11B cell lines) is achieved by TGFβ1/Smads/ Np63/c-Myc pathway with higher proliferative attributes in UMSCC38. However, subsequently TGFβ1 switches it's signaling via PI3K/AKT/ Np63 pathway at the inception stage for EMT/invasive, primarily in the secondary tumor, as seen in the UMSCC11B cell lines (that resulted from relapse and recurrence at the primary site) from. We further conclude that while TGFβ1 has distinct functions in cancer progression, its downstream signaling partners and transcription factors regulate ultimate cancer cell fate and achieve switch from tumor growth to invasion.