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TGF-β1 mediates epithelial to mesenchymal transition via the TGF-β/Smad pathway in squamous cell carcinoma of the head and neck

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Abstract. Development of metastasis is a major cause of death for squamous cell carcinoma of the head and neck (SCCHN) patients. Epithelial to mesenchymal transition (EMT) is now regarded as a correlate of tumor metastasis. Given that transforming growth factor-β1 (TGF-β1) is an important inducer of EMT, we examined the effects of TGF-β1 on the human SCCHN cell line Tu686. We found that TGF-β1 mediated cell morphological changes. Phase-contrast microscopy revealed a loss of the adherent phenotype with cellular elongation, decrease in cell-to-cell contact, and the induction of a fibroblast-like state. Western blotting and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis demonstrated that TGF-β1 could induce down-regulation of the epithelial marker E-cadherin and up-regulation of the mesenchymal marker vimentin in Tu686 cells in a concentration- and time-dependent manner. Wound-healing and transwell invasion assay indicated that TGF-β1 promoted Tu686 cell migration and invasion dramatically. In addition, these changes were mediated via canonical TGF-β/Smad signaling with concomitant up-regulation of phosphorylated Smad2. Smad2 RNAi abrogated both expression and functional effects of TGF-β1 on Tu686 cells. In conclusion, the present study demonstrates that TGF-β1 could induce EMT in the SCCHN cell line via the TGF-β/Smad signaling pathway. More importantly, a cell model for EMT was established, which is valuable for future studies on the metastasis of SCCHN.

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Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common cancer worldwide, which represents a significant public health problem (1). Development of metastasis, including local lymph node or distant metastasis, is a major obstacle to the successful treatment of these patients. The 5-year survival rate is less than 50% for SCCHN patients with lymph node metastasis and around 20% for patients with distant metastasis (2). Thus, the inhibition of invasion and metastasis is of great importance in SCCHN therapies and prognosis.

Metastatic spread of tumor cells is a complicated, multi-step process, in which tumor cells detach from primary tumor, invade into surrounding tissue, intravasate into blood or lymphatic vessels, disseminate in blood stream or lymphatic system, and finally extravasate and outgrow at secondary site (3,4). Many factors have been shown to be involved in the formation of invasion and metastasis, such as loss of proliferative control, architectural changes, extracellular matrix degration and angiogenesis. However, the exact mechanisms of tumor metastasis are not fully understood. Epithelial to mesenchymal transition (EMT), where cells undergo a developmental switch from a polarized epithelial phenotype to a highly motile mesenchymal phenotype, has been considered to be an essential process during embryonic development, fibrosis and cancer progression (5,6). Recently, it is been in the limelight for investigating the onset of cancer cell migration, invasion and metastatic dissemination (7,8). In particular, it has been suggested that EMT is critical for the metastasis of epithelial-oriented tumors, including SCCHN (9). Despite these reports of a number of signals and molecules underlying the initiation and execution of EMT, the exact mechanism of EMT remains elusive. Thus, deciphering the cellular processes and critical signaling network that regulate EMT will have a strong impact in unravelling the mechanisms of tumor metastasis.

Transforming growth factor-β1 (TGF-β1), an effective and ubiquitous mediator of cell proliferation, differentiation and apoptosis, is a potent growth inhibitor and apoptosis inducer for most normal cells. However, there is increasing evidence

that in the later stage of cancer development TGF-β1 not only lose the antiproliferation effects, but contributes to cell growth, invasion and metastasis (10). A large body of work demonstrate that TGF-β1 can initiate and maintain EMT in a variety of biological systems and pathophysiological contex by activating major signaling pathways (11). Several signaling pathways have been implicated in the process of TGF-β1-mediated-EMT, including the Smads. It has been shown that TGF-β1 can mediate EMT by inducing tyrosine phosphorylation of Smad2 and Smad3 with subsequent recruitment of Smad4 (11,12). The capacity of TGF-β1 to induce EMT of carcinoma was previously reported in various cell models such as alveolar epithelial cell line A549 and esophageal adenocarcinoma cell lines (13,14). Nonetheless, there are scarce data on the possibility that TGF-β1 can induce EMT by activating Smad2 in SCCHN cell line. In order to develop a cell model system for studying the molecular basis of EMT in SCCHN and further understanding the metastatic mechanism in this malignant tumor, we investigated the effects of TGF-β1 on SCCHN cell line Tu686. Significant changes in cell morphology, EMT marker protein E-cadherin, vimentin and Smad2, phosphorylated Smad2 expression, migration and invasive capacity were observed in Tu686 cells in response to TGF-β1 treatment.

Materials and methods

Cell culture and TGF-β1 treatment conditions. The SCCHN cell line Tu686 was established from a primary tumor in the base of the tongue and was kindly provided by Dr Zhuo Chen (Emory University Winship Cancer Institute, Atlanta, GA) (15). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (1:1) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 IU/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2.

Recombinant human transforming growth factor-β1 (TGF-β1) (Prepro Tech EC, USA) was prepared in PBS containing 2 mg/ml bovine serum albumin (BSA) at 50 μg/ml. Cells were starved 24 h prior to treatment, then TGF-β1 was added to the cultures to final concentrations of 0, 0.05, 0.5, 5 and 10 ng/ml in serum-free DMEM/F12 medium. Cell morphology was monitored with an inverted phase-contrast microscope (Leica, Germany) equipped with a video camera.

MTT assay. Tu686 cells were plated in 96-well plates at $3x10^3$ per well with complete medium and allowed to adhere to the plate overnight. The cells were then starved for 24 h with serum-free medium, followed by incubation in the presence of various concentrations of TGF-β1 (0-10 ng/ml) for another 48 h at 37˚C in 5% CO_2. At the end of treatment, 20 μl MTT (Sigma) with 5 mg/ml concentration was added to the medium and cultured for another 4 h. Then, the medium was discarded and 150 μl DMSO was added into each well, rocking for 10 min, and the absorbencies of each well were read using a microplate reader at a wavelength of 490 nm. Each condition was performed with 5 wells and each experiment was repeated three times.

RT-PCR. Total RNA of Tu686 cells was isolated by Simply P Total RNA Extraction Kit (Bioer Technology Co. Ltd., China). Total RNA (1 μg) was reverse transcribed by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA) according to manufacturer's instructions. According to Zhau *et al* (16), primers were synthesized by Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). The primers for E-cadherin and vimentin rRNA were as follows: E-cadherin forward, 5'-TCCATTTCTTGGTC TACGCC-3' and reverse, 5'-CACCTTCAGCCAACCTGTTT-3'; Vimentin forward, 5'-TGGCACGTCTTGACCTTGAA-3' and reverse, 5'-GGTCATCGTGATGCTGAGAA-3'. As an internal control, the primers for GAPDH were: forward, 5'-GTCAGTGGTGGACCTGACCT-3' and reverse, 5'-TGAG GAGGGGAGATTCA-3'. RT product (1 μl) was amplified by PCR using the following conditions: 32 cycles of denaturing at 95˚C for 30 sec, annealing at 55˚C for 30 sec, and extending at 72˚C for 1 min. PCR product (6 μl) was then electrophoresed on 1.5% agarose gel. The experiments was repeated three times.

Western blotting. The Western blot assay has been described previously (17). The antibodies for Western blotting were as follows: mouse monoclonal antibody against E-cadherin (1:200, sc-8426; Santa Cruz Biotechnology, CA, USA), mouse monoclonal antibody against vimentin (1:100, sc-32322; Santa Cruz Biotechnology), mouse monoclonal antibody against Smad2 (1:1000; Cell Signaling Technology, Inc.), rabbit ploYclonal antibody against phosphorylated Smad2 (1:800, Ser465/467; Cell Signaling Technology, Inc.). Each experiment was done in triplicate.

Wound healing assay. Tu686 cells were plated in 6-well plates at $2x10^5$ per well with complete medium and allowed to grow to almost confluence, then were placed into serum-free medium for 24 h before wounding. The cell monolayer was wounded with a sterile 200 μl pipette tip. After washing out the floating cells, cells were replenished with fresh serum-free medium with or without 5 ng/ml TGF-β1. Migration of wounded cells was observed and photographed at 0 and 48 h with an inverted Leica phase-contrast microscope. The wound healing rate was quantified with measurements of the gap size over time. Three different areas in each assay were chosen to measure the distance of migrating cells. The experiment was carried out in triplicate.

Invasion assay. The invasion assay has also been described previously (18). Briefly, transwell with a 6.5-mm polycarbonate filter (8 μm pore size, Costar) was coated with 200 μl Matrigel (BD Biosciences) and incubated at 37˚C for 30 min. $2.5x10^4$ Tu686 cells in 100 μl of serum-free medium with or without 5 ng/ml TGF-β1 were seeded in the upper chamber of the transwell and 10% FBS was added as a chemoattractant in the lower chamber. After 48 h of incubation, the upper surface of the membrane was scrubbed with a cotton swab. The invaded cells in the lower membrane were fixed in methanol and stained with H&E. The number of invaded cells was expressed as the average of five random fields under the microscope at x200 magnification. The experiment was conducted four times.

Transient transfection. Tu686 cells were transiently transfected with Smad2 siRNA (sc-38374, Santa Cruz Biotechnology)

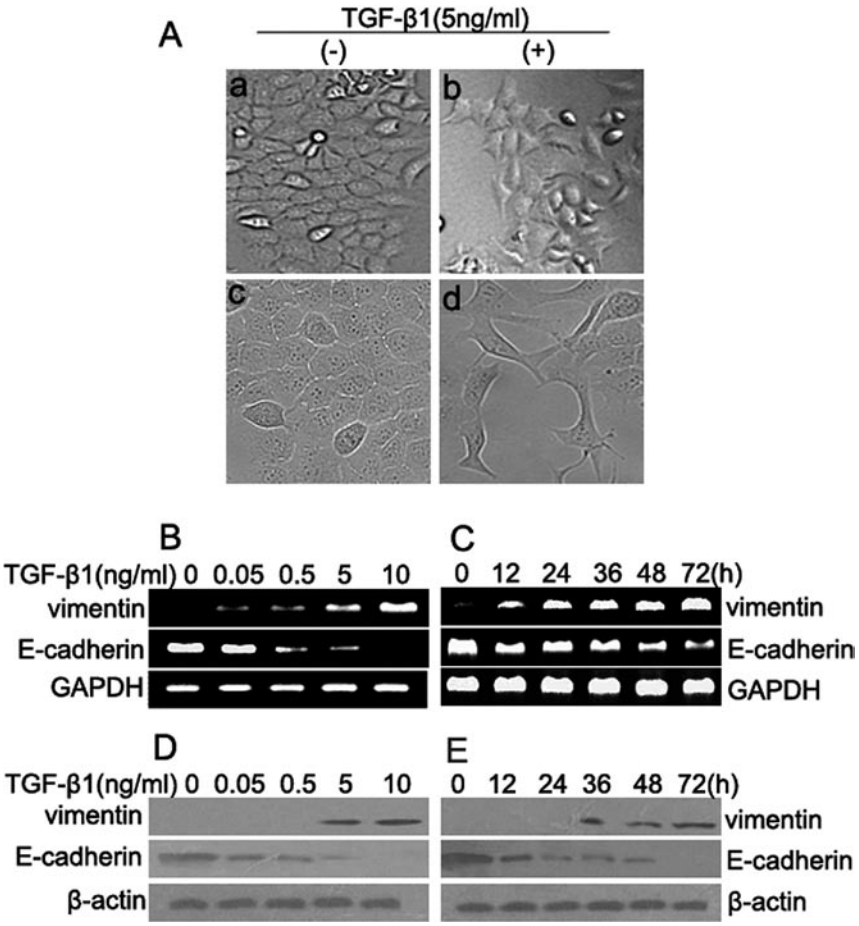

Figure 1. Morphologic and molecular changes induced by TGF-β1 on Tu686 cells. (A) Tu686 cells were incubated with 5 ng/ml TGF-β1 for 48 h, detectable morphological conversion was observed. Untreated cells showed a pebble-like shape and cell-cell adhesions were clearly observed (a, c). TGF-β1-treated cells exhibited loss of the adherent phenotype with cellular elongation, decrease in cell-to-cell contact, and the induction of a fibroblast-like state (b, d). Magnification (a, b) x50; (c, d) x200. (B, D) Tu686 cells were cultured in the serum-free medium with 0, 0.05, 0.5, 5 and 10 ng/ml TGF-β1 for 48 h, RT-PCR (B) and Western blotting (D) were performed to analyse expression levels of E-cadherin and vimentin. (C, E) Tu686 cells were cultured in the serum-free medium with 5 ng/ml TGF-β1 for 0, 12, 24, 36, 48 and 72 h. Also, RT-PCR (C) and Western blotting (E) were performed to analyse expression levels of E-cadherin and vimentin. As shown, in response to TGF-β1 treatment, down-reguration of E-cadherin and up-reguration of vimentin happened in a concentration- and time-dependent manner.

according to the manufacturer's protocol, and control siRNA (SC-36869, Santa Cruz Biotechnology) was used. To determine the efficiency the siRNA knockdown, transfected cells were collected at day 4 and protein levels of Smad2 were assessed with Western blot assay.

Statistical analysis. Statistical analysis was done by using the SPSS software (version 17.0). Results of quantitative data in this study were expressed as the mean ± SD. Statistical differences between groups were compared using two-tailed t test. A p-value <0.05 was considered to be statistically significant.

Results

Effects of TGF-β1 on the proliferation of Tu686 cells. MTT assay was initially performed to evaluate the effect of TGF-β1 on the proliferation of Tu686 cells. After starving for 24 h, Tu686 cells were treated by various concentrations of TGF-β1 (0-10 ng/ml) in serum-free medium and the OD values were gained 48 h later. We found that the effects of TGF-β1 on the proliferations of Tu686 cells were dose-independent and the growth rate was similar between different concentration treatment of TGF-β1 ($p>0.05$) (data not shown). The result demonstrated that TGF-β1 had little affection on the proliferation of Tu686 cells.

TGF-β1-mediated morphological changes of Tu686 cells. Since morphological change is a feature of EMT (6), the effect of TGF-β1 on the morphology of Tu686 cells was obeserved. After treating with TGF-β1 at various concentrations for 12 h, cell morphology was observed under invert phase-contrast microscope. Tu686 cells cultured in the absence of TGF-β1 exhibited a typical pebble-like epithelial morphology and cell-cell adhesions clearly existed. However, exposure to TGF-β1 at concentrations ranging from 0.05 to 10 ng/ml induced obvious morphological changes in Tu686 cells. Phase-contrast microscopy revealed loss of the adherent phenotype with cellular elongation, decrease in cell-to-cell contact, and the induction of a fibroblast-like state (Fig. 1A).

Effects of TGF-β1 on molecular characteristics of Tu686 cells. Subsequently, E-cadherin and vimentin expression were

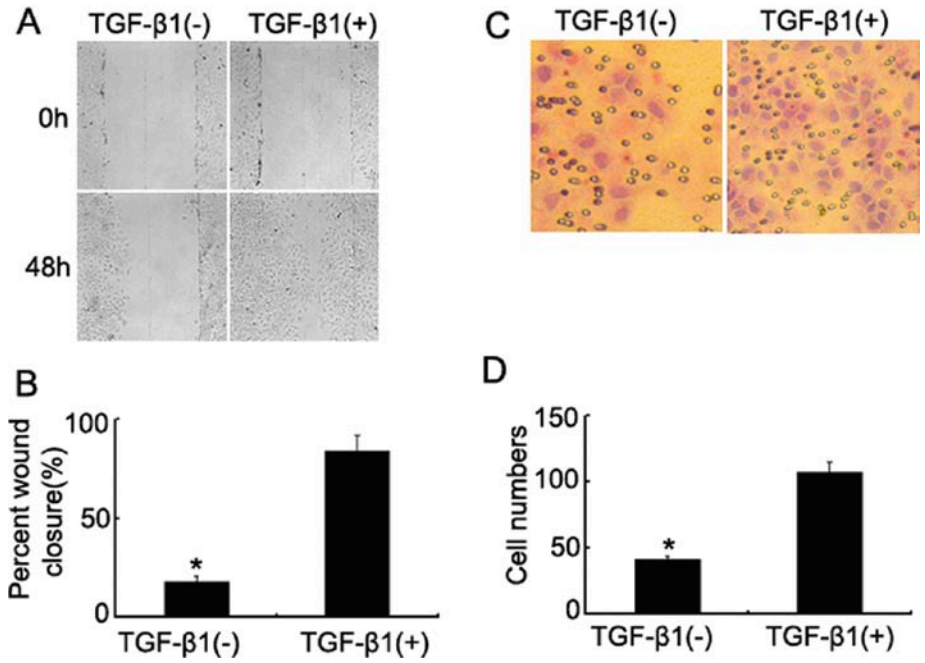

Figure 2. TGF-β1 promoted Tu686 cells migration and invasion (A) The wound healing assay was employed to determine the migration of Tu686 cells. The 'scratch' wounds were created by scraping confluent cell monolayer cultured in 6-well plates with a sterile 200 μl pipette tip. After wounded, the cells were cultured with or without 5 ng/ml TGF-β1 for 48 h. Migration of wounded cells was observed and photographed at 0 and 48 h with an inverted Leica phase-contrast microscope (magnification x100). (B) The wound healing rate was quantified with measurements of the gap size over time. Closures between untreated and TGF-β1-treated cells at 48 h were 18 and 84% ($p<0.05$), respectively. (C) The transwell assay was conducted to determine the invasion ability of Tu686 cells. Tu686 cells ($2.5x10^4$) were seeded into Matrigel-coated transwells. Cells invaded through chambers in the absence or presence of 5 ng/ml TGF-β1 were photographed and counted. (D) The cells, invaded through the pores to the lower surface of the filter, were counted under a microscope at x200 magnification. The number of invaded cells was expressed as the average of five random fields. The cells that invaded through the pores to the lower surface of the filter were 41±3 and 107±8 ($p<0.05$), respectively. Shown are the representative results of the experiment.

measured by using Western blotting and RT-PCR assay as E-cadherin and vimentin are key markers of EMT process (5). We noted that the expression of epithelial marker E-cadherin decreased in a dose-dependent manner with the concentration of 0, 0.05, 0.5, 5 and 10 ng/ml TGF-β1 for 48 h. In contrast, expression of the mesenchymal maker vimentin increased gradually following TGF-β1 treatment (Fig. 1B and D). The loss of E-cadherin expression was revealed at TGF-β1 concentration levels as low as 0.05 ng/ml and more obvious at 5 ng/ml TGF-β1 concentration. In addition, the expression change of E-cadherin and vimentin was time-dependent with TGF-β1 treatment (Fig. 1C and E). The down-regulation of E-cadherin expression was evident at 48 h when treated with 5 ng/ml TGF-β1 concentration.

TGF-β1 promoted Tu686 cell migration and invasion. The migratory and invasive capacity of Tu686 cells were also investigated in response to TGF-β1 treatment. Treated with 5 ng/ml TGF-β1, Tu686 cells showed a significant increase in cell migration. In contrast to the control, the cells exposed to TGF-β1 actively sprouted into the depleted area, the scratch wound was almost closed after 48 h. Closures between untreated and TGF-β1-treated cells at 48 h were 18 and 84% ($p<0.05$), respectively (Fig. 2A and B). The transwell assay was employed to determine the invasion ability of Tu686 cells. Tu686 cells ($2.5x10^4$) were seeded into Matrigel-coated transwells, and then, cells, invaded through chambers in the absence or presence of 5 ng/ml TGF-β1 were photographed and counted at 48 h. The cells that invaded through the pores to the lower surface of the filter were 41±3 and 107±8 ($p<0.05$), respectively (Fig. 2C and D). This indicated that TGF-β1 strongly promoted Tu686 cells invasion.

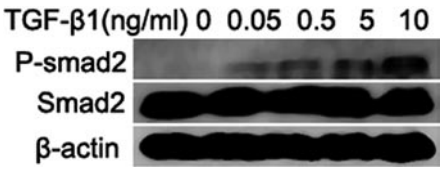

Figure 3. Representative Western blotting probed for total Smad2 and phosphorylated Smad2 for Tu686 cells treated with TGF-β1 over a concentration range of 0-10 ng/ml for 48 h. As shown, in response to TGF-β1 treatment, phosphorylated Samd2 increased gradually, whereas Smad2 level did not increase accordingly.

Effects of TGF-β1 signaling via Smad2 phosphorylation. Considering that TGF-β/Smad singaling pathway is a classical pathway and occurs through phosphorylation of the Smad2/Smad3 (19), we examined the effect of TGF-β1 treatment on Smad2 phosphorylation. Treated with various concentrations of TGF-β1 (0-10 ng/ml), the expression of phosphorylated Smad2 of Tu686 cells increased in a dose-dependent manner, whereas Smad2 level did not increase accordingly (Fig. 3).

To confirm the role of canonical TGF-β/Smad signaling in this system, we then used siRNA to inhabit the expression of Smad2 in Tu686 cells. Western blotting was employed to assess the ability of Smad2 siRNA to down-regulate Smad2

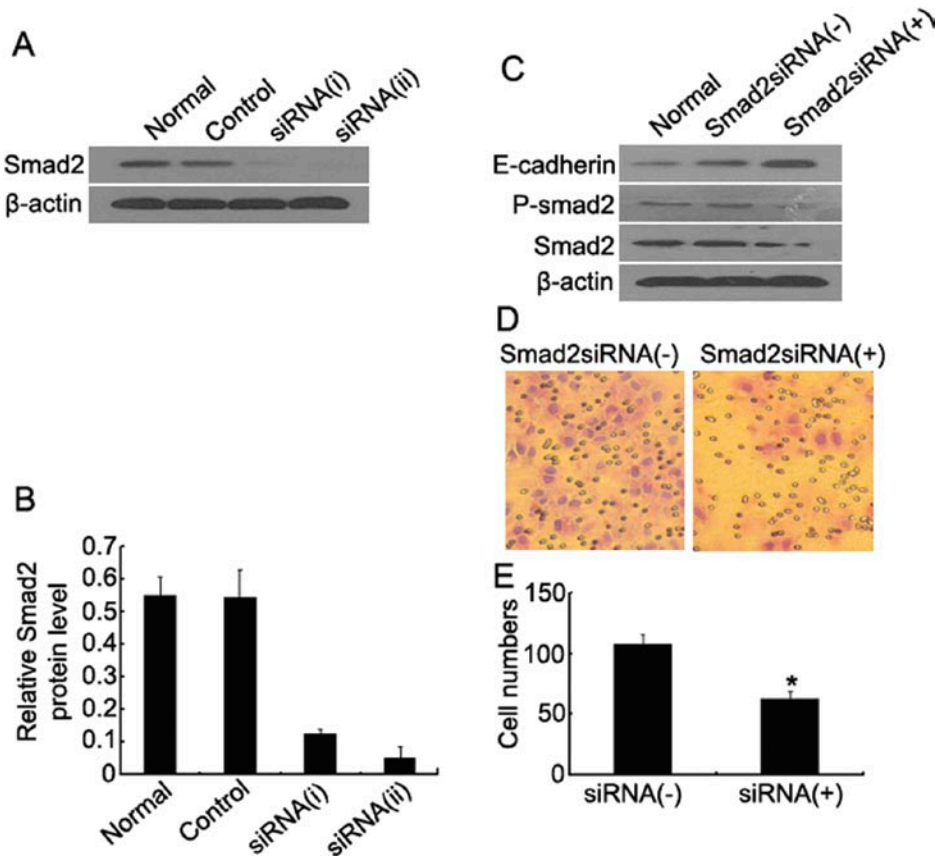

Figure 4. Knockdown of Smad2 by siRNA and its effect on the TGF-β1-mediated changes in Tu686 cells. (A) Western blotting for Smad2 RNAi showing potent silencing of Smad2 with no effect on β-actin (siRNAi, 8 μl siRNA; siRNAii, 10 μl siRNA). (B) Relative expression levels of Smad2 protein in Tu686 cells. As shown, 8 μl siRNA for Smad2 resulted in 78% knockdown, and 10 μl siRNA for Smad2 resulted in a >91% knockdown. (C) Tu686 cells were treated with 5 ng/ml TGF-β1 for 48 h with or without pretreatment with Smad2 RNAi. Then, Western blotting was done for expression of E-cadherin, Smad2, phosphorylated Samd2. E-cadherin expression recovered with low expression of phosphorylated Samd2. (D) For the transwell assay, Tu686 cells, treated with 5 ng/ml TGF-β1 with or without pretreatment with Smad2 RNAi, were seeded into the upper chamber of the transwell for 48 h and the cells that invaded through the pores to the lower surface of the filter were photographed and counted. (E) The cells, invaded through the pores to the lower surface of the filter, were counted under a microscope at x200 magnification. The number of invaded cells was expressed as the average of five random fields. As shown, invasion capacity of Tu686 cells was suppressed (62±7 vs. 108±7, $p<0.05$). Shown are representative results of the experiment.

in Tu686 cells. As shown in Fig. 4A and B, 8 μl siRNA for Smad2 resulted in 78% knockdown, and 10 μl siRNA for Smad2 resulted in a >91% knockdown. So 10 μl Smad2 siRNA was used against Smad2 in Tu686 cells. As shown, Smad2 RNAi abrogated the TGF-β1-induced suppression of E-cadherin expression (Fig. 4C) and suppressed the invasion capacity of Tu686 cells ($p<0.05$, 62±7 vs. 108±7; Fig. 4D and E).

Discussion

Many studies have pointed to the importance of EMT in the process of tumor invasion and metastasis (7,8,20). Compelling evidence for the involvement of EMT in oncogenesis is the ability of multiple EMT regulators to enhance tumor metastasis (21). For example, loss of the classic epithelial marker E-cadherin, a hallmark of EMT, is also implicated in the progression towards metastasic competence (22). Snail, an inducer of EMT in some tumors, can increase the aggressiveness of experimentally induced breast tumors, and high expression of Snail correlates with an increased risk of tumor relapse and poor survival rates in human breast cancer (23,24). In addition, the differential expression of epithelial and mesenchymal makers has been recongized at the invasive margin of tumors (e.g., colorectal and hepatocellular tumors) suggesting that EMT might be involved in tumor metastasis (25,26). EMT has been observed in several epithelial-oriented tumors, which include SCCHN (9) and increasing evidence demonstrates EMT is closely associated with tumor metastasis (16,27-29).

Transforming growth factor-β1 (TGF-β1) was originally isolated as a transformed phenotype inducer (30). Further work revealed that TGF-β1 is very important in malignant progression, invasion and metastasis (10,31,32). TGF-β1 has been investigated as a key mediator of EMT, principally via the activition of TGF-β/Smad signaling, which alters the function of the E-cadherin transcriptional repressors snail and slug (33-35). TGF-β1 has been reported to be able to induce EMT in several malignant cell lines including alveolar epithelial A549 and esophageal adenocarcinoma cell lines (13,14). However, there are scarce data on the possibility that TGF-β1 promotes invasion and metastasis by the induction of EMT in the context of SCCHN.

In the current study, we treated Tu686 cells with different dose of TGF-β1 at different time-points to develop a cellular

EMT model for following metastasis-related studies in SCCHN. TGF-β1 was initially reported as a potent growth inhibitor of a set of cell types. However, in cancer progression, the antiproliferative effects of TGF-β1 are frequently lost (10). In the present study, an MTT assy revealed that TGF-β1 had little effect on the proliferation of Tu686 cells. Our data support that TGF-β1 exposure of Tu686 cells induced EMT characterized by transformation of fibroblast-like morphology, down-regulation of epithelial marker E-cadherin and up-regulation of mesenchymal maker vimentin. In addition, the present data also demonstrated that TGF-β1 exposure of Tu686 cells induced down-regulation of epithelial marker E-cadherin and up-regulation of mesenchymal maker vimentin in a concentration- and time-dependent manner.

The formation of EMT is usually accompanied by less strong adhesions between cells, enhanced migratory and invasion capacity of cells (21). Zhen *et al* have reported that TGF-β1 could enhance cell migraion of human hepatocellular carcinoma cells through EMT formation (36). Similarly, in our study, we demonstrated that TGF-β1 treatment led Tu686 cells to exhibit strong ability to migrate and invade.

TGF-β/Smad singaling pathway is a classical pathway. In this system, TGF-β1 regulates cellular processes by binding and phosphorylating cell-surface receptors (TGF-βRI/TGF-βRII), the activated TGF-βRI phosphorylate Smad2 or Smad3, which then binds to Smad4. The resulting Smad complex then moves into the nucleus, where it interacts in a cell-specific manner with various transcription factors to regulate the transcription of many genes (32). It has been shown that TGF-β1 can mediate EMT by inducing tyrosine phosphorylation of Smad2 and Smad3 in non-malignant and malignant cell lines, including mouse hepatocyte cells (37), human renal proximal epithelial tubular cells (38), esophageal adenocarcinoma cells (14) and breast cancer cells (39). Oft *et al* found that Smad2 plays a critical role in TGF-β-induced differentiation of squamous carcinoma into invasive spindle cell carcinoma *in vivo* (40). In mammary models, overexpression of Smad2 and Smad3 resulted in increased EMT phenotype (12,41). Tian *et al* found that reduction of Smad2 and Smad3 function decreased metastatic potential of xenografted breast cancer cell lines (42). In the present experiment, TGF-β1 treatment activated the canonical TGF-β signaling pathway with induction of phosphorylated Smad2 protein expression. In addition, Smad2 RNAi abrogation of both expression and functional effects of TGF-β1 added further support to the role of canonical Smad signaling in this setting.

Overall, the present study demonstrates that TGF-β1 induced EMT in SCCHN cell line via TGF-β/Smad signaling pathway. A cell model for EMT was established, which is valuable for future studies on metastasis of SCCHN.

Acknowledgements

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