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TGF-β1 -promoted epithelial-to-mesenchymal transformation and cell adhesion contribute to TGF-β1 -enhanced cell migration in SMMC-7721 cells

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ABSTRACT

Transforming growth factor- β 1 (TGF- β 1), a multi-function polypeptide, is a double-edged sword in cancer. For some tumor cells, TGF- β 1 is a potent growth inhibitor and apoptosis inducer. More commonly, TGF- β 1 loses its growth-inhibitory and apoptosis-inducing effects, but stimulates the metastatic capacity of tumor cells. It is currently little known about TGF- β 1-promoted cell migration in hepatocellular carcinoma (HCC) cells, let alone its mechanism. In this study, we found that TGF- β 1 lost its tumor-suppressive effects, but significantly stimulated cell migration in SMMC-7721 human HCC cells. By FACS and Western blot analysis, we observed that TGF- β 1 enhanced the expression of α 5 β 1 integrin obviously, and subsequently stimulated cell adhesion onto fibronectin (Fn). Furthermore, we observed that TGF- β 1 could also promote SMMC-7721 cells adhesion onto laminin (Ln). Our data also provided evidences that TGF- β 1 induced epithelial-to-mesenchymal transformation (EMT) in SMMC-7721 cells. First, SMMC-7721 cells clearly switched to the spindle shape morphology after TGF- β 1 treatment. Furthermore, TGF- β 1 induced the down-regulation of E-cadherin and the nuclear translocation of β -catenin. These results indicated that TGF- β 1-promoted cell adhesion and TGF- β 1-induced epithelial-to-mesenchymal transformation might be both responsible for TGF- β 1-enhanced cell migration.

Key words: *TGF- β 1 , cell migration, epithelial-to-mesenchymal transformation, α 5 β 1 integrin.*

INTRODUCTION

TGF- β 1, a polypeptide with multi-function, modulates a variety of cellular processes, such as proliferation, differentiation and apoptosis[1,2]. TGF- β 1 is a potent growth inhibitor and apoptosis inducer for most normal cells. However, many tumor cells are nevertheless sensitive to the tumor-suppressive effects of TGF- β 1. It is known that TGF- β 1 could strongly stimulate the invasive and metastatic capacity of tumor cells[3]. The role of TGF- β 1 in HCC cells is not well understood. Previous studies demonstrated that TGF- β 1 also lost the tumor-suppressive effects in many HCC cells[4]. Moreover, TGF- β 1 concentration increased in the plasma of HCC patients[5,6]. The study on the role for TGF- β 1 in invasive and metastasis of hepatocellular carcinoma is scarce.

It is well known that TGF- β 1 could modify the expression of many different integrins, and subsequently alter cell adhesion and migration[7]. Furthermore, cell migration is dependent on cell adhesion[8]. EMT correlates with the metastatic potential of tumor cells[3,9]. Previous studies demonstrated that TGF- β 1 could induce the EMT of tumor cells with epithelial origin[10-14]. However, there are no reports about TGF- β 1-promoted EMT of HCC cells.

Here we investigated the role for TGF- β 1 in SMMC-7721 human HCC cells with low differentiation [15]. We found that TGF- β 1 had no effects on the growth and apoptosis of SMMC-7721 cells. At the meantime, TGF- β 1 significantly enhanced cell migration on Fn and Ln. We intended to study whether TGF-

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Abbreviations used: TGF- β 1, transforming growth factor- β 1; HCC, hepatocellular carcinoma; FACS, flow cytometric analysis; Fn, Fibronectin; Ln, Laminin; EMT, epithelial-to-mesenchymal transformation; ECM, extracellular matrix.
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β1-enhanced cell migration correlated with TGF-β1-promoted cell adhesion and EMT in SMMC-7721 cells.

MATERIALS AND METHODS

Cell lines, antibodies and reagents

SMMC-7721 human HCC cell line was obtained from Shanghai No.2 Military Medical University (Shanghai, China)[15]. Fn and Ln were all obtained from Sigma. Human recombinant TGF-β1 was from PeproTech EC Ltd. Anti-E-cadherin polyclonal antibody, anti-integrin α5 subunit monoclonal antibody, anti-β-actin monoclonal antibody, anti-β-catenin monoclonal antibody were purchased from Santa Cruze. Anti-integrin β1 subunit monoclonal antibody was from BD Transduction Laboratories. Secondary antibodies conjugated with HRP were purchased from Watson Biotech (Shanghai).

Cell culture, TGF-β1 treatment and cell morphology examination

SMMC-7721 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum in 37 ºC and 5% CO_2, and grown to 50-70% confluence prior to TGF-β1 treatment. TGF-β1 was stored in PBS containing[2] mg/ml bovine serum albumin (BSA), and added into the serum-free medium to a final concentration. Cell morphology was monitored on a phase contrast microscope equipped with a video camera.

Cell migration assay

In vitro wound healing assay[16]

Subconfluent cells were detached and $5x10^5$ cells were seeded in Falcon six-well tissue culture plates coated with 10 mg/ml Fn. After 12 h, cells were cultured in serum-free medium for 24 h (grown to confluence to form a monolayer), and were treated with or without 10 ng/ml TGF-β1. In vitro, 'Scratch' wounds were created by scraping confluent cell monolayers with a sterile pipette tip to make an approximately 1.0 mm gap. The cells scraped down were washed by serum-free medium. After 24 h and 48 h, migration was quantified by counting cell numbers that had advanced into the cell-free space from a number of randomly chosen 1mm segments of the initial wound border. Each point stands for Mean±SD from at least 4 wounds of every experiment, and 4 separate experiments were carried out.

Assay of random migration using the agarose drop method [17]

Cellls to be tested were removed from culture dishes by tryspinization, washed and centrifuged into a pellet. To the cell pellet was added 100 ml RPMI 1640 medium containing 10% serum and 16.7 μl 2% (w/v) agarose to be the agarose-cell suspension containing 0.3% agarose. Prior to use, the agarose-cell suspension was kept in a water bath at 37 ºC for preventing the solidification. 1.5 μl droplets of the agarose-cell suspension were delivered with a sterile micropipette into the 24-wells tissue culture plates. The dish was then placed in a refrigerator for 10 min to allow the agarose to solidify. The radius of the droplet (r) was measured using inverted microscope fitted with a rule in eyepiece. After cultured in the RPMI 1640 medium containing 1% serum for 6-8 h, the droplets were washed gently with serum-free medium, and were treated with or without TGF-β1 in serum-free medium for 24 and 48 h. At 24 and 48 h, the radius of the droplet was measured under eight sides of each droplet, and the average value (r') was calculated. Cells' random migration potential was determined by the value of (r'-r). Five drops were used for each point.

Flow cytometric analysis of integrin and E-cadherin expression on cell membrane

$1x10^6$ cells were incubated with PBS containing 1% BSA alone or with primary antibodies against integrin α5 subunit, integrin β1 subunit and E-cadherin respectively for 45 min in 4 ºC. Cells were washed extensively (3 times in PBS) and incubated with appropriate FITC-conjugated second antibody for 45 min in 4ºC in the dark. After washing the cells extensively, they were analyzed on a FACScan[?] (Becton-Dickinson & Co., Mountain View, CA). For each sample, data from 10,000 cells were collected. Cells as control were incubated with secondary antibody alone to show background fluorescence.

Cell adhesion assay

Cell adhesion assay was carried out as previously described []. Briefly, 96-well plates (Nunc) were coated at 37 ºC for 1 with 100 μl 5 μg/ml Fn, 20 μg/ml Ln and 100 μg/ml Poly-lysine in PBS. The plates were washed twice with PBS and blocked with 100 μl 1% BSA for 1 h at 37 ºC. Wells were washed twice with PBS and stored at 4 ºC before use. Cells were collected and resuspended in complete RPMI without FCS. A total of 10,000 cells in 100 ml were added into each substrate coated well, and plates were incubated for 30 min at 37ºC in 5% CO_2. Unattached cells were gently washed away with PBS. The attached cells were fixed with 4% formaldehyde, stained with 0.5% crystal violet overnight, destained with distilled water, solubilized in 36% acetic acid and quantified by the micro-titer plate reader.

Nuclear protein extracts

Nuclear protein extracts were prepared by the mini-extraction method as described previously[19]. Cells were washed with ice cold PBS and harvested by being scraped in 1.5 ml PBS. Cells were then pelleted and resuspended in 400 μl of 10 m*M* Hepes-potassium hydroxide, pH 7.9, 1.5 m*M* magnesium chloride, 10 m*M* potassium chloride, 0.5 m*M* dithiothreitol, 0.2 m*M* PMSF. After 10 min of incubation on ice nuclei were pelleted by being spun for 10s and resuspended in 50 ml of 20 m*M* Hepes-potassium hydroxide, pH 7.9, 25% glycerol, 420 m*M* sodium chloride, 1.5mM magnesium chloride, 0.2 m*M* EDTA, 0.5 m*M* dithiothreitol, 0.2 m*M* PMSF. Tubes were incubated for 20 min on ice and then centrifuged to clear the cellular debris. Nuclear extracts were stored at -70ºC.

Protein preparation

Cells were washed with PBS and lysated in modified loading buffer containing 50 m*M* Tris-HCl pH 6.8, 2% SDS, 10% glycerol, phosphatase inhibitors (100 m*M* Na_3VO_4, 10 m*M* NaF, 10 m*M* $Na_4P_2O_7$), and protease inhibitors (1 m*M* PMSF). The samples were boiled for 10 min and centrifuged at 12,000 rpm for 10 min, and insoluble material was removed.

Western blot analysis

Equal amount of protein were loaded on a SDS-PAGE and transferred to PVDF membrane. After blocked with 3% BSA in PBS (containing 0.05% Tween 20), the membranes were incubated with the specific primary antibodies, followed by HRP-conjugated secondary antibodies. Proteins were visualized by fluorography using an enhanced chemiluminescence system (Perfect Biotech. Shanghai). To reprobe another primary antibody, membranes were incubated in stripping buffer (62.5 m*M* Tris pH 6.7, 10 0 m*M* 2-mercaptoethanel, 2% SDS) at 70 °C for 30 min, washed and then used for further study. Signal intensity of each band was quantified by densiometric analysis using a scanning imager (Pharmacia Co.)

RESULTS

TGF-β1 promoted the migration of SMMC-7721 cells

TGF-β1 could inhibit cell growth and induce apoptosis in normal liver cells. However, for most HCC cells with low differentiation, TGF-β1 lost these functions. For example, we found that TGF-β1 lost its growth-inhibitory and apoptosis-inducing effects in SMMC-7721 cells with low differentiation (Data not shown). Since TGF-β1 lost its tumor-suppressive properties, we wondered whether TGF-β1 could promote cell migration. Agarose drop method and wound healing assay were performed to measure the migration of SMMC-7721 cells in vitro. As shown in Fig 1, by agarose drop method, we found that contrast to control, cell migration was 1.39-fold at 24 h, and 1.42-fold at 48 h with the concentration of 10 ng/ml TGF-β1. Using wound healing assay, we also found that cell migration was 1.61-fold at 24 h, and 1.80-fold at 48 h respectively (Fig 2). These results indicated that TGF-β1 could promote the metastatic potential of SMMC-7721 cells in vitro.

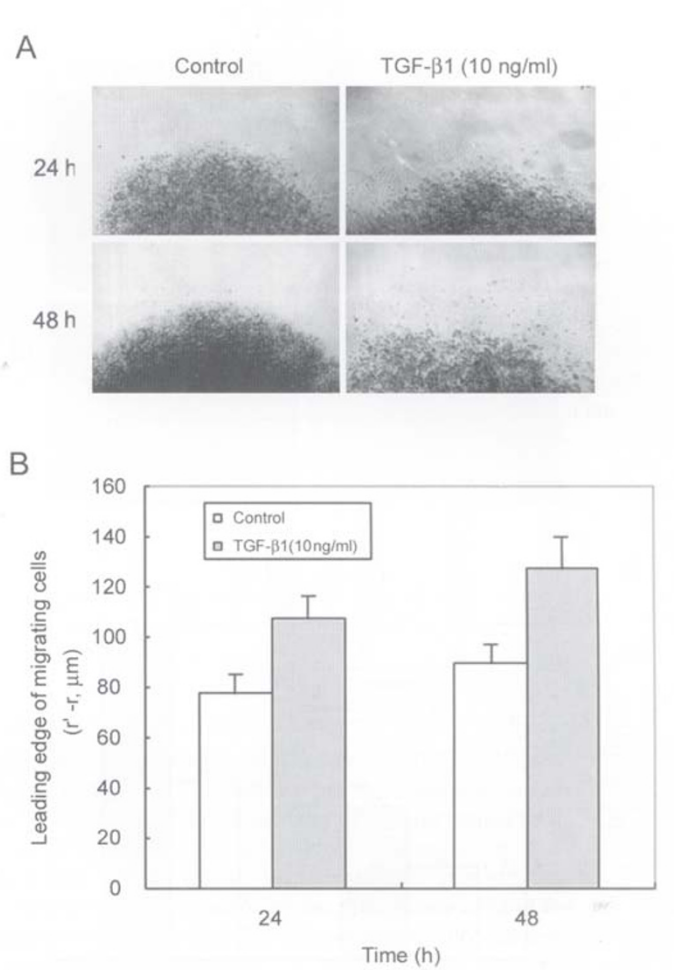

Fig 1. Assay of random migration using the agarose drop method. (A) Random migration of SMMC-7721 cells out of agarose drops explants treated with or without 10 ng/ml TGF-β1. At 24 h and 48 h, the migration of cells out from the drops was measured using inverted microscope fitted with a rule in eyepiece. (B) Cell migration rate was measured by the distance of the leading edge of migrating cells from the edge of the agarose droplet (r'-r). Each date represents Mean±SE.

TGF-β1 stimulated the expression of α5β1 integrin and promoted cell adhesion

Cell migration through ECM is generally mediated by integrins on cell membrane. Efficient evidences emerged that TGF-β1 could modify many integrins and subsequently alter the aspects of cell adhesion, migration. Furthermore, cell migration depends on cell adhesion. By FACS we found that contrast to control, α5 subunit was about 1.77-fold, and β1 subunit was about 1.65-fold with the concentration of 10ng/ml TGF-β1 for 48 h (Fig 3). By Western blot analysis, we also found that the expression of α5 subunit and β1 subunit were apparently enhanced with the concentration of 0, 1, 5, 10, 20 ng/ml TGF-β1 for 48 h (Fig 4). The β1 subunit appeared as two bands implying two forms of β1 subunit (seen in discussion).

α5β1 integrin is a cell adhesion receptor for Fn, and cell adhesion onto Fn is mediated by α5β1 integrin. Since TGF-β1 could enhance the expression of α5β1 integrin, we wanted to determine whether TGF-β1 could promote cell adhesion onto Fn. As shown in Fig 5, cell adhesion onto Fn was 1.60-fold at 24 h, and 1.87-fold at 48 h with the concentration of 10 ng/ml TGF-β1. We observed that TGF-β1 could also promote cell adhesion onto another ECM protein Ln.

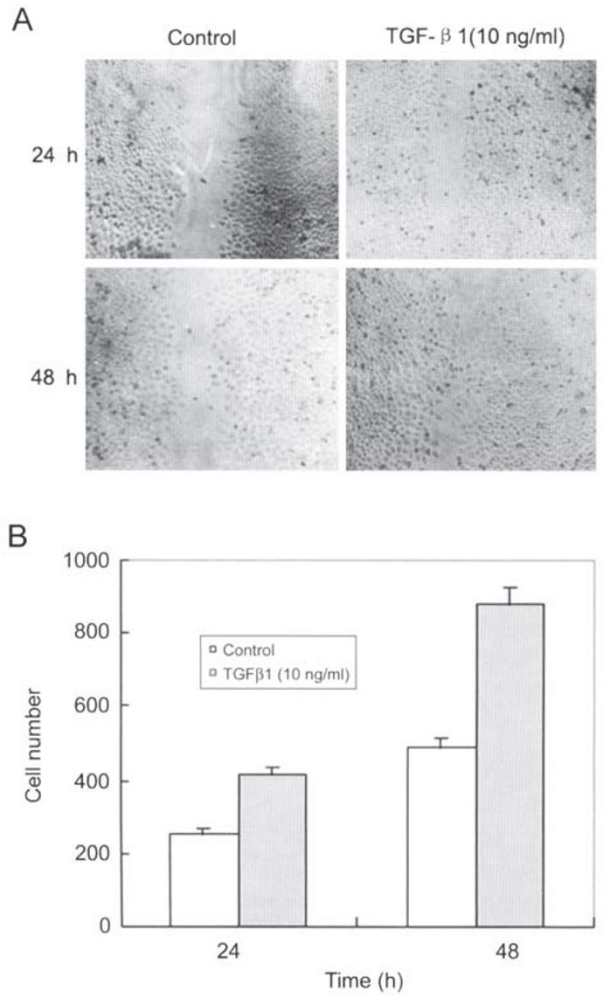

Fig 2. Wound healing assay. (A) In vitro, "scratch" wounds were created by scraping confluent cell monolayer cultured in Fn-coated dishes with a sterile pipette tip. Cells were cultured with or without 10 ng/ml TGF-β1 for 24 and 48 h. (B) Cell migration rate was measured by counting cell numbers that advanced into the cell-free space. Each date represents Mean (cells/mm2) ±SE.

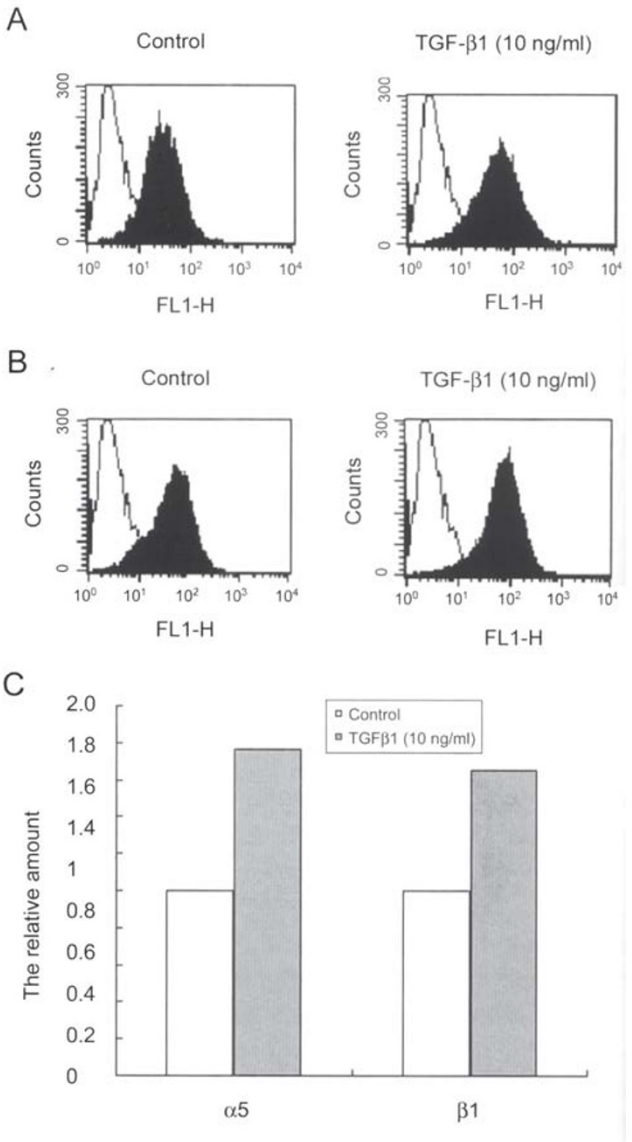

Fig 3. FACS analysis of the effect of TGF-β1 on the expression of α5 subunit and β1 subunit on cell membrane. SMMC-7721 cells were cultured in the serum-free medium with or without 10 ng/ml TGF-β1. After 48 h, cells were labeled by primary and secondary antibodies and then assayed by FACS. (A) The expression of α5 subunit treated with or without TGF-β1. (B) The expression of β1 subunit treated with or without TGF-β1. (C) The relative amount of α5 subunit and β1 subunit. Results are representative of 3 repeated experiments.

TGF-β1 induced the EMT of SMMC 7721 cells

EMT could contribute to the metastatic potential of tumor cells. Previous studies demonstrated that TGF-β1 could induce the EMT of tumor cells[10-14]. The EMT of cells is characterized by a clear switch to the spindle shape morphology, accompanied by down-regulation of E-cadherin and nuclear translocation of β-catenin. The development of tumor cells metastatic phenotype correlates with down-regulation of E-cadherin expression.

By microscopic examination, we found that SMMC-7721 cells underwent a clear switch to the spindle morphology with the concentration of 10 ng/ml TGF-β1 for 48 h (Fig 6). FACS analysis showed that the expression of E-cadherin on cell membrane was about 62% after treatment with 10 ng/ml TGF-β1 for 48 h (Fig 7). By

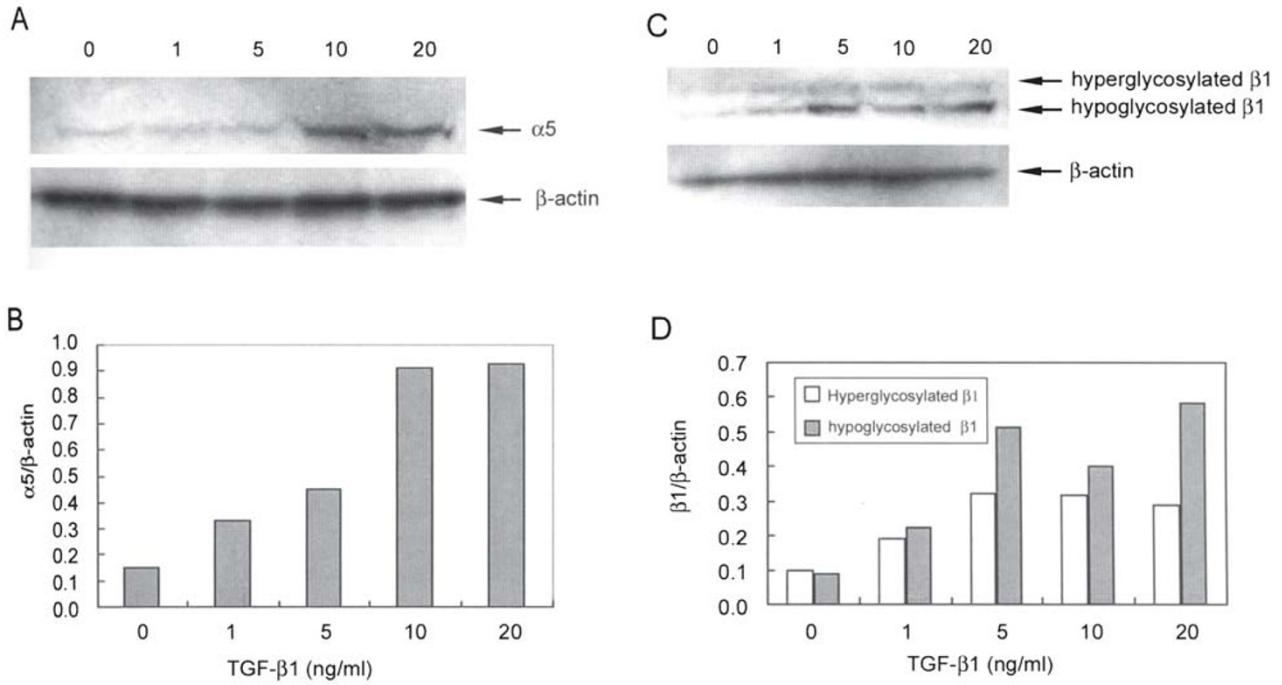

Fig 4. Western blot analysis of the effect of TGF-β1 on the expression of α5 subunit and β1 subunit. SMMC-7721 cells were cultured in the serum-free medium with 0, 1, 5, 10, 20 ng/ml TGF-β1 for 48 h, and Western blot assay was carried out. (A) The expression of α5 subunit treated with or without TGF-β1. (C) The expression of β1 subunit treated with or without TGF-β1. Two forms of β1 integrin were due to the different levels of β1-chain glycosylation. The hypoglycosylated form (lower band) was tentatively identified as biosynthetic precursor of β1 subunit; the hyperglycosylated form (upper band) was mature β1 subunit, exposed mainly on cell menbrane. The protein level of β-actin showed the loading amounts in each well. (B, D) α5/β-actin and β1/β-actin show the relative amount of α5 subunit and β1 subunit respectively. Results are representative of 3 repeated experiments.

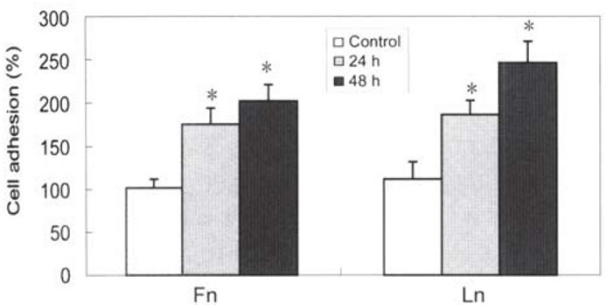

Fig 5. TGF-β1 enhanced cell adhesion onto Fn and Ln. After treated with 10 ng/ml TGF-β1 for 24 and 48 h, cells were plated onto 96-well plates coated with poly-lysine (100 ng/ml), Fn (10 mg/ml), Ln (30 mg/ml) and BSA (1%). Cell adhesion assay was performed as previously described. Each data represents Mean±SE (*p<0.01).

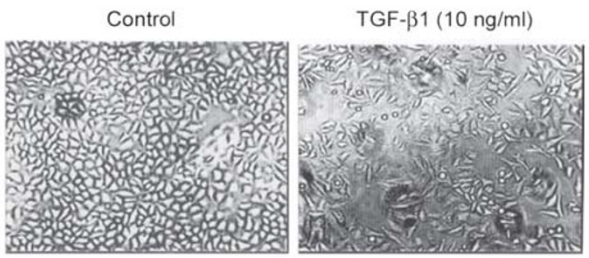

Fig 6. TGF-β1 treatment affected cell morphology. Phase-contrast microphotographs of SMMC-7721 cells treated with or without 10 ng/ml TGF-β1 for 48 h.

Western blot analysis, we noted that the expression of nuclear β-catenin increased in a dose-dependen manner with the concentration of 0, 1, 5, 10, 20 ng/ml TGF-β1 for 48 h (Fig 8). In contrast the xpression of total β1-catenin was not altered. We provided evidences that TGF-β1 induced the EMT of SMMC-7721 cells, which also could contribute to TGF-β1-promoted cell migration.

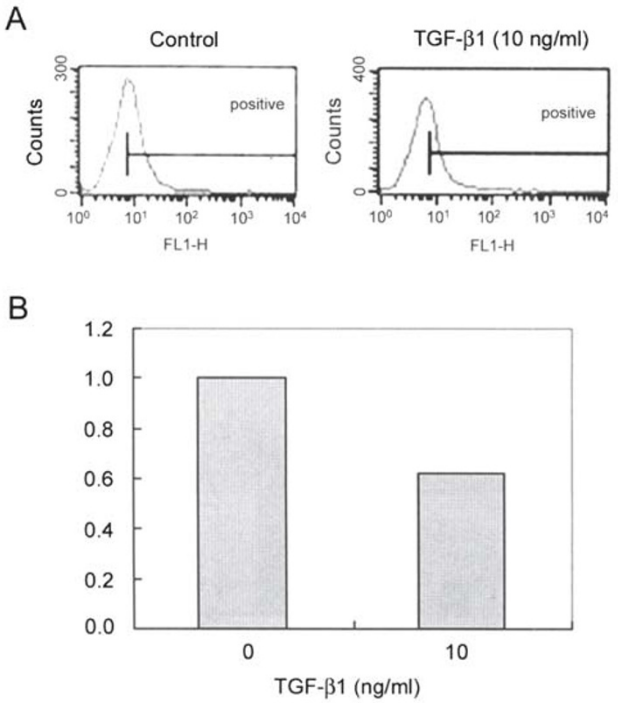

Fig 7. FACS analysis of the effect of TGF-β1 the expression of E-cadherin on cell membrane
(**A**) SMMC-7721 cells were cultured in the serum-free medium with or without 10 ng/ml TGF-β1. After 48 h, cells were labeled by primary and secondary antibodies and then assayed by FACS. (**B**) The relative amount of E-cadherin. Results are representative of 3 repeated experiments.

DISCUSSION

TGF-β1 is growth inhibitory and apoptosis inducing to most normal cell types. However, the majority tumor cells are nevertheless sensitive to the tumor-suppressive effects of TGF-β1, due to loss-of-function mutation of components in the TGF-β1 signaling pathways or up-regulation of the MAP kinase pathway during carcinogenesis[3]. Furthermore, previous evidences demonstrated that TGF-β1 could promote the invasive and metastatic capacity of human tumor, in vivo and in vitro [3]. It is not currently well understood the role of TGF-β1 in HCC cells. The fact that high serum concentration of TGF-β1 in HCC patients indicates that TGF-β1 loses the tumor-suppressive effects in most HCC cells [5,6]. However, whether TGF-β1 could enhance the metastatic capacity of HCC cells is not known yet, let alone its mechanism. Giannelli et al reported that TGF-β1 played an important role in the invasiveness of HCC cells by stimulating the expression of α3β1 integrin[20].

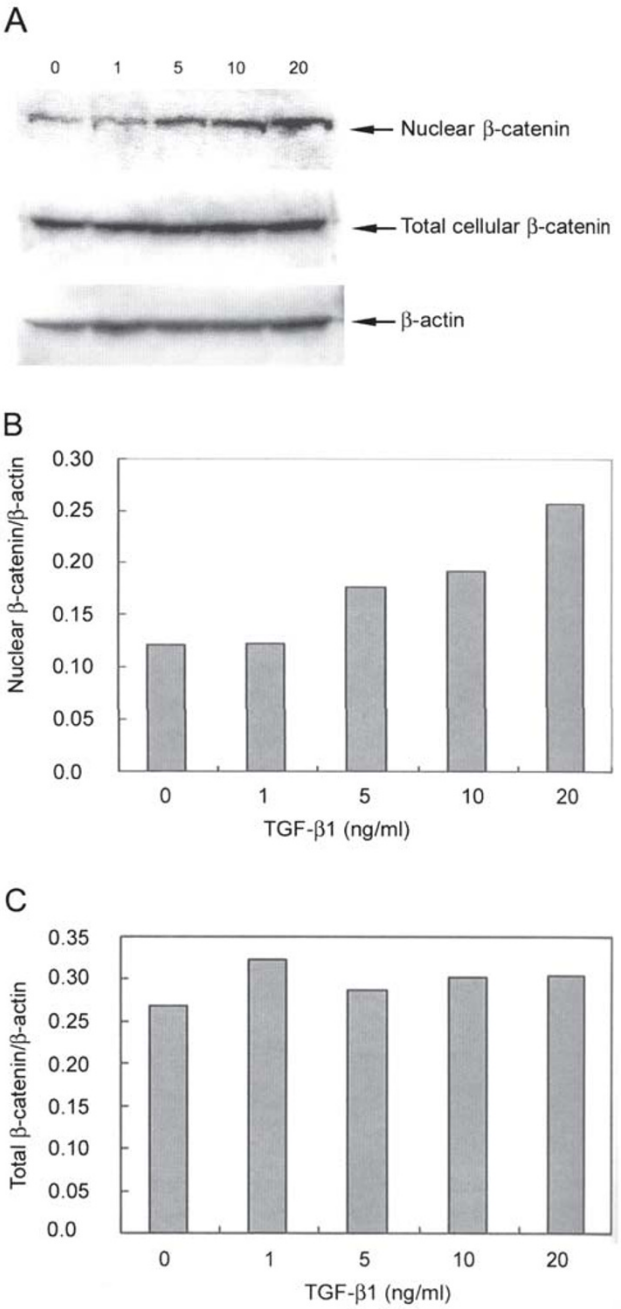

Fig 8. TGF-β1 promoted nuclear translocation of β-catenin. (**A**) SMMC-7721 cells treated with the concentration of 0, 1, 5, 10, 20 ng/ml TGF-β1 for 48 h. Nuclear extracts and total protein were prepared, and Western blot assay was performed. The protein level of β-actin showed the loading amounts in each well. (**B, C**) Nuclear β-catenin/β-actin and total β-catenin/β-actin show the relative amount of nuclear β-catenin and total β-catenin respectively. Results are representative of 3 repeated experiments.

Previous studies demonstrated that cell adhesion and EMT both played important roles in the matastatic phe-

notype of tumor cells[1,3]. TGF-β1 could regulate several integrins on many types of tumor cells, subsequently affected cell adhesion mediated by integrins[7]. A role for TGF-β1 to induce EMT has been reported in squamous skin carcinomas[12], Ras-transformed mammary carcinoma cells and ovarian adenosarcoma cells[14]. Although the cellular machinery of TGF-β1-induced EMT remains unclear, previous studies demonstrated that EMT during embryonic development correlated with down-regulation of E-cadherin and nuclear translocation of β-catenin.

In this repoet, we found that TGF-β1 lost the tumor-suppressive effects in SMMC-7721 human HCC cells. At the meantime, TGF-β1 promoted cell migration in vitro. We focused on investigating the possible mechanism of TGF-β1-stimulated cell migration and found that SMMC-7721 cells underwent a clear switch to the spindle morphology after treatment with TGF-β1.The fibro- blastoid phenotype correlated with down-regulation of E-cadherin expression and nuclear translocation of β-catenin. These results indicated that TGF-β1 could induce the EMT of SMMC-7721 cells, which possibly contributed to TGF-β1-promoted cell migration.

By FACS and Western blot analysis, we observed that TGF-β1 obviously increased the expression of integrin α5β1. In Western blot analysis, the β1 subunit appeared as two bands suggesting two forms of β1 subunit. It maybe caused by variable post-translational modification (mainly N-glycosylation). The hypoglycosylated form (lower band in Fig 4C) was tentatively identified as biosynthetic precursor of β1 subunit. The band with lower migration rate (upper band in Fig 4C) was the hyperglycosylated mature form of integrin β1 subunit, which mainly located at the plasma membrane [21-23]. The correlation between α5β1 integrin and cell migration has been previously reported. For example, Beauvais et al found that over-expression of α5β1 integrin in transfected sarcoma S180 cells enhanced their mobility on Fn in vitro, and changes their migratory properties in vivo[24]. We also found that TGF-β1 could promote SMMC-7721 cells adhesion onto Fn mediated by α5β1 integrin. Furthermore, TGF-β1 promoted SMMC-7721 cells adhesion onto another ECM protein Ln. The latter result indicated that some type of integrins (such as α5β1 and α3β1; the receptors of Ln) were possibly also up-regulated by TGF-β1. So we hypothesized that TGF-β1 could regulate the expression of several integrins, and subsequently promote cell adhesion onto ECM, which might also contribute to TGF-β1-promoted cell migration.

Take together, TGF-β1-promoted EMT and cell adhesion might be both responsible for TGF-β1-enhanced cell migration in SMMC-7721 cells.

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