



# TGF- $\beta$ 1 regulates the invasive and metastatic potential of mucoepidermoid carcinoma cells

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**BACKGROUND:** Patients with mucoepidermoid carcinoma exhibit poor long-term prognosis because of the lack of therapeutic strategies that effectively block tumor progression. We have previously characterized the Ms cells as a highly metastatic mucoepidermoid carcinoma cell line that expresses high levels of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1). Here, we studied the effect of suppressing TGF- $\beta$ 1 by RNA silencing on the invasive and metastatic potential of mucoepidermoid carcinoma.

**METHODS:** Cell motility, substratum adhesion, and transmembrane invasion were estimated by migration, matrigel adhesion, and matrigel invasion assay. Matrix metalloproteinase (MMP)-2 and MMP-9 activity were determined using gelatin gel zymography. Balb/c nu/nu nude mice lung metastatic model was used to test the metastatic ability of the Ms cells. Lung metastatic tumors were experimentally induced by mice tail vein inoculation of cancer cells.

**RESULTS:** TGF- $\beta$ 1 silencing inhibits cell motility, substratum adhesion, and transmembrane invasion. In vivo, a significant decrease in lung metastasis was observed when mice received tail vein injections of TGF- $\beta$ 1-silenced mucoepidermoid carcinoma cells, as compared to controls.

**CONCLUSION:** These results unveil a critical role for TGF- $\beta$ 1 in the progression of mucoepidermoid carcinomas and suggest that patients with this malignancy may benefit from therapeutic inhibition of the effectors of the TGF- $\beta$ 1 pathway.

J Oral Pathol Med (2011) 40: 762–768

**Keywords:** extracellular matrix; matrix metalloproteinase; metastasis; salivary gland cancer; tumor progression

## Introduction

Mucoepidermoid carcinoma is the most frequent salivary gland cancer, accounting to approximately 30% of all salivary gland malignancies. Primary mucoepidermoid carcinomas are not sensitive to radiotherapy and chemotherapy (1). The most common cause of death is the lack of response to therapy, leading to recurrence or distant metastasis. As a consequence, the long-term prognosis for patients with this malignancy is invariably poor (2). The understanding of mechanisms involved in the invasive behavior of mucoepidermoid carcinoma cells is critical for the development of mechanism-based therapies for this cancer.

Transforming growth factor- $\beta$  (TGF- $\beta$ ) belongs to a family of structurally related, dimeric, disulfide-linked polypeptides, which includes five TGF- $\beta$  isoforms (TGF  $\beta$ 1–5), activins, inhibins, Mullerian-inhibiting substance, bone morphogenetic proteins, and products of the *Xenopus Vg1* and *Drosophila decapentaplegic* genes. In mammals, three isoforms of TGF  $\beta$  (TGF  $\beta$ 1–3) have been identified (3). They are known to regulate cell proliferation and differentiation, positively or negatively depending on the cell type, and have been implicated in diverse physiological events such as angiogenesis, immune function, steroidogenesis, and tissue remodeling and repair. TGF- $\beta$  plays a critical role in the progression of many epithelial cancers. TGF- $\beta$ 1 is a potent growth inhibitor for epithelial cells, and this function contributes greatly to its role in tumor suppression (4). Paradoxically, TGF- $\beta$ 1 is overexpressed in many malignant human tumors (5, 6) and in various cancers in experimental animals, including skin tumors (7). Studies have shown that TGF- $\beta$ 1 overexpression at early stages of carcinogenesis provides tumor-suppressive effects primarily via growth inhibition (8), whereas TGF- $\beta$ 1 overexpression at late stages promotes tumor progression, metastasis, and epithelial to mesenchymal transition (EMT), potentially via loss of adhesion molecules, angiogenesis, proteinase activation, and immune suppression (9–12).

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Accepted for publication April 12, 2011

It is known that TGF- $\beta$ 1 modulates the motile behavior of several tumor cells including human glioma (13), oral squamous carcinoma (14), prostate cancer (15), breast cancer (16), and lung cancer cells (17). However, the role of TGF- $\beta$ 1 in salivary gland mucoepidermoid carcinoma cells is not currently well understood. We have observed that the metastatic Ms mucoepidermoid carcinoma cell line expresses high levels of TGF- $\beta$ 1 (18). We hypothesize that TGF- $\beta$ 1 may contribute to the invasive and metastatic potential of mucoepidermoid carcinomas. Here, we report that silencing of TGF- $\beta$ 1 effectively decreases invasion and metastasis of Ms cells.

## Methods

*Construction of a vector-expressing shRNA for TGF- $\beta$ 1*  
The pWH1 was used for the construction of a vector-expressing shRNA for TGF- $\beta$ 1. Human TGF- $\beta$ 1-specific sequences 5'-GATCCCCTGCTGCCGCTGCTGTACCTcaagagaGGTAGCAGCAGCGGCAGCATTTTGGAAA-3' and 5'-AGCTTTTCCAAAATGCTGCCGCTGCTACCTctctttaaGGTAGCAGCAGCGGCAGCAGGG-3' were cloned into *Bgl*/*Hind* sites of the pWH1 vector, which was generously provided by Dr. Wu YM.

### *Cell culture and transfection conditions*

Ms cells (mucoepidermoid carcinoma spindle code metastatic cell lines), established and characterized in our laboratory, showed a higher metastatic potential than the parental Mc3 cells' *in vitro* and *in vivo* behavior (19). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Langley, OK, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 100  $\mu$ g/ml streptomycin and 100-U/ml penicillin at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were periodically determined as mycoplasma free by Immuno-Myco test kit. Twenty-four hours before transfection, cells were seeded onto 24-well plates with antibiotics-free growth medium at a density of  $5 \times 10^5$  cells/well, so that the confluence would reach approximately 50–70% at the time of transfection. Cells were transfected with 1  $\mu$ g/well of shRNA vector targeting TGF- $\beta$ 1 using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) reagent following the protocol provided by the manufacturer. Control transfection was generated by transfecting pWH1-puro into Ms cells as shRNA-C. Control cells were incubated with DMEM alone without shRNA or Lipofectamine 2000 as control. After 6 h, medium was replaced by antibiotics-free DMEM. Cells were harvested and plated on 100-mm culture dish and cultured for 48 h, and clones stably expressing shRNA were selected using 600  $\mu$ g/ml G418 (Gibco) for 3 weeks. Clones were screened by RT-PCR, immunocytochemistry, and Western blotting (20).

### *Soft agar colony formation assay*

To evaluate whether cells were able to grow in soft agar, 24-well plates were prepared with 0.8-ml feeder layer of 5 g/l agar in medium plus 20% FBS and a 3 g/l agar

top layer in the same medium that contained recently trypsinized log-phase growing cells (500 cells/well). Two weeks after seeding, colonies with more than 60  $\mu$ m in diameter were counted under an inverted microscope.

### *Matrigel adhesion assay*

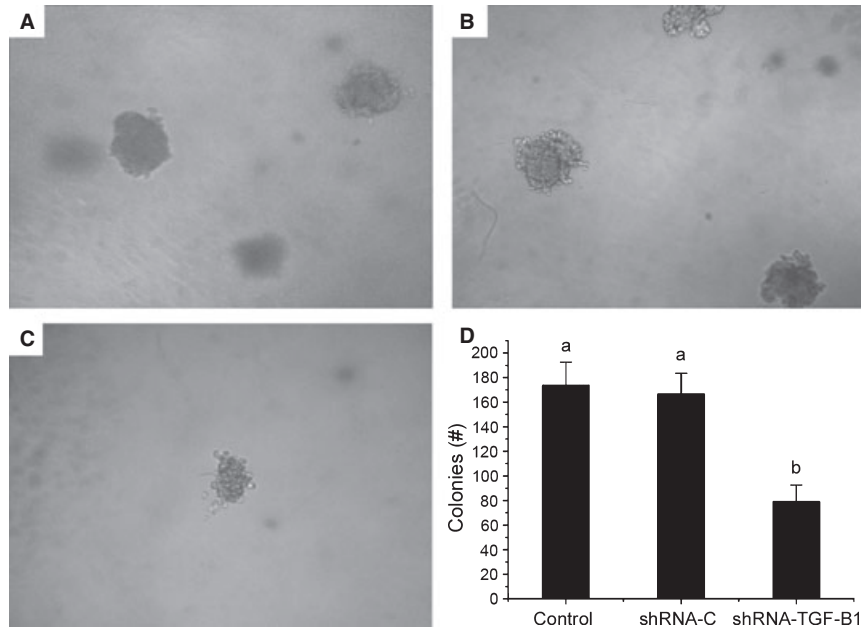
The effect of TGF- $\beta$ 1 on the adhesive capacity of Ms cells was investigated in a Matrigel adhesion assay, as described (21). Wells from 96-well plates were covered with five-times-diluted Matrigel Basement Membrane Matrix (Becton Dickinson, Franklin Lakes, NJ, USA), and incubated at room temperature for 1 h. Coated wells were further incubated for 1 h with DMEM plus 10 g/l bovine serum albumin (BSA) to saturate any substrate-free space. Wells coated with 10 g/l BSA alone were used as a substrate control. MTT was prepared in PBS (5 mg/ml) and further diluted (10%) in DMEM. To analyze the ability of cells to adhere to Matrigel,  $1 \times 10^5$  cells in 0.1 ml DMEM with 10 g/l BSA were plated onto coated wells. After 1 h of incubation at 37°C, each well was washed twice in PBS to remove non-adherent cells. Attached cells were treated with 250  $\mu$ l sterile MTT dye for 4 h at 37°C. The excess of MTT solution was removed and DMSO was added and thoroughly mixed for 10 min. The absorbance of the DMSO solution was measured spectrometry at 570 nm. The rate of adhesion (%) = (A in experiment group/A in a BSA substrate control-1)  $\times$  100.

### *Migration assay*

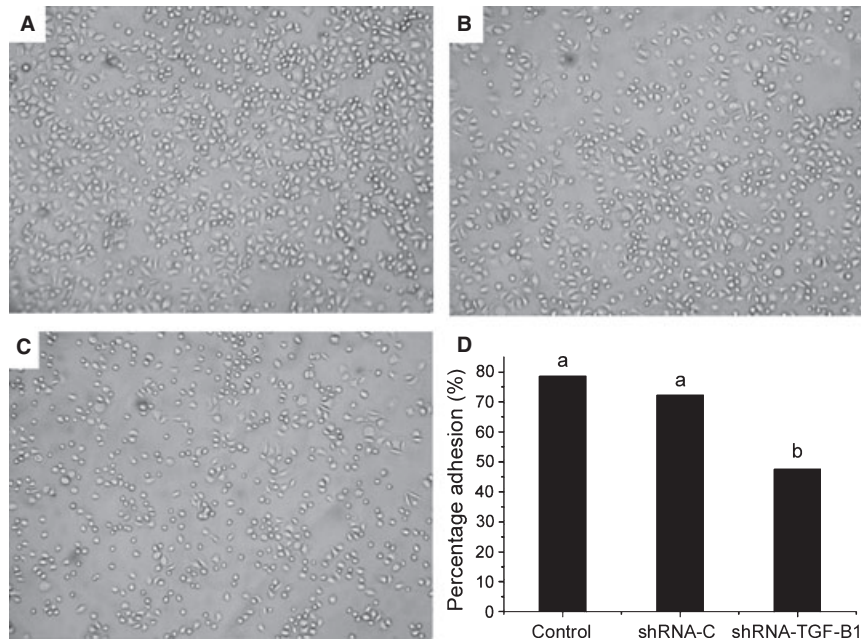
To determine the effects of TGF- $\beta$ 1 shRNA transfection on the motility of Ms cells, an *in vitro* scratch assay was used. Briefly,  $1 \times 10^5$  cells were seeded into 96-well plates in which were coated Matrigel in DMEM supplemented with 10% FBS. When cells reached confluence, the cell monolayer was then wounded by scraping the surface with a plastic tip to leave an approximately 600- $\mu$ m-wide clearing. After wounding, cultures were washed in DMEM plus 10 g/l BSA three times to remove residual FBS and cells detached during the wounding process and then cultured in DMEM with 10 g/l BSA. After 24-h incubation, medium was replaced with fresh DMEM supplemented with 10% FBS. Cell motility was determined by measuring the distance of cell movement toward the wounded space after 48-h culture.

### *Matrigel invasion assay*

The invasion ability of the cells was assayed using a Millicell (Millipore) separated by a polycarbonate filters (8  $\mu$ m pore size). The polycarbonate filter was coated with 75  $\mu$ l of Matrigel diluted ( $5 \times$ ) in cold DMEM and incubated at room temperature for 1 h. The 600  $\mu$ l NIH3T3 fibroblast-conditioned medium (obtained by incubation of NIH3T3 cells for 24 h in serum-free DMEM) was used as a chemoattractant in the lower compartment. DMEM was used as negative control. The coated Millicell were further incubated for 1 h with assay medium (DMEM with 10 g/l BSA) to saturate any substrate-free space. Confluent cultures from three group cells were detached and resuspended in DMEM.



**Figure 1** shRNA-TGF- $\beta$ 1 affects characteristics of anchorage-independent growth from MS cells in soft agar after 14 days. Cells were plated in 24-well plates at 500 cells per well. Representative images of colonies arising from (A) control, (B) shRNA-C, and (C) shRNA-TGF- $\beta$ 1 (100  $\times$ ). (D) The diameter of colonies greater than 60  $\mu$ m was scored. (a: Control compared to shRNA-C,  $P > 0.05$ ; b: shRNA-TGF- $\beta$ 1 compared to control and shRNA-C,  $P < 0.05$ .)



**Figure 2** The adherence assay using Matrigel substrates for (A) control, (B) shRNA-C, and (C) shRNA-TGF- $\beta$ 1. Cell adhesiveness was observed under inverted microscope (100  $\times$ ). (D) The strength of attachment to substratum was estimated by the rate of adhesion. (a: Control compared to shRNA-C,  $P > 0.05$ ; b: shRNA-TGF- $\beta$ 1 compared to Control and shRNA-C,  $P < 0.05$ .)

A suspension of tumor cells ( $1 \times 10^5$  cells/well) was placed in the upper compartment. After 48-h culture, the cells attached to the upper surface of the filters were removed by wiping with a cotton swab, and those attached to the lower surface of the filters were fixed with 10% neutral buffered formalin and stained with

Trypan blue. Then the migrated cells were counted in 10 randomly selected fields per filter at 200 magnification.

*Gelatin zymography analysis*

Analysis of MMP-2 and MMP-9 was conducted using modified sodium dodecyl sulfate polyacrylamide gel



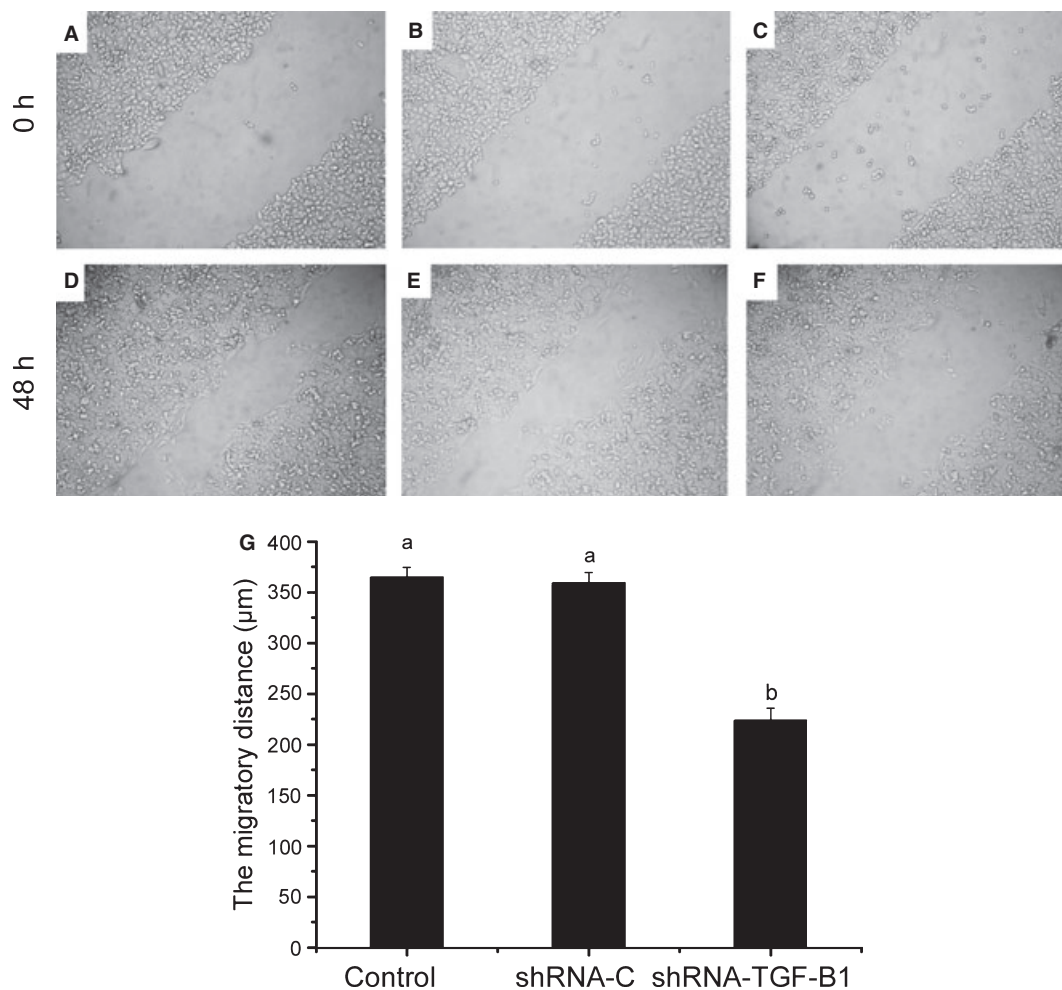
electrophoresis (SDS-PAGE). This technique was performed on 10% polyacrylamide gel copolymerized with 2 mg/ml gelatin type I. Cells were placed in 25-cm<sup>2</sup> culture vessels and incubated with DMEM containing 10% FBS until they reached 70% confluence. Cells were then washed and fed with fresh serum-free medium. The conditioned media were collected after 24-h incubation, centrifuged, and concentrated. Serum-free medium conditioned by U937 cells, which secrete MMP-2 and MMP-9, was used as positive control for zymographic analysis. After electrophoresis, gels were washed three times in 2.5% (v/v) Triton X-100 for 30 min at room temperature to remove SDS and allowed proteins to renature and then transferred to a substrate buffer that contained 0.1 M Tris-HCl/0.2 M NaCl/10 mM CaCl<sub>2</sub> buffer (pH 8.0) at 37°C for 24 h. Clear bands of gelatinolytic activity were visualized after staining the gels with 0.25% (w/v) Coomassie Brilliant Blue R-250 and then were destained by 90% methanol and 10% acetic acid. Enzyme-digested regions were visualized as clear bands on a blue background.

#### In vivo metastasis assay

All the experiments were carried out using 6- to 8-week-old Balb/c nu/nu nude mice obtained from the Animal Care Area of the Silaike in Shanghai. Cells were washed and resuspended in serum-free DMEM before inoculation. In each of the nude mice ( $n = 8$ ),  $2 \times 10^5$  cells in 200  $\mu$ l culture medium were inoculated into tail vein. Six weeks after inoculation, all animals were euthanized and the lungs were removed. Anti-tumor effect was evaluated by counting the number of metastatic tumor clones on the surface of the lungs. All animal experiments were approved by the local animal ethics committee at Medical University.

#### Statistical analysis

The results were expressed as the mean  $\pm$  standard deviation (SD) from triplicate specimens per condition. Three independent experiments were performed to verify reproducibility of the data. One-way ANOVA was performed using the SPSS 13.0 software.  $P$ -values were considered significant at the 0.05 level, and all tests were two-sided.



**Figure 3** Suppression of TGF- $\beta$ 1 decreased migration of Ms cells after 48 h. (A, D) control, (B, E) shRNA-C, and (C, F) shRNA-TGF- $\beta$ 1. The micrographs of cell migrate under inverted microscope (100 $\times$ ). (G) The migratory distance of three group cells. (a: Control compared to shRNA-C,  $P > 0.05$ ; b: shRNA-TGF- $\beta$ 1 compared to Control and shRNA-C,  $P < 0.05$ .)

## Results

### *TGF- $\beta$ 1 is required for Ms anchorage*

It has been reported that the ability of cells to form colonies in a semisolid medium is generally considered as a marker of anchorage independence and is positively associated with metastatic potential (22). Here, the effect of TGF- $\beta$ 1 on colony formation of mucoepidermoid carcinoma cells was determined by soft agar assay. Colonies with more than 60  $\mu$ m in diameter were scored. After 2 weeks, shRNA-TGF- $\beta$ 1-transfected Ms cells (Ms-shRNA-TGF- $\beta$ 1) showed reduced capacity of colony formation in soft agar (Fig. 1). TGF- $\beta$ 1 shRNA inhibited the colony formation by 54.5% and 52.6%, as compared to control and shRNA-C. There was no significant difference in colony formation between control and shRNA-C.

The ability of cells to adhere to the extracellular matrix was analyzed in a Matrigel adhesion assay. The Ms-shRNA-TGF- $\beta$ 1 cells had less adhesiveness to Matrigel than the control cells (Fig. 2). Notably, adhesion to plastic surfaces was similar in three group cells (data not shown).

### *Silencing of TGF- $\beta$ 1 expression inhibits migration of Ms cells*

An *in vitro* scratch assay was performed to analyze the effect of TGF- $\beta$ 1 on migration of Ms cells. Cells in control and shRNA-C groups migrated rapidly, while Ms-shRNA-TGF- $\beta$ 1 cells showed delayed migration (Fig. 3). Forty-eight hours after the initial wounding, the control, shRNA-C, and Ms-shRNA-TGF- $\beta$ 1 cells covered 60.8%, 60.0%, and 37.3% of the cell-free area, respectively.

### *TGF- $\beta$ 1 regulates the invasive phenotype of Ms cells*

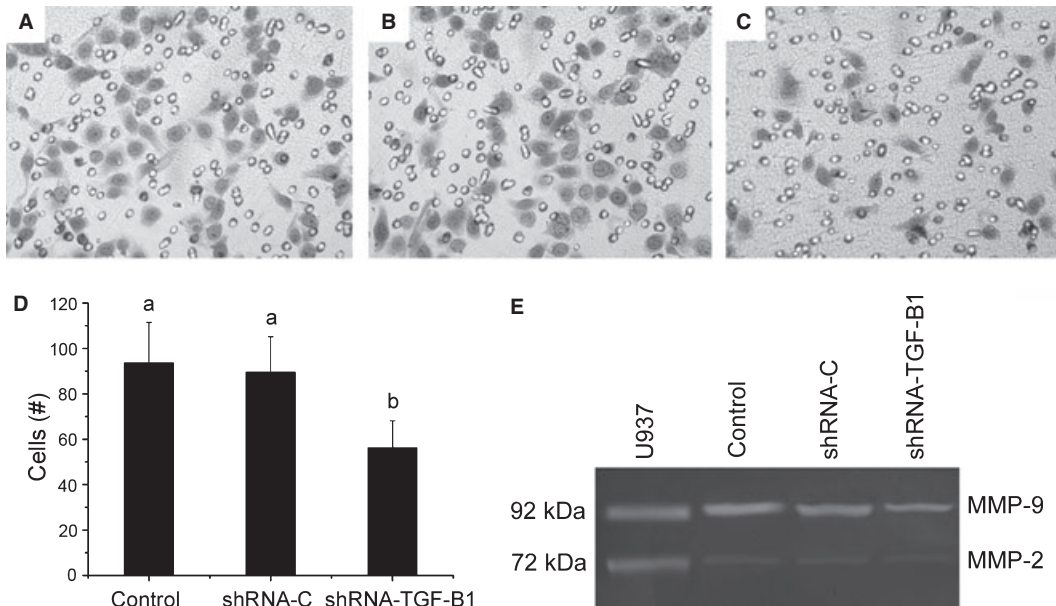
To evaluate the role of TGF- $\beta$ 1 on mucoepidermoid carcinoma cell invasion, we performed the Millicell assay with Ms cells. Cells with downregulated TGF- $\beta$ 1 showed markedly less invasive potential than control cells (Fig. 4A–D). To understand potential mechanisms responsible for the effect of TGF- $\beta$ 1 on invasion of mucoepidermoid carcinoma cells, we evaluate the impact of TGF- $\beta$ 1 silence on the amounts of MMP-2 and MMP-9 enzymatic by gelatin zymography. Both MMP-9 (92 kDa) and MMP-2 (72 kDa) were found in Ms cells. However, the amount of MMP9 was significantly higher than the activity of MMP-2 in control cells. The amount of MMP-9 was observed to be reduced markedly in MS-shRNA-TGF- $\beta$ 1 compared with control and shRNA-C. TGF- $\beta$ 1 silencing correlated with reduced MMP-9 activity in Ms cells, but did not change MMP-2 activity (Fig. 4E).

### *TGF- $\beta$ 1 silencing inhibited the metastatic potential of Ms cells*

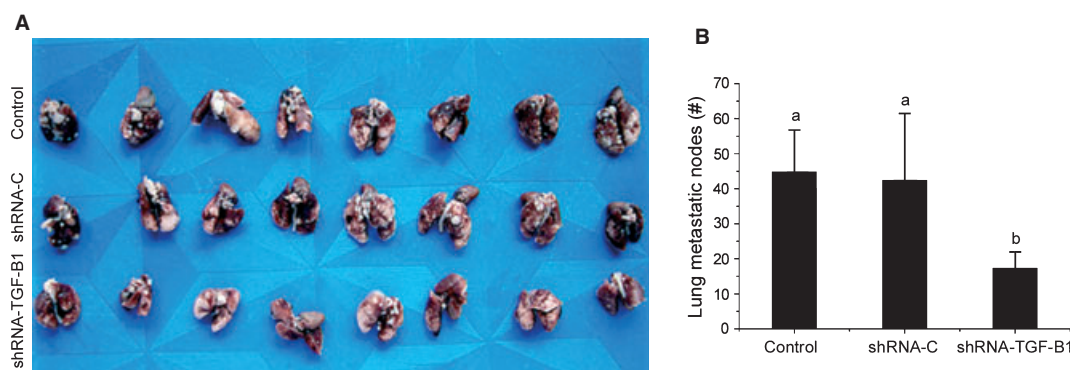
To investigate the effect of TGF- $\beta$ 1 on Ms metastasis, we injected cells into the tail vein of nude mice and evaluated the presence of metastatic nodes in the lungs after 6 weeks (Fig. 5). We observed a significant reduction in the number of metastatic nodes in the mice that received Ms cells stably transduced with TGF- $\beta$ 1, as compared to controls (Fig. 5B).

## Discussion

Tumor progression is the process by which tumor cells acquire malignant properties, such as aggressive growth,



**Figure 4** With matrigel polycarbonate filter,  $1 \times 10^5$  cells were seeded in Millicell upper chamber. Representative images of the cells that invade Matrigel (200 $\times$ ). (A) control, (B) shRNA-C, and (C) shRNA-TGF- $\beta$ 1. (D) The number of cells that invaded through the chamber was averaged. The invading cells were counted as a sum of 10 fields of vision under a microscope. (a: Control compared to shRNA-C,  $P > 0.05$ ; b: shRNA-TGF- $\beta$ 1 compared to control and shRNA-C,  $P < 0.05$ .) (E) The amounts of MMP-2 and MMP-9 were examined by 10% SDS-PAGE gelatin zymography.



**Figure 5** A volume  $2 \times 10^5/200 \mu\text{l}$  was injected into each of the nude mice through tail vein. (A) The macroscopic view of lung metastatic nodes after inoculation with cancer cells. (B) The number of lung metastatic nodes. (a: Control compared to shRNA-C,  $P > 0.05$ ; b: shRNA-TGF- $\beta$ 1 compared to control and shRNA-C,  $P < 0.05$ .)

invasion, and metastasis. Tumor islands are often surrounded by basement membrane, which suggests that the cellular capacity of degrading matrix components is an essential regulator of tumor progression. The ability of MMP-2 and MMP-9 to initiate basement membrane degradation and further to degrade the collagenous and non-collagenous components of the ECM are essential in tumor cell metastasis (23). In head and neck squamous cell carcinoma cell lines, it has been shown that TGF- $\beta$  induces MMP-9 expression (24). However, the effect of TGF- $\beta$ 1 in the invasive and metastatic potential of mucoepidermoid carcinoma cells has not been studied before.

To define the role of TGF- $\beta$ 1 in metastasis and invasion more precisely, we have used here several approaches involving gene silencing. Ms are mucoepidermoid carcinoma cells that exhibit a highly metastatic potential (19). Our work has previously demonstrated that TGF- $\beta$ 1 is strongly expressed in Ms cells (18). In this study, the inhibitory effect of suppressing TGF- $\beta$ 1 by RNA silencing on the metastatic and invasive potential of the mucoepidermoid carcinoma Ms cell line was clearly demonstrated. Our results showed that stably suppressing TGF- $\beta$ 1 inhibited cell motility, substratum adhesion, transmembrane invasion ability, and MMP-9 activation. The results also suggested that increased activation of MMP-9, but not MMP-2, by TGF- $\beta$ 1 correlates with the invasive potential of salivary gland mucoepidermoid carcinoma cells. We postulate here that TGF- $\beta$ 1 is involved in the maintenance of MMP-9 enzymatic activity in the highly metastatic Ms cells. Collectively, these observations identify TGF- $\beta$ 1 as a key regulator of the invasive and metastatic potential of mucoepidermoid carcinoma cells and suggest that patients with this malignancy may benefit from targeted therapies blocking effectors of this signaling pathway.

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## Acknowledgements

This work was supported by the grants from National Natural Science Foundation of PR China (No. 30371551) the Fundamental Research Funds for the Central University (No. Lzujbky-2009-96) and the Medical Subject Fund of Lanzhou University (No. LZUYX200707).

## Conflict of interest

No conflict of interest.