

# Role of matrix metalloproteinase-9 in transforming growth factor- $\beta$ 1-induced epithelial–mesenchymal transition in esophageal squamous cell carcinoma

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**Abstract:** Epithelial–mesenchymal transition (EMT) is thought to be a crucial event during the early metastasis of tumor cells. Transforming growth factor (TGF)- $\beta$ 1 is involved in the process of EMT in a variety of human malignancies. Matrix metalloproteinase (MMP)-9 plays an important role in tumor invasion and metastasis, and its expression is regulated by various growth factors, including TGF- $\beta$ 1, in different cell types. To date, the role of MMP-9 in TGF- $\beta$ 1-induced EMT in esophageal squamous cell carcinoma (ESCC) remains unclear. In this study, we aimed to elucidate the mechanism underlying MMP-9-mediated TGF- $\beta$ 1 induction of EMT in ESCC. We analyzed the expression of MMP-9, E-cadherin, and vimentin, in ESCC cells (EC-1), before and after the treatment with exogenous TGF- $\beta$ 1 or a broad spectrum MMP inhibitor, GM6001. Additionally, we analyzed the activity of MMP-9 in these cells and performed *MMP-9* knockdown experiments. The results obtained in this study demonstrated that the treatment of EC-1 cells with TGF- $\beta$ 1 can induce EMT, together with the upregulation of vimentin and downregulation of E-cadherin expression in a time-dependent manner. The treatment with GM6001 was shown to attenuate TGF- $\beta$ 1-induced EMT. Furthermore, the exposure of EC-1 cells to TGF- $\beta$ 1 increased the expression and activity of MMP-9, while *MMP-9* knockdown blocked TGF- $\beta$ 1-induced EMT and inhibited cell invasiveness and migration. Additionally, treatment with the recombinant human MMP-9 was shown to induce EMT and enhance ESCC cell invasion and metastasis. The obtained data suggest that the regulation of MMP-9 by TGF- $\beta$ 1 may represent a novel mechanism underlying TGF- $\beta$ 1-induced EMT in ESCC.

**Keywords:** epithelial–mesenchymal transition, esophageal squamous cell carcinoma, matrix metalloproteinase-9, transforming growth factor- $\beta$ 1

## Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the predominant malignant tumors in the world. Its incidence is very high in China, especially in Henan Province. Although a large number of different antitumor therapies are used for the treatment of ESCC patients, the prognosis is usually poor.<sup>1,2</sup>

Among the most prominent features of malignant tumors are the invasion and metastasis of cancer cells. Numerous studies showed that the epithelial–mesenchymal transition (EMT) is an important process in the embryonic development, which also plays a key role in the invasion and metastasis of a variety of malignant tumors originating from the epithelial cells.<sup>3,4</sup> EMT is characterized by the dissociation of cell-to-cell contacts and the loss of cell adhesion and cell polarity, resulting in cells that

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gain mesenchymal properties.<sup>5</sup> During the process of EMT, epithelial markers, including E-cadherin and  $\beta$ -catenin, were shown to be downregulated, while the markers related to the mesenchymal phenotype, including vimentin, N-cadherin, and fibronectin, are upregulated,<sup>5</sup> which results in the induction of migratory and invasive properties of cells.<sup>6</sup> Therefore, it is very important to elucidate the mechanism underlying the EMT during the invasion and metastasis of ESCCs.

Transforming growth factor (TGF)- $\beta$ 1 is one of the pivotal factors regulating EMT, which is responsible for the initiation and maintenance of the EMT.<sup>6–8</sup> Recently, a number of studies demonstrated that TGF- $\beta$ 1 is involved in the EMT in a wide variety of human malignancies, including oral, liver, pancreatic, and esophageal cancers.<sup>9–13</sup> Furthermore, it was recently demonstrated that TGF- $\beta$ 1 can lead to an increase in the expression of matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, in breast and oral cancers.<sup>14–17</sup> However, the mechanism of TGF- $\beta$ 1-induced EMT has not been completely understood. MMPs represent a family of zinc-containing proteolytic enzymes that break down the protein components of the extracellular matrix (ECM) and endothelial cell basement membrane.<sup>18</sup> MMPs and their specific inhibitors (TIMPs) play an important role in the progression of different types of solid and hematological malignancies by regulating cell growth, cancer cell activation, and immune functions.<sup>19,20</sup> In addition to the ability of MMPs to remodel ECM, some of these molecules, such as MMP-3, MMP-9, MMP-14, and MMP-28, are known to induce EMT directly.<sup>21–25</sup> However, to date, the role of MMP-9 in TGF- $\beta$ 1-induced EMT in ESCC remains unclear.

In this study, we showed that MMP-9 is involved in TGF- $\beta$ 1-induced EMT. MMP-9 is a downstream mediator of TGF- $\beta$ 1, and we demonstrated that the inhibition of its expression prevents TGF- $\beta$ 1-induced EMT and reduces cell invasion and metastasis. Furthermore, the upregulation of MMP-9 expression is sufficient to induce EMT and enhance cell invasion and metastasis in EC-1 cells. These data indicate that TGF- $\beta$ 1-induced EMT is mediated by MMP-9, which represents a previously unknown mechanism underlying TGF- $\beta$ 1-induced EMT in ESCC.

## Materials and methods

### Cell lines and cell culture

EC-1 cells (kindly provided by Professor Shihua Cao, University of Hong Kong) were cultured in RPMI 1640 medium (Solarbio, Beijing, People's Republic of China) with 10% fetal bovine serum (FBS; Gemini Bio, West Sacramento, CA, USA), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin and incubated at 37°C in a humid atmosphere

with 5% CO<sub>2</sub>. The cells were originally purchased from ATCC (Manassas, VA, USA) and that the research had been reviewed and approved by the Zhengzhou University Life Science Ethics Review Committee.

### Cell treatment

EC-1 cells were seeded at a low density in six-well plates and maintained for 24 h in the complete medium. Afterward, they were starved overnight and washed in phosphate-buffered saline (PBS), and the medium was replaced with serum-free RPMI 1640 medium, serum-free medium containing 10 ng/mL recombinant human TGF- $\beta$ 1 (PeproTech, Rocky Hill, NJ, USA), serum-free medium containing 25  $\mu$ mol/L broad spectrum MMP inhibitor GM6001 (Millipore, Darmstadt, Germany), and serum-free medium containing both TGF- $\beta$ 1 and GM6001. Additionally, to explore the role of MMP-9 in EMT, EC-1 cells were seeded at low density ( $2 \times 10^5$ ) in six-well plates for 24 h in the complete medium and starved overnight. Recombinant human MMP-9 protein (R&D Systems, Minneapolis, MN, USA) was activated using p-aminophenylmercuric acetate (APMA; Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 1 mM at 37°C overnight, and the cells treated with this protein were incubated at 37°C for 48 h. Gelatin zymography was used to determine the presence of the active form of MMP-9.

### Stable transfection

Human MMP-9 short hairpin RNA (shRNA) (GenePharma, Shanghai, People's Republic of China) was used, and the target sequence was 5'-GCAGATTCCAAACCTTTGATT-3'. Briefly, 1 day before transfection,  $2 \times 10^5$  cells/mL were seeded into six-well plates. When the confluence of 60%–80% was reached, EC-1 cells were transfected with 3  $\mu$ g of MMP-9 shRNA or control shRNA, using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and serum-free medium, according to the manufacturer's instructions. After 6 h of incubation, the medium was replaced with fresh RPMI 1640 with 10% FBS, or medium containing TGF- $\beta$ 1, and the cells were cultured for additional 48 h.

### Real-time polymerase chain reaction (PCR)

Total RNA was isolated from EC-1 cells incubated with different molecules using RNAPure total RNA Kit (Aidlab, Beijing, People's Republic of China), according to the manufacturer's instructions. RNA was reverse transcribed into complementary DNA (cDNA) using HiScript II Q RT SuperMix Kit (Vazyme Biotech, Jiangsu Sheng, People's Republic of China). Primer sequences used in

this study are as follows: MMP-9 (forward [F]: 5'-CCAACTACGACACCGACGAC-3', reverse [R]: 5'-TGGAAGATGAATGGAAACTGG-3'), E-cadherin (F: 5'-TGATTC TGCTGCTCTTGCTG-3', R: 5'-CAAAGTCCTGG TCCTCTTCTCC-3'), vimentin (F: 5'-ATGTGGATGAGT CCAAGCCTGAC-3', R: 5'-GAGTGGGTATCAACCAGAG GGAGT-3'), and  $\beta$ -actin (F: 5'-TGACGTGGACATC CGCAACAAAG-3', R: 5'-CTGGAAGGTGGACAG CGAGG-3'). All reactions were performed using 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), with qPCR SYBR Green Master Mix (Vazyme Biotech), and normalized to  $\beta$ -actin messenger RNA (mRNA) levels.

## Western blot analysis

Total proteins were isolated from the treated EC-1 cells using RIPA buffer (Solarbio) and stored at  $-80^{\circ}\text{C}$ , according to the manufacturer's instructions. Protein concentration was determined by measuring the absorbance (optical density, OD562), using BCA Protein Assay Kit (Biotech Well, Shanghai, People's Republic of China). Briefly, 40  $\mu\text{g}$  of total protein isolated from each sample was separated on 10%–15% sodium dodecyl sulfate (SDS)-polyacrylamide gels (PAGE) and then transferred to polyvinylidene difluoride membrane (Millipore) at 100 mV for 1.5–2 h. The blots were blocked and incubated with primary antibodies overnight at  $4^{\circ}\text{C}$ , using anti-MMP-9 (1:200; Cell Signaling Technology, Danvers, MA, USA), anti-E-cadherin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-vimentin (1:400), and anti- $\beta$ -actin (1:1,000; ZsBio, Beijing, People's Republic of China) antibodies. After washing thoroughly ( $3\times 10$  min) with Tris-buffered saline–Tween 20 (TBST), the samples were incubated with goat anti-mouse secondary immunoglobulin G (IgG) (1:2,000; Bioss, Shanghai, People's Republic of China) or goat anti-rabbit secondary IgG (1:2,000; Bioss) for 2 h at room temperature. The bands were detected using enhanced chemiluminescence method (BeyoECL Plus). All experiments were repeated three times.

## Immunofluorescence analysis

ESCC EC-1 cells were seeded in six-well plates containing glass coverslips, and they were incubated overnight. After incubating EC-1 cells with different treatments for additional 48 h, cells were washed three times with PBS. Following this, they were fixed in 4% paraformaldehyde for 30 min and then permeabilized in 0.4% Triton X-100 for 10 min, washed three times with PBS, and blocked with 5% bovine serum albumin (BSA) for 30 min at  $37^{\circ}\text{C}$ . Next, the samples were incubated with primary anti-E-cadherin (1:100) and

anti-vimentin (1:100) antibodies, at  $4^{\circ}\text{C}$  overnight, followed by the incubation with a CY-3-conjugated goat anti-rabbit secondary antibody (Biotech Well) for 2 h at  $37^{\circ}\text{C}$  and DAPI (Biotech Well) for 5 min at room temperature, in order to detect E-cadherin, vimentin, and cell nuclei. The cells were visualized using fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

## Gelatin zymography

EC-1 cells were treated with TGF- $\beta$ 1, GM6001, or the combination of these two molecules for 48 h. Afterward, MMP-9 activity was determined by gelatin zymography, using Gelatin Zymo Electrophoresis Kit (Genmed Scientifics, Arlington, MA, USA), according to the manufacturer's instructions.

## Cell invasion assay

Cell invasion assay was performed using transwell inserts (Costar Transwell, Corning, People's Republic of China). Matrigel-coated polycarbonate membranes (8  $\mu\text{m}$  pore size) separated the upper and lower compartments of a transwell chamber. EC-1 cells, not transfected or transfected with MMP-9 shRNA, were incubated with previously described treatments, including serum-free medium, and afterward seeded into the upper compartments, while RPMI 1640 with 10% FBS was added to the lower compartments, as a chemoattractant to induce invasion. Afterward, the plates were incubated at  $37^{\circ}\text{C}$  for 48 h, the cells were fixed with methanol, stained for 20 min with 0.4% Trypan blue (Solarbio), and the number of cells that invaded through the membranes was counted using an optical microscope. Each experiment was performed in duplicate and repeated three times.

## Scratch assay

Cell migratory ability was examined using the scratch assay. EC-1 cells, untransfected or transfected with MMP-9 shRNA, were incubated with different treatments and seeded in six-well plates. When 80%–100% of confluence was reached, cell monolayer was scratched with a 200  $\mu\text{L}$  tip at the center of the wells. Following this, the cells were incubated in the presence or absence of TGF- $\beta$ 1 for 48 h, washed with PBS, and RPMI 1640 containing 0.2% FBS was added. After 48 h, the images were captured at  $200\times$  magnification under a inverted light microscope. At least three replicates for each treatment were used.

## Statistical analysis

All statistical analyses were performed with analysis of variance (ANOVA) and two-tailed Student's *t*-test, using SPSS 17.0 (IBM Corp., Armonk, NY, USA) software

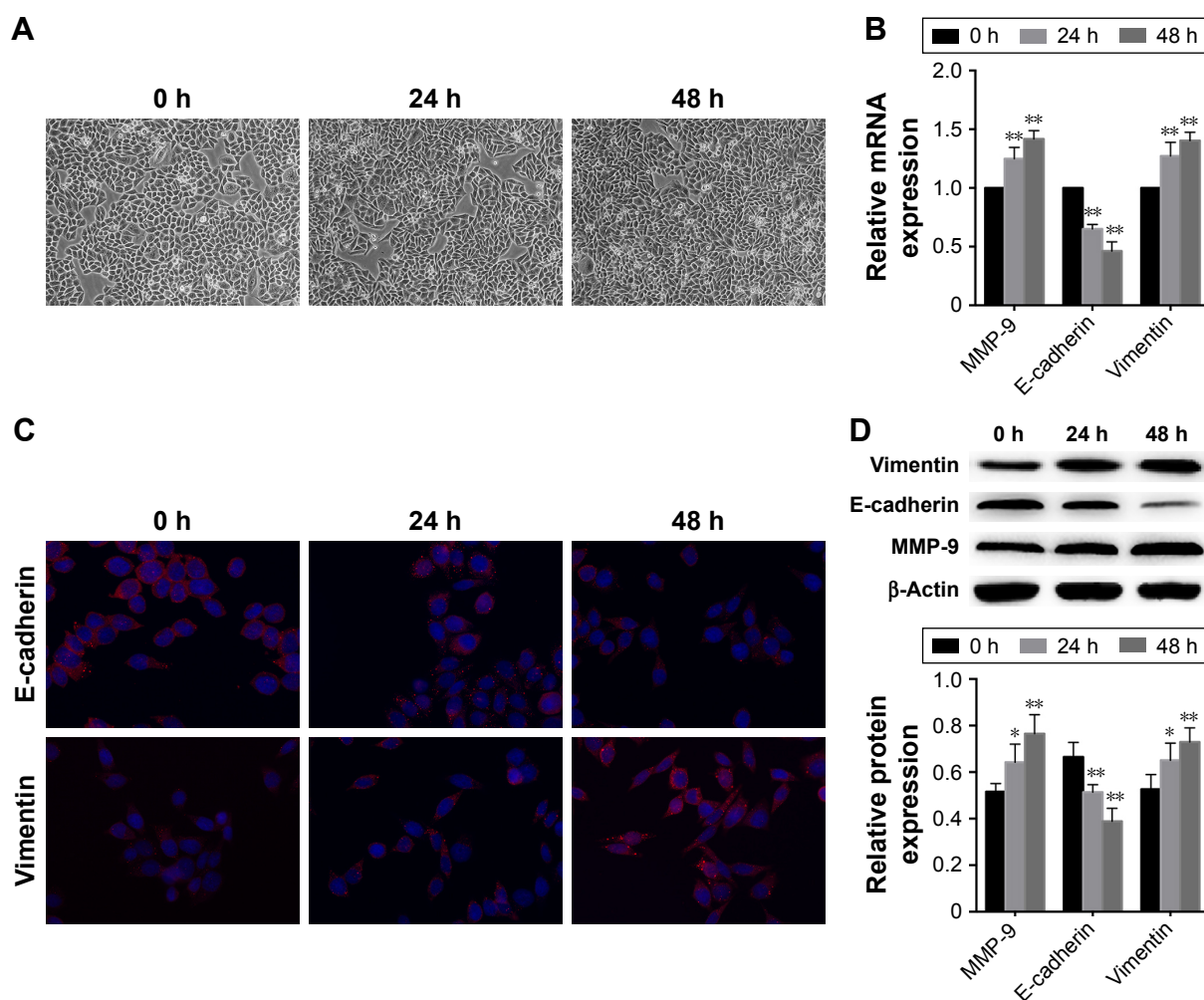
package. The results are presented as mean  $\pm$  standard deviation (SD) of three replicate assays.  $P$ -values  $<0.05$  were considered statistically significant.

## Results

### TGF- $\beta$ 1 induces change in the EMT-associated gene expression levels and alters ESCC cell morphology

In order to determine whether TGF- $\beta$ 1 induces EMT, we treated EC-1 cells with TGF- $\beta$ 1 and incubated them for 0 h, 24 h, and 48 h. Compared with the cells incubated for 0 h (control), EC-1 cells treated for 24 h or 48 h displayed morphological changes typical for EMT, as demonstrated

by phenotypic transformation from the endothelial cobblestone shape to fibroblastic spindle-shaped morphology (Figure 1A). Real-time PCR analysis showed that E-cadherin levels were downregulated and vimentin expression was upregulated in EC-1 cells treated with TGF- $\beta$ 1, in comparison with those in the cells treated for 0 h (control) (Figure 1B). Immunofluorescence and Western blot analysis indicated that TGF- $\beta$ 1 treatment significantly decreased E-cadherin expression and increased vimentin expression as well (Figure 1C and D). The changes in E-cadherin and vimentin expression, at both mRNA and protein levels, in response to TGF- $\beta$ 1, were shown to be considerably time-dependent.



**Figure 1** TGF- $\beta$ 1 induces EMT in EC-1 cells.

**Notes:** (A) Changes in cell morphology: compared with the cells incubated for 0 h (control), EC-1 cells treated for 24 h or 48 h displayed morphological changes typical for EMT, as demonstrated by the phenotypic transformation from the endothelial cobblestone shape to fibroblastic spindle-shaped morphology. (B) *MMP-9*, *E-cadherin*, and *vimentin* mRNA levels at 0 h, 24 h, and 48 h of incubation: the expression of *E-cadherin* was downregulated, while the levels of *MMP-9* and *vimentin* were upregulated in EC-1 cells treated with TGF- $\beta$ 1, in comparison with those at 0 h (control). (C) *E-cadherin* and *vimentin* immunofluorescence analysis in EC-1 cells treated with TGF- $\beta$ 1. (D) Western blot analysis of *MMP-9*, *E-cadherin*, and *vimentin* levels: the protein expression of *E-cadherin* was reduced, while the expressions of *MMP-9* and *vimentin* were increased in EC-1 cells treated with TGF- $\beta$ 1, in comparison with those at 0 h (control).  $\beta$ -Actin was used as an internal control. Scale bar: 50  $\mu$ m. Original magnification,  $\times 200$ . The data in (B and D) represent mean  $\pm$  SD of the results obtained in three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the controls.

**Abbreviations:** TGF, transforming growth factor; EMT, epithelial-mesenchymal transition; mRNA, messenger RNA; MMP, matrix metalloproteinase; SD, standard deviation.

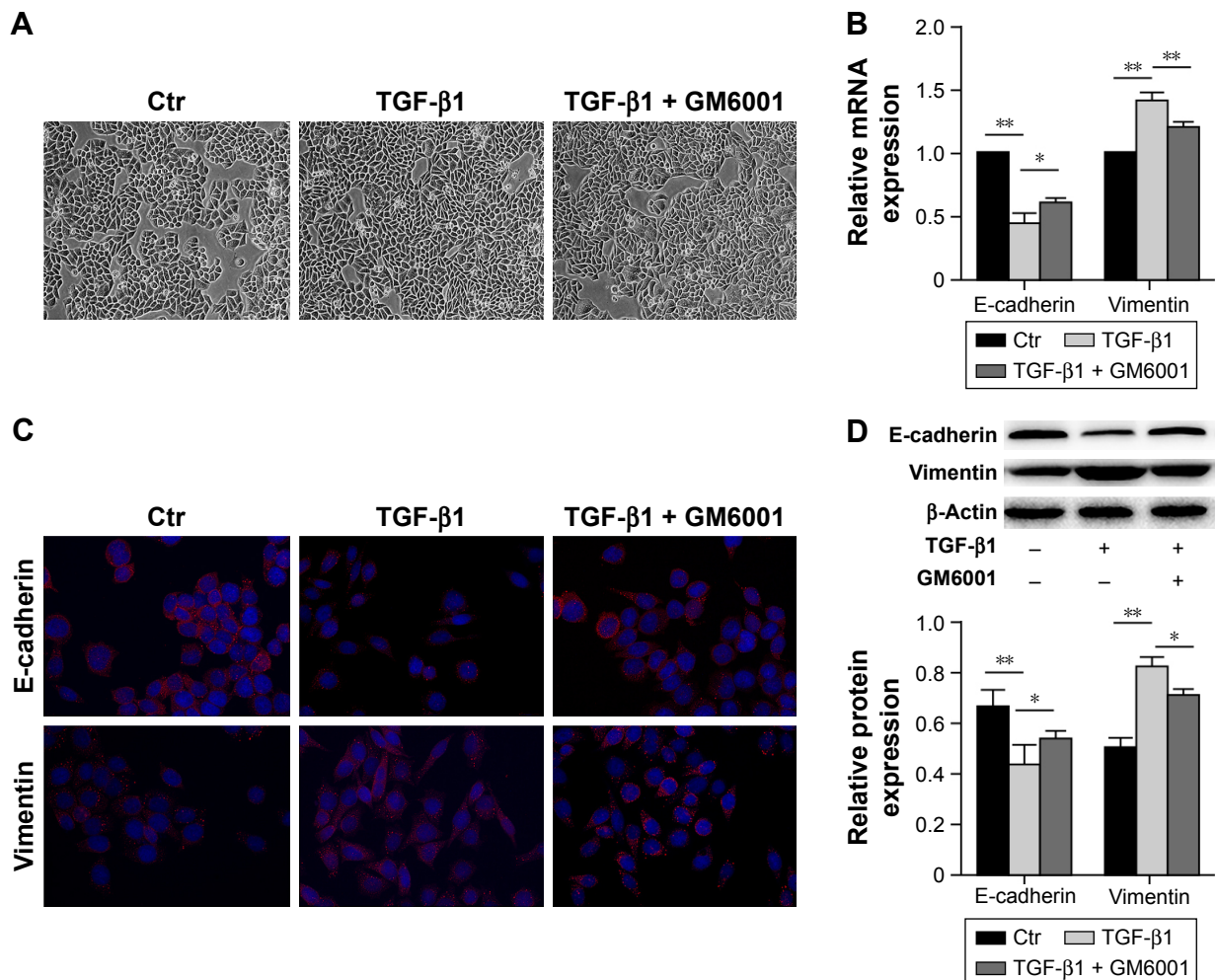
## TGF- $\beta$ 1-induced EMT is abrogated by MMP inhibitor GM6001

To elucidate whether MMPs mediate TGF- $\beta$ 1-induced EMT, a broad spectrum MMP inhibitor GM6001 was used. Following the treatment of cells with this inhibitor for 48 h, we observed that TGF- $\beta$ 1-induced EMT was abrogated. EC-1 cells generally maintained endothelial cobblestone shape, but some cells showed fibroblastic spindle-shaped morphology (Figure 2A). Real-time PCR analysis showed that GM6001 inhibited the TGF- $\beta$ 1-induced decrease in E-cadherin expression and increase in vimentin expression compared with those in the TGF- $\beta$ 1-treated cells (Figure 2B). Consistent with this, immunofluorescence staining and Western blot analysis

showed that the downregulation of E-cadherin and the induction of vimentin expression were inhibited by GM6001 (Figure 2C and D).

## MMP-9 mediates TGF- $\beta$ 1-induced EMT in ESCC

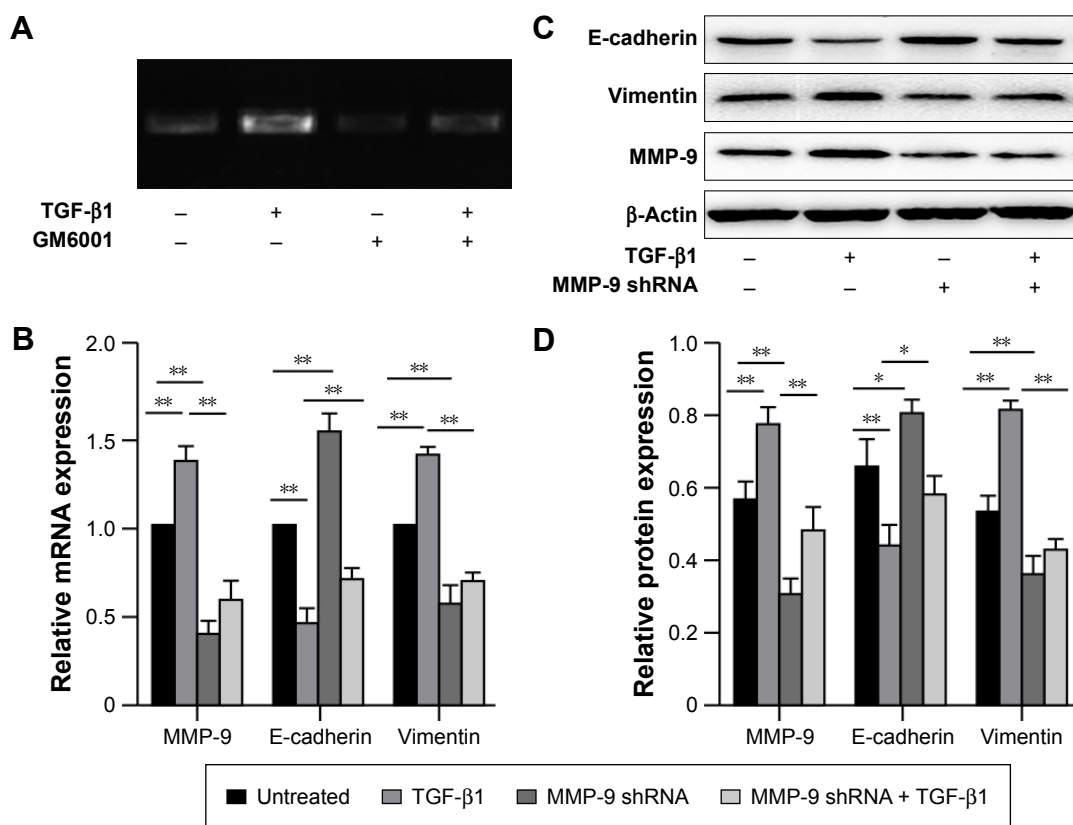
Gelatin zymography showed that MMP-9 activity was significantly increased following the treatment with TGF- $\beta$ 1. However, after the cells were treated with GM6001, MMP-9 activity was shown to be reduced (Figure 3A). Furthermore, real-time PCR and Western blot analysis demonstrated that a significant increase in MMP-9 mRNA and protein levels is induced in EC-1 cells treated with TGF- $\beta$ 1 in comparison with the untreated cells (Figure 1B and D).



**Figure 2** MMP inhibitor GM6001 inhibits TGF- $\beta$ 1-induced EMT.

**Notes:** (A) Changes in cell morphology: after the exposure to TGF- $\beta$ 1, EC-1 cells displayed morphological changes typical for EMT; GM6001 partially reversed this. (B) E-cadherin and vimentin mRNA levels: the expression of E-cadherin was upregulated and the expression of vimentin was downregulated in EC-1 cells treated with GM6001, in comparison with those in the TGF- $\beta$ 1-treated cells. (C) Immunofluorescence analysis of E-cadherin and vimentin expression in the treated cells and controls (Ctr). (D) Western blot analysis of E-cadherin and vimentin expression: the expression of E-cadherin was upregulated and the expression of vimentin was downregulated in EC-1 cells treated with GM6001, in comparison with those in the TGF- $\beta$ 1-treated cells.  $\beta$ -Actin was used as an internal control. Scale bar: 50 $\mu$ m. Original magnification,  $\times$ 200. The data in (B and D) represent mean  $\pm$  SD obtained in three independent experiments. \* $P$ <0.05, \*\* $P$ <0.01.

**Abbreviations:** MMP, matrix metalloproteinase; TGF, transforming growth factor; EMT, epithelial–mesenchymal transition; mRNA, messenger RNA; SD, standard deviation.



**Figure 3** MMP-9 mediates TGF- $\beta$ 1-induced EMT in ESCC.

**Notes:** (A) MMP-9 activity, determined in EC-1 cells treated for 48 h with medium, TGF- $\beta$ 1, GM6001, or the combination of TGF- $\beta$ 1 and GM6001. (B) MMP-9, E-cadherin, and vimentin expression in *MMP-9* knockdown cells, in the presence or absence of TGF- $\beta$ 1. (C and D) MMP-9, E-cadherin, and vimentin protein expression in EC-1 cells transfected with *MMP-9* shRNA in the presence or absence of TGF- $\beta$ 1.  $\beta$ -Actin was used as an internal control. The data in (B and D) represent mean  $\pm$  SD of three independent experiments. \* $P$ <0.05, \*\* $P$ <0.01.

**Abbreviations:** MMP, matrix metalloproteinase; TGF, transforming growth factor; EMT, epithelial–mesenchymal transition; ESCC, esophageal squamous cell carcinoma; shRNA, short hairpin RNA; SD, standard deviation; mRNA, messenger RNA.

### MMP-9 expression knockdown attenuates TGF- $\beta$ 1-induced EMT and inhibits cell invasiveness and migration

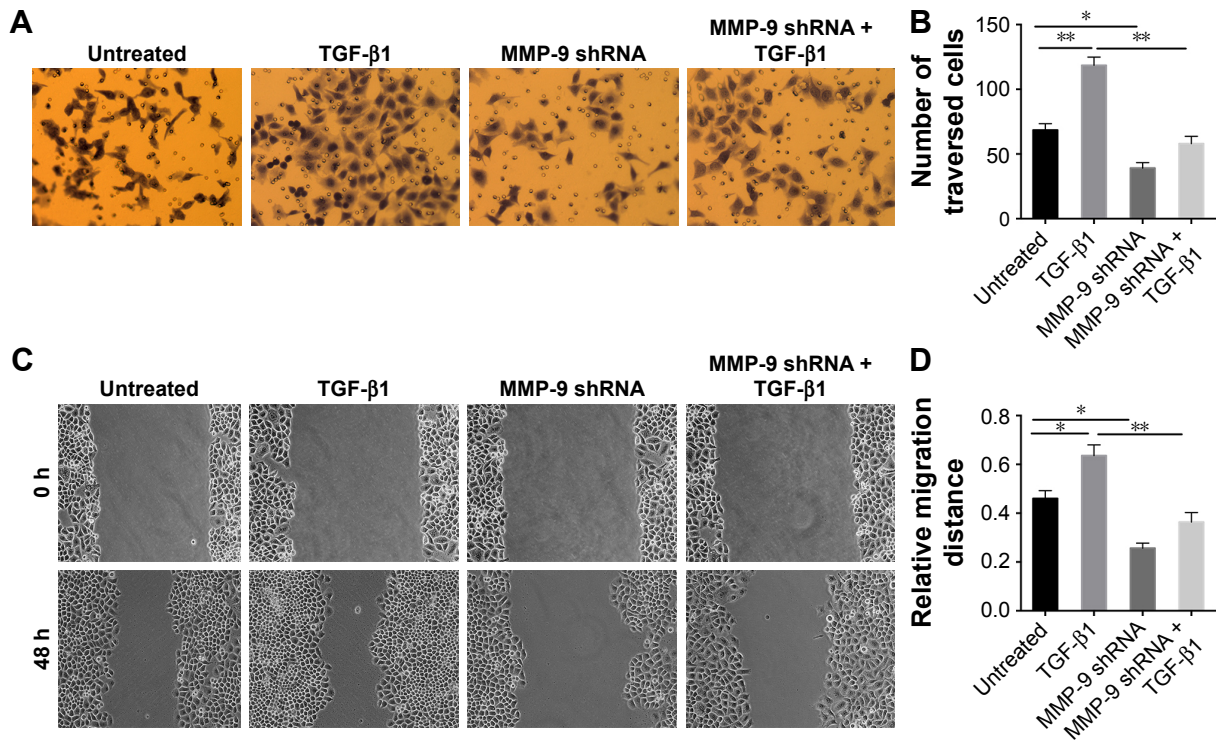
MMP-9 shRNA was used to inhibit the expression of endogenous MMP-9 in EC-1 cells. The inhibition of MMP-9 expression was confirmed in EC-1 cells at both mRNA and protein levels (Figure 3B–D). Furthermore, real-time PCR and Western blot analysis showed that *MMP-9* knockdown significantly increases the expression of E-cadherin and decreases vimentin expression compared with those in the untreated cells. MMP-9 knockdown was shown to inhibit the effects of TGF- $\beta$ 1 on E-cadherin and vimentin expression (Figure 3B–D).

Next, we wanted to determine the role of MMP-9 in TGF- $\beta$ 1-induced tumor cell invasion and metastasis, using invasion assay and scratch assay, respectively. These experiments demonstrated that *MMP-9* knockdown dramatically decreased invasive and metastatic potential of cells and blocked TGF- $\beta$ 1-induced cell invasion and metastasis (Figure 4A–D).

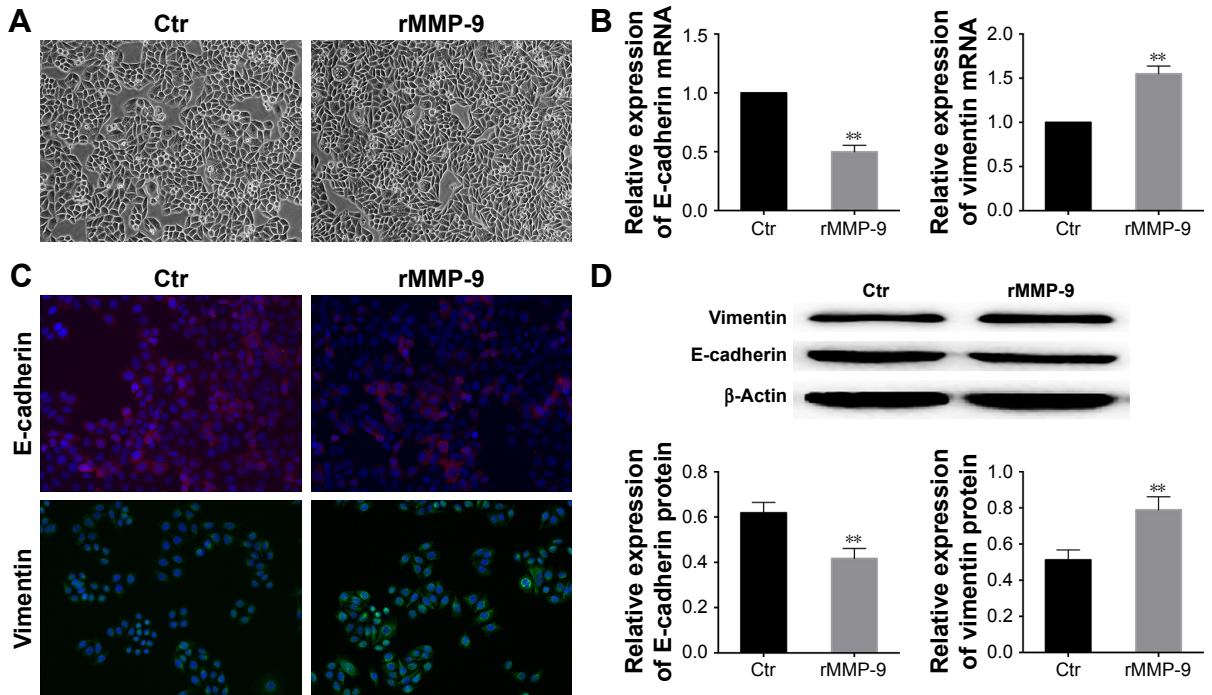
### MMP-9 induces EMT and enhances cell invasion and metastasis in ESCC

We investigated whether MMP-9 expression is sufficient to induce EMT. After treating the cells with recombinant human MMP-9 for 48 h, we observed morphological changes in cells, which changed from cuboid clustered epithelial cells to fibroblastic spindle-shaped cells (Figure 5A). Real-time PCR analysis showed a significant downregulation of E-cadherin and upregulation of vimentin expression (Figure 5B). Immunofluorescence and Western blot analysis showed similar results, and E-cadherin levels were shown to be decreased, while the levels of mesenchymal marker vimentin were increased in comparison with those in the control cells (Figure 5C and D).

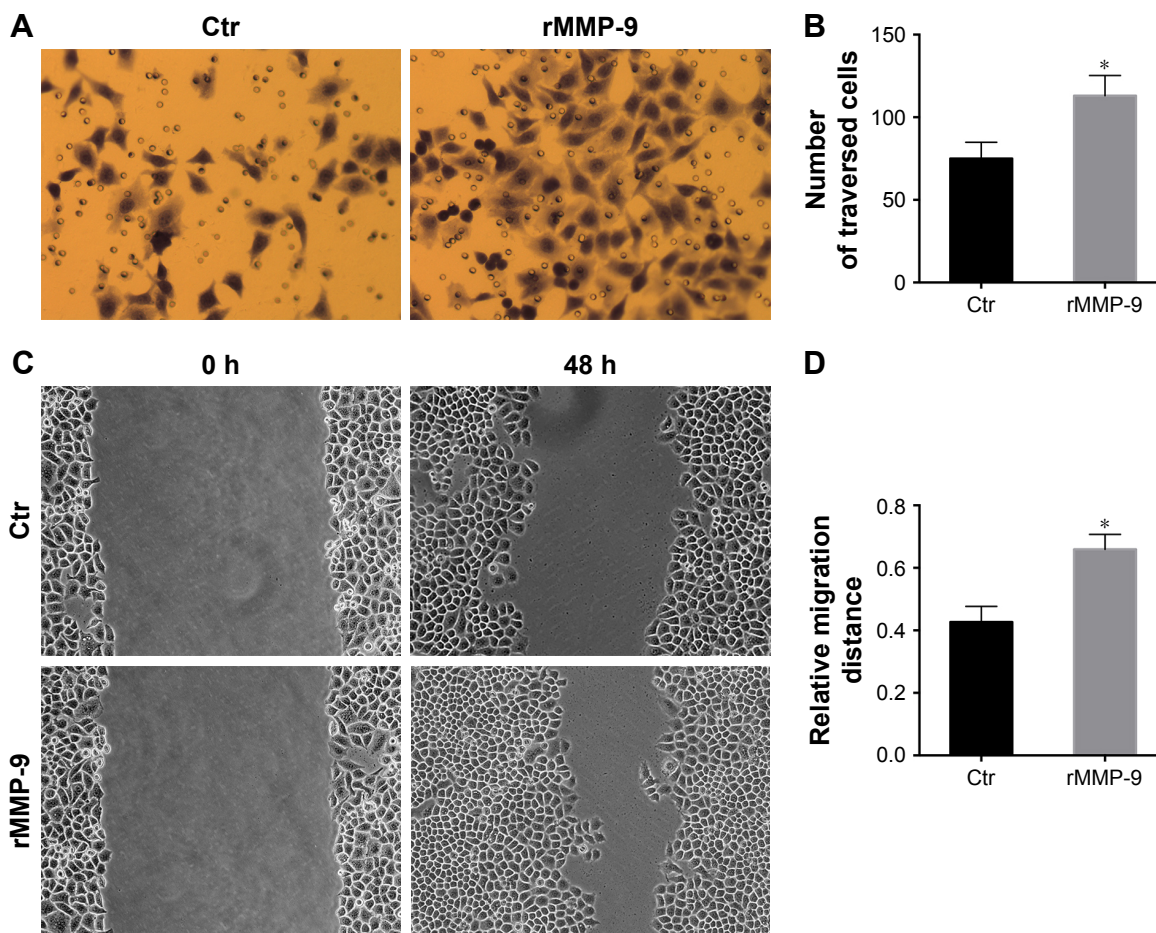
Cell invasion assays showed that the invasiveness of EC-1 cells treated with recombinant MMP-9 significantly increases, compared with that of the control cells (Figure 6A and B). Scratch assay results demonstrated that EC-1 cells treated with recombinant MMP-9 repopulated the wound area faster than the control cells (Figure 6C and D).



**Figure 4** MMP-9 knockdown inhibits invasive and metastatic potential of EC-1 cells and blocks TGF-β1-induced cell invasion and metastasis. **Notes:** (A) Representative images of cell invasion assay, showing cells migrating through the Matrigel-coated membranes. (B) Analysis of cell invasion assay results. (C) Representative images of cell scratch assay. (D) Analysis of scratch assay results. Scale bar: 50 μm. Original magnification, ×200. The data in (B and D) represent mean ± SD of the results obtained in three independent experiments. \*P<0.05, \*\*P<0.01. **Abbreviations:** MMP, matrix metalloproteinase; TGF, transforming growth factor; SD, standard deviation; shRNA, short hairpin RNA.



**Figure 5** Upregulation of MMP-9 expression induces EMT. **Notes:** (A) Representative images of changes in cell morphology, consistent with the induction of EMT. (B) Analysis of E-cadherin and vimentin levels in cells treated with rMMP-9 and controls. (C) Representative images of E-cadherin and vimentin immunofluorescence analysis, in cells treated with rMMP-9 and controls. (D) Western blot analysis of E-cadherin and vimentin expression. β-Actin was used as an internal control. Scale bar: 50 μm. Original magnification, ×200. The data in (B and D) represent mean ± SD of the results obtained in three independent experiments. \*\*P<0.01, compared with the controls. **Abbreviations:** MMP, matrix metalloproteinase; EMT, epithelial–mesenchymal transition; rMMP-9, recombinant MMP-9; SD, standard deviation; Ctr, control; mRNA, messenger RNA.



**Figure 6** Upregulation of MMP-9 expression enhances EC-I invasiveness and metastatic potential.

**Notes:** EC-I cells were untreated or treated with rMMP-9. (A) Representative images of cells invading through Matrigel-coated membranes. (B) Analysis of the invasion assay results. (C) Representative images, showing the migration of EC-I cells. (D) Analysis of scratch assay results. Scale bar: 50  $\mu$ m. Original magnification,  $\times$ 200. The data in (B and D) represent mean  $\pm$  SD of the results obtained in three independent experiments. \* $P$ <0.05 compared with the controls.

**Abbreviations:** MMP, matrix metalloproteinase; rMMP-9, recombinant MMP-9; SD, standard deviation; Ctrl, control.

## Discussion

Recent investigations demonstrated that EMT contributes to cancer progression, invasion, and metastasis.<sup>6,26</sup> A growing number of studies showed that several growth factors, including TGF- $\beta$ ,<sup>27,28</sup> epidermal growth factor (EGF),<sup>29,30</sup> vascular endothelial growth factor (VEGF),<sup>31</sup> and hepatocyte growth factor (HGF),<sup>32</sup> induce the process of EMT, which is characterized by the loss of epithelial markers and the induction of the expression of mesenchymal markers. TGF- $\beta$ 1 signaling is induced following the binding of this protein to TGF- $\beta$  receptor types I and II, which promotes the phosphorylation of SMAD protein family members (SMAD2 and SMAD3), and it binds a cytoplasmic protein SMAD4 and translocates to the nucleus, further activating multiple cellular responses, such as MMP expression.<sup>33</sup> Here, we demonstrated that TGF- $\beta$ 1 induces changes in the morphology of ESCC cells and their transition from

epithelial to mesenchymal phenotype. This change was shown to be accompanied by the downregulation of E-cadherin and upregulation of vimentin expression, which is consistent with the data obtained in the previous studies.<sup>12,13</sup>

A previous study showed that TGF- $\beta$ 1 induces the expression of SNAIL in OKF4, OKF6, and UMSCC-1 cells, but not in SCC-25 cells, while SNAIL induces the expression of MMP-9 in oral squamous cell carcinoma (OSCC) cell.<sup>15</sup> Additionally, in OSCC cells, TGF- $\beta$ 1 effects on SLUG expression were shown to be mediated by ERK1/2-dependent pathways, and not PI3-kinase signaling, and SLUG was shown to promote OSCC cells invasion by increasing the expression of MMP-9.<sup>16</sup> Furthermore, TGF- $\beta$ 1 can promote breast cancer and liver cancer metastases by upregulating MMP-9 expression.<sup>34,35</sup> In our study, we showed that TGF- $\beta$ 1 can induce the expression of MMP-9. Furthermore, gelatin zymography results demonstrated that MMP-9 activity is



significantly increased in EC-1 cells after the treatment with TGF- $\beta$ 1. Following the treatment of cells with GM6001, MMP-9 activity was shown to be decreased, but MMP-2 expression was not detected (data not shown). The role of MMP-9 in TGF- $\beta$ 1-induced EMT was further examined by MMP-9 shRNA knockdown, and the obtained results showed that this significantly increases E-cadherin and decreases vimentin levels, inhibiting cell invasiveness and migration as well. Taken together, these data suggest that MMP-9 is the downstream target of TGF- $\beta$ 1 and that TGF- $\beta$ 1-induced downregulation of E-cadherin expression is largely mediated by MMP-9. One previous report indicated that MMP-9 is involved in TGF- $\beta$ 1-induced EMT in renal tubular cells, which agrees with our results.<sup>36</sup> However, the mechanism underlying the effects of TGF- $\beta$ 1 on MMP-9 expression remains undetermined. Interestingly, an increasing number of studies demonstrated that MMPs, such as MMP-2, MMP-9, and MMP-14, can stimulate TGF- $\beta$ 1 activity by cleaving latent TGF- $\beta$ -binding protein-1.<sup>37</sup> MMP-8 was reported to cooperate with TGF- $\beta$ 1, stimulating EMT and enhancing the invasion and metastasis of hepatocellular carcinoma.<sup>38</sup>

MMPs are divided into six categories: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs.<sup>19</sup> The expression and activity of MMPs were shown to be increased in almost every type of human cancer; and this increase was shown to correlate with the advanced tumor stage, increased invasion and metastasis, and shortened survival.<sup>20</sup> MMP-9, also known as gelatinase B, plays a critical role in the processes that represent the hallmarks of cancer, including migration, invasion, and angiogenesis.<sup>39–41</sup> During the EMT, MMPs are crucial for the regulation of cell behavior. The overexpression of stromelysin-1 (MMP-3) can induce EMT in mammary epithelial cells and increase the expression of RAC1, which can result in an increase in cellular reactive oxygen species levels and the subsequent upregulation of the EMT transcription factor SNAIL.<sup>22</sup> Epilysin (MMP-28) was shown to induce EMT and cell invasion through the proteolytic processing of latent TGF- $\beta$  complexes into the active form in lung carcinoma cells.<sup>25</sup> Several other MMPs, including MMP-3, MMP-7, MMP-9, ADAM10, and MT1-MMP, were shown to be involved in E-cadherin cleavage.<sup>42–46</sup> MMP-2, MMP-9, MMP-13, and MT1-MMP have also been implicated in the regulation of osteoblast, chondrocyte, and epithelial cell functions through TGF- $\beta$  activation as well.<sup>47–50</sup>

In this study, we reported that the treatment of cells with the recombinant MMP-9 is sufficient for the reduction of E-cadherin levels and it induced migratory and invasive

phenotype of these cells. This indicates that the upregulation of MMP-9 expression can promote the invasion and metastasis of ESCC. It was reported that MMP-8 regulates TGF- $\beta$ 1 expression through the activation of PI3K/Akt/Rac1 signaling,<sup>38</sup> but it remains unknown whether MMPs can also induce TGF- $\beta$ 1 expression.

## Conclusion

The results obtained in this study showed a novel mechanism of EMT induction in ESCC, showing that TGF- $\beta$ 1 and MMP-9 play significant roles in the EMT and that MMP-9 is a mediator of TGF- $\beta$ 1-induced EMT in ESCC.

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## Disclosure

The authors report no conflicts of interest in this work.

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