

## Inhibition of Migration and Invasion by Tet-1 Overexpression in Human Lung Carcinoma H460 Cells

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In the present study, we found that lung cancer cell line (H460 cells) expressing Tet1 showed higher levels of adhesion, and Tet1 inhibited H460 cell proliferation. In addition, these cells showed a significantly reduced ability of collagen degradation and Smad2/3 phosphorylation compared to controls. Furthermore, vimentin was found to be highly expressed in larger metastatic cancer area. Tet1 overexpression was reduced in the epithelial marker E-cadherin. Moreover, Tet1 repressed cancer cell metastasis in nude mice. Collectively, these findings suggest that Tet1 expression plays a critical role in metastasis of lung cancer cells by suppression of invasion and epithelial–mesenchymal transition (EMT).

**Key words:** Tet1; Demethylation; Lung cancer; Epithelial–mesenchymal transition (EMT); Metastasis

### INTRODUCTION

Recent studies have shown that DNA demethylation can be catalyzed by a class of methylcytosine dioxygenases termed the ten-eleven translocation (Tet) family (1). The mouse Tet genes, including Tet1, Tet2, and Tet3, have a CD domain (Cys-rich and DSBH regions). Tet1 and Tet3 also have a CXXC domain, though Tet2 does not. CXXC domains typically bind unmethylated CpG dinucleotides (2). Tet encourages DNA demethylation by catalyzing the conversion of 5-methylcytosine (5-mC) primarily to 5-hydroxymethylcytosine (5-hmC) in addition to 5-formylcytosine or 5-carboxylcytosine (1). The activity of Tet1 and its role in mouse embryonic stem cells was confirmed by another independent group (1). Tet proteins potentially regulate the active DNA demethylation process. They have also been shown to prevent DNA methylation by binding to DNA (3). Moreover, an inverse relationship between 5-hmC levels and cell proliferation was observed with lack of 5-hmC in proliferating cells. Additionally, 5-hmC levels

were profoundly reduced in several cancers compared to normal tissues (3). However, the mechanisms of its tumor-suppressing activity remain unclear.

Cell invasion is one of the crucial steps in both primary tumor growth and metastasis initiation (4). Transformed cells must first escape from the primary tumor by breaking down the basement membrane to allow cancer cells to intravasate into blood vessels. Tumor cells can then migrate to distant tissues, extravasate out of the circulatory system, and subsequently colonize remote sites. The ability of malignant cells to undertake these two stages of metastasis, intravasation and extravasation, is thought to explain the substantial upregulation of matrix metalloproteinase (MMP) activity in cancer cells. MMPs are enzymes that can degrade extracellular matrix (ECM) proteins and allow invasion through the basement membrane (5). In normal cells, adhesion molecules on the cell surface must be bound to their ligand to activate intracellular downstream signals without which cells will stop proliferating and undergo

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apoptosis (6). However, metastatic cells must break away from their original organ and migrate to distant sites. This process, which presumably requires cells to avoid normal adhesion-mediated survival signaling, enhances migration and invasiveness. Epithelial–mesenchymal transition (EMT) is a morphogenetic transformation during which epithelial cells downregulate their epithelial properties and upregulate mesenchymal characteristics (7). EMT is important for efficient metastasis colonization in epithelium-derived cancer (8). Several oncogenic pathways induce EMT, and a critical molecular event is the downregulation of the cell adhesion molecule E-cadherin (9). In recent years, accumulating evidence indicates that EMT is a critical process not only in development but also in tumorigenesis (9).

A recent report suggested that Tet1 was associated with a more aggressive metastatic cancer cell type. This study was designed to determine whether this finding holds true for the H460 lung cancer cell line.

## MATERIALS AND METHODS

### *Plasmid Construction*

The mouse Tet1 gene was amplified from mouse embryonic stem cells. A *Sac*II, *Bam*HI (TaKaRa, Kyoto, Japan) fragment containing the full-length mouse Tet1 cDNA was cloned into the pIRES2-EGFP vector driven by the CMV promoter. This expression cassette was confirmed by digesting the recombinant vector with *Nhe*I (New England Biolabs Inc., Ipswich, MA, USA). The plasmid DNA was purified using a midi-prep kit (Qiagen, Venlo, Netherlands).

### *Cell Culture and Transfection*

H460 cells were grown on 10-cm dishes in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (Life Technologies). Cells were transfected by using Fugene (Life Technologies). The pIRES2-EGFP was used as a control (GFP-labeled empty vector transfected H460 cells).

### *RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*

Total RNA was isolated by RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions, and the single-strand cDNA was synthesized from 1 µg of total RNA using cDNA synthesis kit (Enzymomics, Daejeon, Korea). The primers used were Tet1 forward primer: 5'-ATG GCA GTC GAA CGA C-3', Tet1 reverse primer: 5'-GCG GCA TCG CAG CTC C-3'. The *GAPDH* gene was used as an internal control.

### *Flow Cytometry*

Cultured cells were harvested by exposure to trypsin/EDTA, suspended in ice-cold 1% FBS/PBS solution and

analyzed using a flow cytometer FACSVerse™ (BD Biosciences, Franklin Lakes, NJ, USA) equipped with a laser that provided an excitation wavelength of 488 nm. BD FACSuite software (BD Biosciences) was used to quantify the fluorescent signals and to set the logical electronic-gating parameters. Expression of GFP in original H460 and GFP-labeled H460 cells was analyzed by flow cytometry.

### *Western Blot Analysis*

Proteins were extracted by lysing cells in extraction buffer, and the protein concentration was determined by using Nano drop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Antibodies against TET methylcytosine dioxygenase 1 (Millipore, Billerica, MA, USA), phospho Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), vimentin antibody (Abcam, Cambridge, UK), E-cadherin (Abcam), and β-actin (Santa Cruz Biotechnology) were used. Secondary antibodies were from GE Health Life Sciences (Buckinghamshire, UK), and signals were detected using ECL Plus (GE Health Life Sciences). The intensity of the protein bands was quantified by using ImageJ software developed by the National Institutes of Health (<http://imagej.nih.gov/>).

### *Adhesion to Fibronectin*

Microwell plates were coated with fibronectin (FN, 1 µg/ml) according to the manufacturer's instructions (Collaborative Biomedical Products, Bedford, MA, USA). Plates were washed three times with water, incubated for 1 h with 0.1% heat-inactivated BSA for blocking nonspecific binding sites. Cells were trypsinized, resuspended in SFM, and maintained in suspension at 37°C for 30 min before plating onto FN-coated 96-well plates at 37°C for the indicated times. They were stained with 0.01% crystal violet for 10 min. Bound staining was dissolved with Triton X-100, and optical density (OD) was read at 595 nm using a microplate reader.

### *Cell Viability Test (CCK-8 Assay)*

H460 cells ( $1 \times 10^5$  cells/well in a 96-well plate) were seeded and incubated. For the cell counting assay, cells were resuspended by pipetting and collected. The cell numbers were then counted using trypan blue (WelGENE Inc., Daegu, Korea) staining on a hemocytometer. For Cell Counting kit (CCK)-8 assay, 10% CCK-8 solution (Dojindo, Tabaru, Japan) was added to each well and incubated at 37°C in a 5% CO<sub>2</sub> incubator for 2 h. Absorbance at 450 nm was measured using microplate reader.

### *In Vitro Invasion Assay*

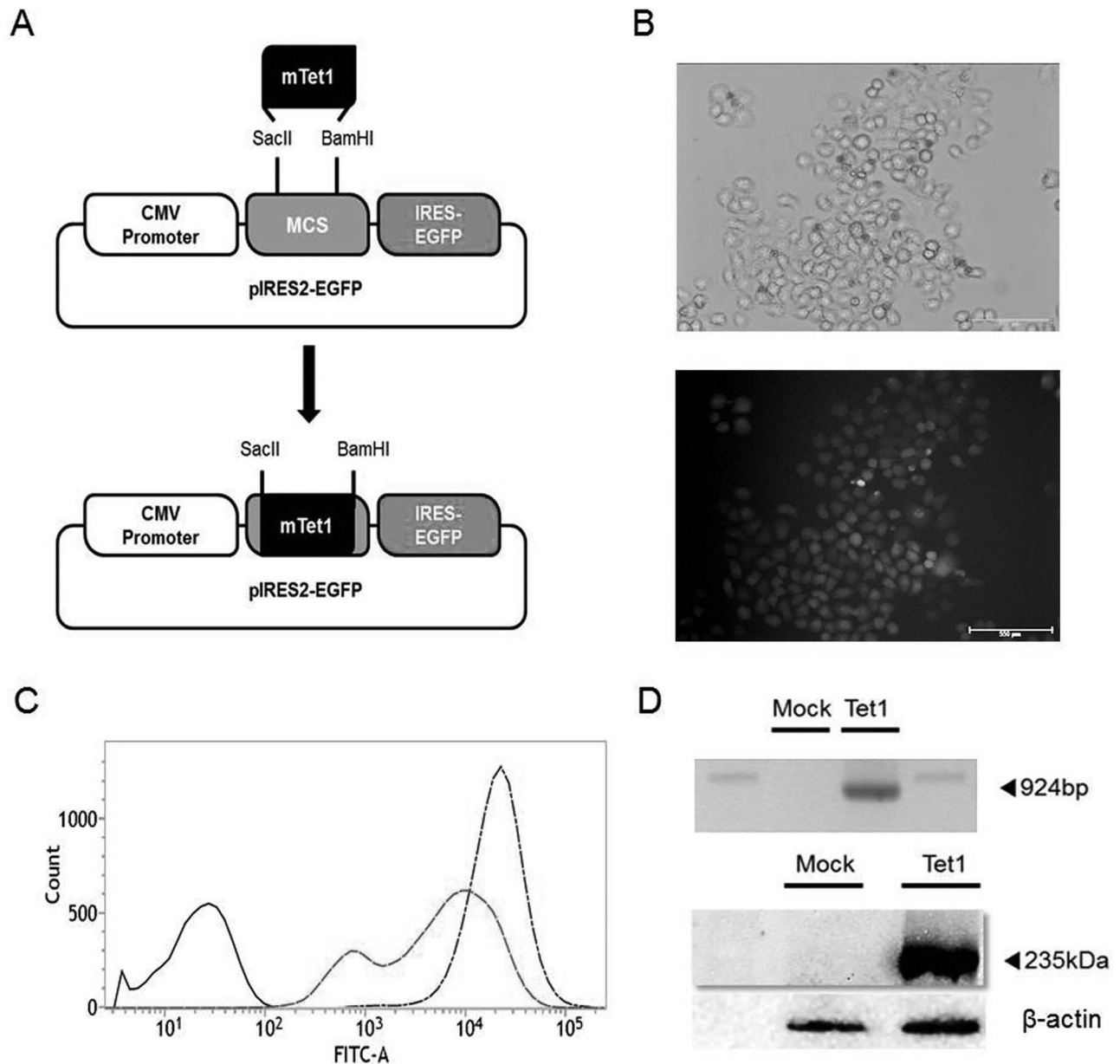
Invasion assays were carried out by using the 96-well QCMTM 96-well cell invasion assay (Millipore)

according to the manufacturer's recommendations. Briefly,  $2 \times 10^5$  cells in 100  $\mu$ l of serum-free DMEM were added to the ECMatrix™ cell culture inserts and control inserts, while DMEM with chemoattractant was added to the lower well. After 24 h at 37°C, noninvading cells were carefully removed. The cells on the bottom of the inserts were washed, incubated with detachment solution for 10 min, and stained. Each chamber was transferred to a

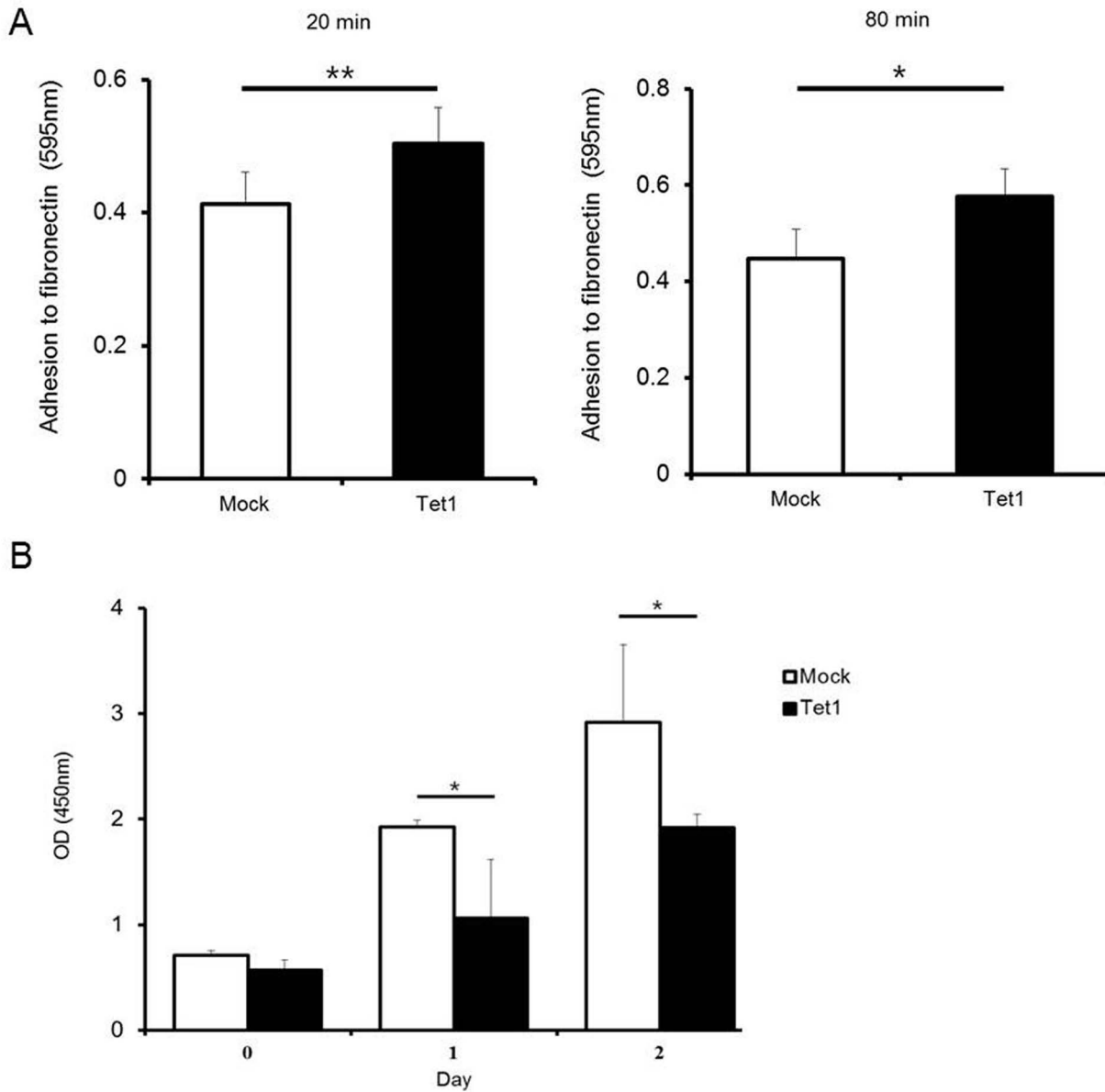
new 96-well plate, and the OD was measured at 595 nm using a microplate reader.

#### Gelatin Zymography

MMP-9 activity in the culture supernatant was determined by substrate gel electrophoresis. Zymography, in SDS polyacrylamide gel containing 0.1% gelatin, was performed. Culture supernatants were collected after 24 h



**Figure 1.** Establishment of the Tet1-overexpressing cell line. mTet1 mRNA, protein, and GFP expression were confirmed in mock and mTet1-transfected H460 cells. (A) Construct of the mock and Tet1 vectors. Tet1 cDNA was synthesized by PCR and inserted into the pIRES2-EGFP vector. (B) Confirmation of GFP expression in transfected H460 cells. (C) Analysis of GFP expression by FACS (wild type: solid line on left; mock-H460: dashed line on right; Tet1-H460: dashed dotted line). (D) mRNA and protein levels were quantified by RT-PCR and Western blotting, respectively. The following primers were used to detect a 924-bp fragment by genomic PCR. Tet1 forward primer: 5'-ATG GCA GTC GAA CGA C-3', Tet1 reverse primer: 5'-GCG GCA TCG CAG CTC C-3'.



**Figure 2.** Adhesion and proliferation of Tet1-overexpressing H460 cells. (A) Tet1 affects H460 cell adhesion activity. H460 cells were cultured on FN (1  $\mu\text{g}/\text{ml}$ )-coated 96-well plate. After incubation, H460 cells were washed out. Amounts of adherent cells were measured by crystal violet staining, and color density was read at 595 nm. (B) H460 cells were seeded and incubated at 37°C in a 5%  $\text{CO}_2$  incubator for 48 h. Proliferation was measured using CCK-8 assay. \* $p < 0.05$ ; \*\* $p < 0.01$ .

of incubation. The gels were then sequentially treated with 2.5% Triton X-100 for 40 min to remove SDS and incubated in digestion. The gels were finally stained with 0.1% Coomassie Brilliant Blue R-250 (Biosesang, Seongnam, Korea) and destained to visualize the bands.

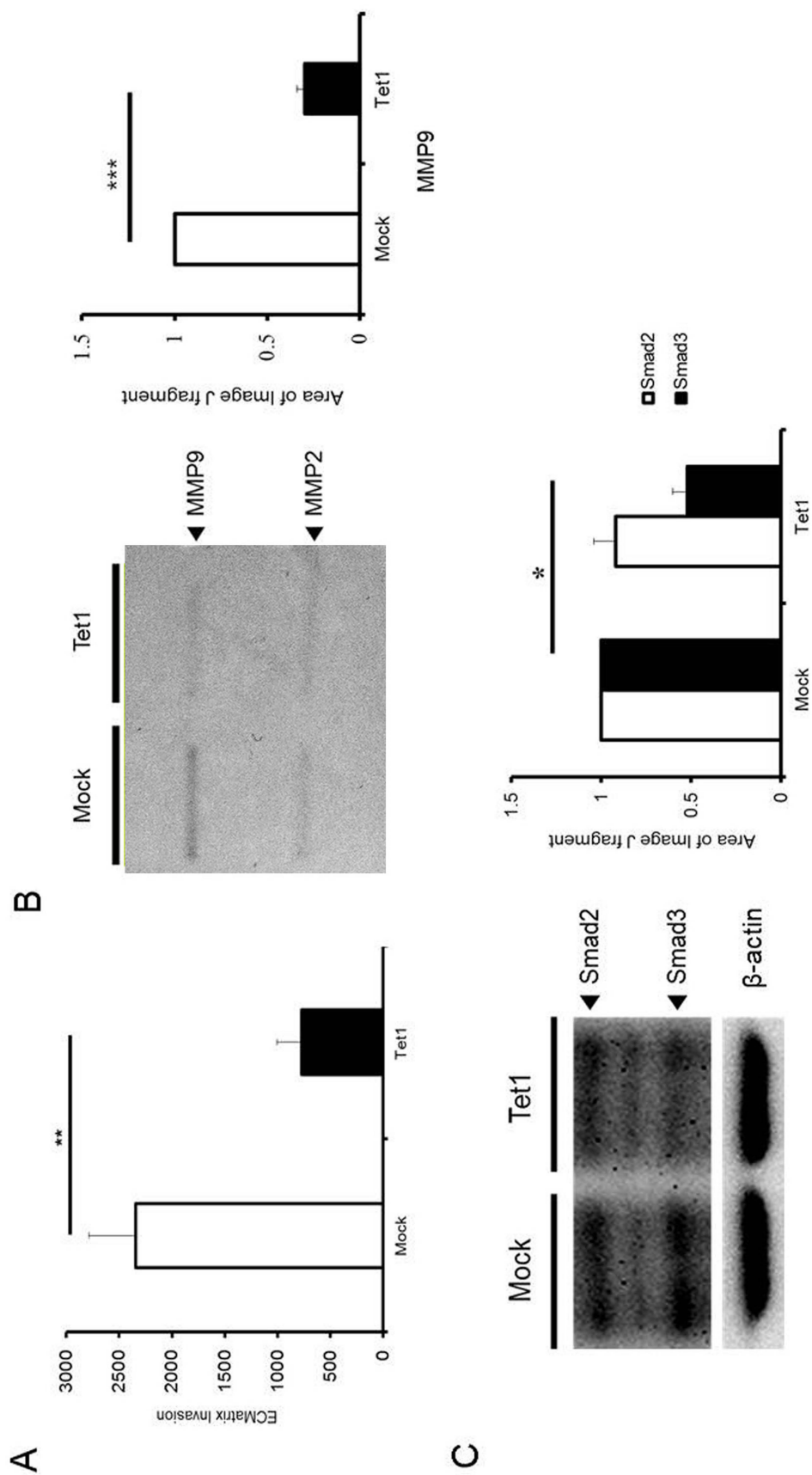
#### *In Vivo Metastasis*

Male BALB/c nu/nu nude mice were housed in a specific pathogen-free environment and temperature-controlled room at 22°C. All mice received humane care,

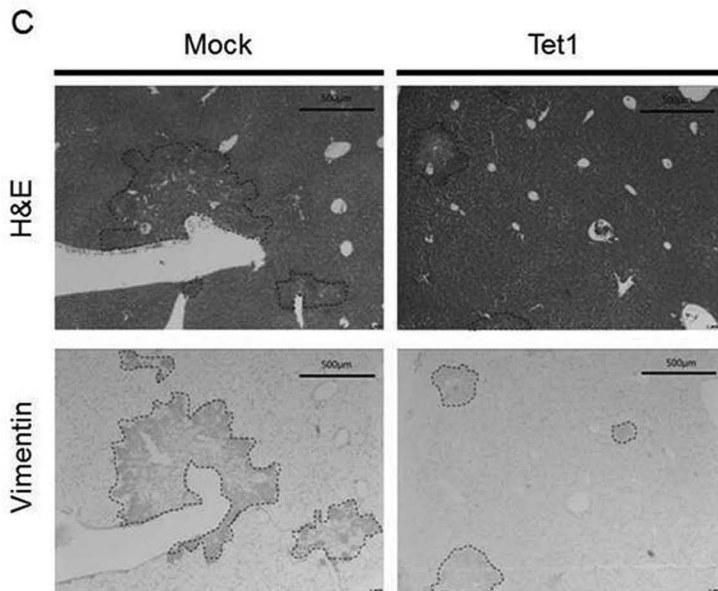
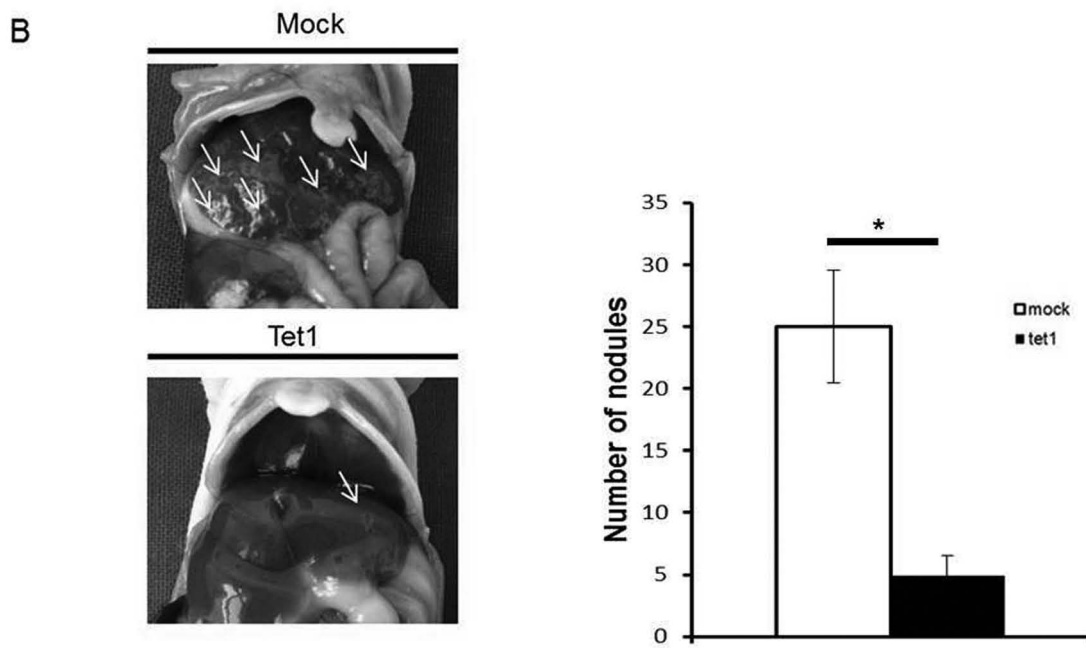
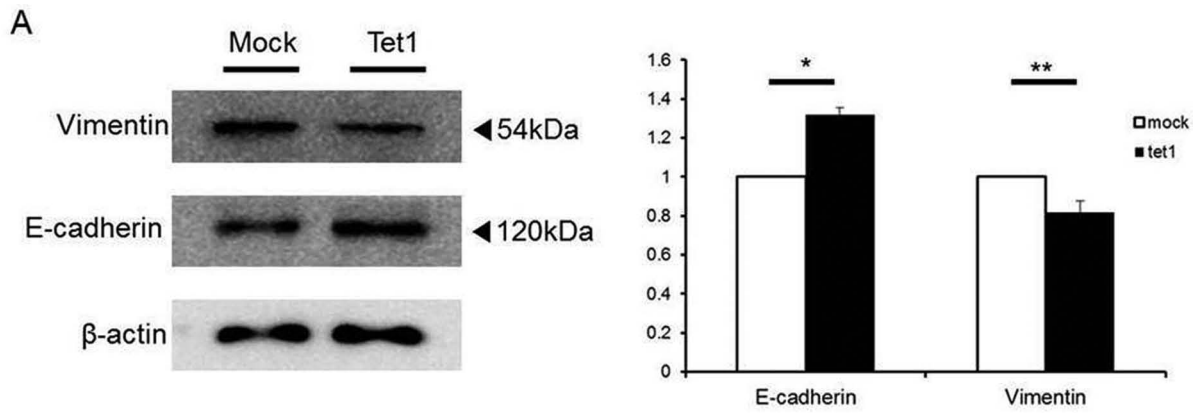
and the study protocols comply with the Kyung-pook National University Intramural Animal Use and Care Committee. Cells ( $2 \times 10^6$ ) were subcutaneously injected into 6-week-old nude mice. Mice were euthanized 28 days after injection under anesthesia. Six mice were used in each group. Sites of metastasis were visualized and counted.

#### *Histological Analysis*

The livers were collected at the time of euthanasia. Thin sections (5  $\mu\text{m}$ ) were stained with H&E. For



**Figure 3.** Tet1 reduces H460 cell invasion ability. (A) Invasion ability was measured using an ECMatrix invasion chamber. OD was measured at 595 nm. (B) Culture supernatants were collected after 48 h of incubation for the analysis of MMP-9/MMP-2 activity using gelatin zymography. MMP-9 activity was quantified by using ImageJ. (C) Western blot analysis of Phospho-Smad2/3 expression levels. Smad2/3 activity was quantified by using ImageJ. \* $p < 0.001$ ; \*\* $p < 0.005$ ; \*\*\* $p < 0.01$ .



immunohistochemical analysis, the sections were incubated with a rabbit monoclonal anti-vimentin antibody (Abcam) diluted in blocking solution overnight at 4°C. The secondary antibody was an anti-rabbit IgG-HRP (Cell Signaling Technology, Danvers, MA, USA). Samples underwent successive diaminobenzidine (DAB) staining and dehydration and were mounted on cover glass. Serial sections were incubated with the same quantity of nonimmune rabbit IgG instead of the primary antibody as negative controls.

#### Statistical Analysis

The results are expressed as the mean and standard error (mean±SE) of at least three independent experiments. The significance of differences between groups was calculated using a two-tailed Student's *t*-test. Differences with a value of  $p < 0.05$  were considered statistically significant.

## RESULTS

#### Stable Cell Line Establishment

Tet1 expression vectors were constructed as described in Materials and Methods (Fig. 1A). The Tet1 vector, pIRES-EGFP-Tet1, was transfected into lung cancer H460 cells for 48 h. The GFP expression intensity was observed by fluorescent microscopy (Fig. 1B). To confirm that vector-targeted GFP was indeed expressed in the transfected H460 cell line, GFP-expressing cells were selected under a microscope, and GFP expression was measured by FACS (Fig. 1C). Tet1 expression levels were confirmed by RT-PCR. Western blot analysis showed a single band of 235 kDa in transfected cell lysates (Fig. 1D). The data showed that Tet1 vector was specifically expressed in H460 cells.

#### Increased Cell Adhesion and Decreased Cell Proliferation

Various types of cancer cells exhibit decreased levels of intercellular adhesiveness, enhancing their ability to overcome cell–cell adhesion, which might underline the characteristics associated with metastasis in vivo (10). We then examined whether the Tet1 tumor cell line exhibited any changes in adhesion characteristics. Adhesion assays were performed to assess the ability of the cell lines transfected with and without Tet1 to bind to FN (Fig. 2A).

Tet1-expressing cells displayed higher levels of adhesion compared to control cells.

To explore Tet1 effects on lung cancer cell growth, the CCK-8 assay was conducted in mock-H460 and Tet1-H460 cells. The results showed that Tet1 inhibited cancer cell proliferation (Fig. 2B).

#### Impaired Tumor Cell Invasion

To determine if the increase in adhesion ability affected the cell ability to invade a collagen matrix, the penetration of cells through an ECMatrix was assessed. As expected, Tet1-expressing cells presented a reduced ability to infiltrate the matrix, further indicating that these cells presented a reduction in invasion ability in vitro (Fig. 3A). We wondered whether the reduced invasion in Tet1-expressing cells affected their ability to degrade matrix proteins as this enables the cell to invade through the basement membrane. To this end, we first measured MMP-2 and MMP-9 activity using an in vitro matrix degradation activity assay. MMP family members have been shown to play a pivotal role in tumor cell invasion and metastasis (11). MMP-2 and MMP-9, which are gelatinases, mainly degrade type IV and V collagens and have been shown to correlate with the invasiveness of various cell types (12). Interestingly, we found that the ability of Tet1-expressing cells to degrade collagen was significantly reduced by almost threefold compared to that of mock-H460 (Fig. 3B).

#### Phosphorylation of Smad2/3 Was Inhibited in Tet1-Expressing Cells

Members of the transforming growth factor (TGF)- $\beta$  signaling pathway are considered as predictive biomarkers for progressive tumorigenesis as well as molecular targets for prevention and treatment of cancer and metastasis (13). We also wondered whether there was a change in TGF- $\beta$  downstream signaling (Fig. 3C). To test this hypothesis, Smad2/3 phosphorylation state was determined. Smad3 phosphorylation was substantially inhibited in cells expressing Tet1 compared to controls.

#### Tet1-Repressive Effects on Cancer Cell Metastasis in Nude Mice

We then focused on EMT. E-cadherin is a transmembrane glycoprotein involved in cell–cell adhesion and

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**Figure 4.** Tet1 reduces spontaneous metastasis and colonization of distant organs. (A) Western blot analysis of mock and Tet1-transfected cell lines using specific antibodies to vimentin, E-cadherin, and  $\beta$ -actin. Vimentin and E-cadherin activities were quantified by using ImageJ. (B) Nude mice metastatic nodules in the liver. Fewer and smaller metastases were observed in the mock-transfected H460 cell line. The number of liver metastatic nodules in nude mice transplanted with Tet1-transfected cells was significantly reduced. Arrow: metastatic nodule. (C) Representative images of H&E staining and immunohistochemical staining of vimentin on liver tissues from immunodeficient mice injected with mock or Tet1-transfected cell lines ( $2 \times 10^6$ ). The dotted line represents the tumor outline. \* $p < 0.0001$ ; \*\* $p < 0.002$ .

EMT. Vimentin is highly expressed in mesenchymal cells and is positively correlated with increased metastasis (14). It has been reported that vimentin can be targeted for tumor inhibition due to its specific upregulation in tumor vasculatures (15).

We performed Western blot using mock-H460 and Tet1-H460 cell lysates and vimentin and E-cadherin antibodies (Fig. 4A). Vimentin expression was downregulated, and E-cadherin expression was upregulated in Tet-H460 compared to that in mock-H460.

We then confirmed whether Tet1 regulates H460 cell metastasis in nude mice. Nude mice were euthanized 4 weeks after subcutaneous injection of mock-H460 and Tet1-H460. Metastatic tumors were detected in the liver (Fig. 4B). The effect of inhibiting metastasis by Tet1 overexpression was assessed based on the metastatic tumor number. The number of cases of liver metastasis in the Tet1-H460-injected mice was less than that in the mock-H460-injected mice. Immunohistochemistry was performed to investigate the effect of Tet1 overexpression on vimentin expression in metastatic liver cancer. Vimentin was highly expressed in larger metastatic cancer area in mock-H460-injected mice (Fig. 4C).

These results indicated that Tet1 expression reduced lung cancer metastasis *in vivo*. It strongly supports the effects observed *in vitro*, indicating that Tet1 plays a critical role in invasion and migration of lung cancer cells.

## DISCUSSION

Several recent reports show a considerable loss of Tet1 gene expression and/or 5-hmC in cancer cell lines (16). However, the mechanism by which Tet1 is involved in metastasis is not well understood. We found that Tet1 overexpression in a lung cancer cell line (H460) induces a decrease in metastasis *in vivo*. Consequently, there is evidence from a number of groups suggesting that Tet1 is a possible tumor suppressor. There has also been a considerable interest in the ability of Tet1 to modulate cell adhesion. Metastatic cells must break away from their progenitor tissue/organ and migrate to distant sites, a process that presumably requires the cell to avoid normal adhesion-mediated survival signaling. Additionally, Tet2 is inactivated by mutation in about 15% of myeloid cancers (17), including 22% of acute myeloid leukemia (AML). The other Tet family member, Tet3, mediates hydroxymethylation of epigenetically silenced genes and contributes to bone morphogenic protein 7 (BMP-7)-induced reversal of kidney fibrosis (18). Thus, TET proteins have the potential to be important regulators of epigenetic status in various cells. Here we showed that the adhesion ability of H460 lung cancer cells is increased after Tet1 transfection and that metastasis is reduced by Tet1 in Tet1-H460-injected nude mice relative to mock-H460-injected nude mice.

Previous studies have shown that tumor growth was inhibited by FN as well as by fibrinogen polymer (19). FN plays a major role in cell adhesion, growth, migration, and differentiation. It is also important for processes such as wound healing and embryonic development (20). Cancer cells exhibit decreased levels of intercellular adhesiveness, enhancing their capabilities to overcome cell–cell adhesion, which might underline the characteristics associated with metastasis *in vivo* (10). Consequently, adhesion signaling downregulation, but not the deficiency of adhesion ability, is thought to be important for metastatic tumor cells to move through the blood vessels or lymphatic system (21). Our data show that Tet1-expressing cells displayed higher levels of adhesion on FN-coated plate compared to control cells, suggesting that Tet1 prevents metastasis of lung cancer via promoting cell–cell adhesion.

MMPs are a family of proteases that are capable of degrading all kinds of ECM proteins. Among them, MMP-2 and MMP-9, which are gelatinases, have been demonstrated to promote tumor cell invasion and metastasis (22). *In vivo*, one of the most important barriers preventing a cancer cell from becoming metastatic is the basement membrane. Once a cancer cell breaks through the basement membrane, it is able to enter the circulatory system and move from the primary tumor site. This is thought to account for the upregulation of MMPs in many cancers (23). Consequently, we measured the ability of cells to invade through a SDS polyacrylamide gel and tested the enzyme activity of secreted MMP-2 and -9. Tissue inhibitors of metalloproteinases (TIMPs) are originally known to inhibit MMP activities. Members of the TIMP family regulate the activity of multifunctional metalloproteinases. TIMP is downregulated in a variety of cancer cell lines. The loss of expression and the promoter hypermethylation of the TIMP-2 gene have been reported in prostate tumor cells (24). Overexpression of TIMPs reduced experimental melanoma metastasis (25). Moreover, a recent study shows that Tet1 suppresses cancer invasion by activating TIMPs (16). Importantly, in the cancer cell line tested in this study, overexpression of Tet1 resulted in a decreased capacity of MMP-9 to degrade a gelatin matrix, suggesting a suppressive role of Tet1 in tumor cell invasion ability.

In order to determine if Tet1 is involved in the regulation of metastasis, we generated a stable cell line by expressing exogenous Tet1 and tested Tet1 overexpression ability to alter a range of *in vitro* characteristics that are thought to mimic the phenotype of metastatic cancer cells. EMT occurs in different cancers of epithelial origin. Cancers are usually confined from motility by the basement membrane (26), but the process of converting toward a cancer cell that has mesenchymal characteristics allows cancer cells to infiltrate the circulatory systems, causing motility that will later lead to metastasis (27). In our study, Tet1-expressing cancer cells exhibited



increased adhesion ability and enhanced activation of EMT-associated signaling, detected through Smad2/3 phosphorylation and E-cadherin and vimentin expression. Smad signaling is essential for TGF- $\beta$ -induced EMT (28). In lung cancer, Smad3 has been found to have biological effects on malignant properties such as EMT and metastasis. These effects can be inhibited by silencing Smad3 (29). Thus, Smad3 transcription factors are important for TGF- $\beta$ -induced EMT. As a cancer progresses, the hallmark change that characterizes EMT is the loss of the epithelial protein, E-cadherin, and the upregulation of mesenchymal proteins such as vimentin (30). We observed that Smad3 was substantially inhibited, vimentin expression was downregulated, and E-cadherin expression was upregulated in Tet1-expressing cells compared to control cells. Furthermore, a decrease in in vivo metastasis was observed when compared to controls.

In conclusion, Tet1 expression in a lung cancer cell line plays a critical role in metastasis. We demonstrated that Tet1 expression is associated with decrease levels of invasion, proliferation, and EMT markers, which is consistent with our previous observations in tumorigenic cells expressing exogenous Tet1 (16). These results suggest that Tet1 could be a promising target candidate for anti-metastasis therapy in certain types of cancer.

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