Translationally Controlled Tumor Protein induces epithelial to mesenchymal transition and promotes cell migration, invasion and metastasis

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Translationally controlled tumor protein (TCTP), is a highly conserved protein involved in fundamental processes, such as cell proliferation and growth, tumorigenesis, apoptosis, pluripotency, and cell cycle regulation. TCTP also inhibits Na,K-ATPase whose subunits have been suggested as a marker of epithelial-to-mesenchymal transition (EMT), a crucial step during tumor invasiveness, metastasis and fibrosis. We hypothesized that, TCTP might also serve as an EMT inducer. This study attempts to verify this hypothesis. We found that overexpression of TCTP in a porcine renal proximal tubule cell line, LLC-PK1, induced EMT-like phenotypes with the expected morphological changes and appearance of EMT related markers. Conversely, depletion of TCTP reversed the induction of these EMT phenotypes. TCTP overexpression also enhanced cell migration via activation of mTORC2/Akt/GSK3β/β-catenin, and invasiveness by activating MMP-9. Moreover, TCTP depletion in melanoma cells significantly reduced pulmonary metastasis by inhibiting the development of mesenchymal-like phenotypes. Overall, these findings support our hypothesis that TCTP is a positive regulator of EMT and suggest that modulation of TCTP expression is a potential approach to inhibit the invasiveness and migration of cancer cells and the attendant pathologic processes including metastasis.
The reported reduction in Na,K-ATPase expression during TGF-β1 mediated EMT process suggested to us a possible relationship between TCTP which inhibits Na,K-ATPase and EMT and led us to hypothesize that TCTP induces EMT and contributes to metastasis by promoting EMT process. In this study we describe our attempts to test this hypothesis by focusing on the roles of and interrelationship between TCTP, and EMT in metastasis.

**Results**

**Ectopic overexpression of TCTP promotes EMT and enhances cell migration.** Several studies showed that TCTP levels increase in colon cancer14, prostate cancer15 and hepatocellular carcinoma (HCC)16. In addition, a strong correlation between the expression levels of TCTP and degree of metastasis was observed in ovarian cancer17, colon cancer cell18, and human glioma19. It has been well established that TCTP acts as an anti-apoptotic protein and contributes to malignancy19. Although TCTP is clearly associated with cancer progression and metastasis, the exact role of TCTP on cancer metastasis is unclear. We tested our hypothesis that TCTP increases metastasis by inducing EMT, employing LLC-PK1-renal proximal tubular epithelial cells transiently altered by adenoviral vector to overexpress TCTP. Phase contrast microscopic studies indicated that the TCTP-overexpressing cells lost cell-cell contacts and acquired dispersed appearance, which are hallmarks of cellular/morphologic changes during EMT (Figure 1a)20. Immunoblotting studies demonstrated alterations in the epithelial and mesenchymal markers in these cells. We also observed reduction in the epithelial marker; E-cadherin, and increases in the mesenchymal markers, EMT.

![Figure 1](https://www.nature.com/scientificreports)
fibronectin, vimentin, α-smooth muscle actin (α-SMA) and N-cadherin, hallmarks of EMT induced by ectopic expression of TCTP (Figure 1b). Because of the demonstrated role of transcriptional repressors in the loss of E-cadherin21, we also examined the expression levels of E-cadherin transcription repressors such as ZEB1, slug and twist, by immunoblotting, and found that these repressors were elevated by TCTP overexpression (Figure 1c). Furthermore, we also confirmed that TCTP induces the expression of mesenchymal markers in cancer cell lines, A549 and HeLa cells (Figure S1).

Next, we immunostained E-cadherin and mapped the changes in the localization of E-cadherin caused by TCTP overexpression. E-cadherin was found localized in areas of cell-cell contact in the control cells, in contrast TCTP overexpressing cells which showed reduced membrane localization of E-cadherin (Figure 1d).

We also examined whether TCTP overexpression induces cytoskeletal reorganization of actin cytoskeleton, which has been shown to lead to morphologic and motility changes22. Control cells exhibited cortical actin staining, and actin filament bundles below the plasma membrane, whereas TCTP overexpressing cells showed F-actin stress fibers (Figure 1e). Because acquisition of EMT phenotype alters migratory ability of epithelial cells, we examined the effect of TCTP overexpression on cell migration using both wound-healing and transwell migration assays. In wound healing assay, cells were allowed to starve enough to minimize the effect of TCTP on cell proliferation and complicate the interpretation of migration assays. We found that the wound healing capacities of TCTP overexpressing cells increased for 48 hours after wounding (Figure 1f). Transwell migration assays revealed that the migration capacity of the TCTP overexpressing cells increased over two (2.14) fold (Figure 1g). These results indicate that ectopic expression of TCTP induces all the EMT phenotypes examined, and thereby promotes cell migration.

Silencing TCTP reverses EMT phenotypes and suppresses cell migration. Next, we examined whether down-regulation of TCTP can reverse EMT phenotypes and suppress migration of LLC-PK1 cells. For this, we depleted TCTP using shRNA transfection. As shown in Figure 2, the TCTP-depleted cells exhibited: 1. Cobblestone-like cell clusters (Figure 2a); 2. Elevated expression of E-cadherin, as well as reduced expression of mesenchymal markers in immunoblotting experiment (Figure 2b); 3. Decreased expression of ZEB1 and Twist, transcription repressors of E-cadherin (Figure 2c). These results were also confirmed in both A549 and HeLa cells (Figure S2); and 4. Significantly slower serum-induced directional migration, compared with control cells in transwell migration assays (Figure 2d). These findings clearly show that depletion of TCTP inhibits the development of mesenchymal-like phenotypes and confirm TCTP’s role in positively regulating EMT.

TCTP enhances cell invasion through MMP-9 activation. EMT is implicated in the remodeling of the cytoskeleton and the formation of structures which promote cell invasion23. Therefore, we examined whether TCTP overexpression increases the cell’s invasive ability. Performing matrigel-coated transwell invasion assay, we observed over two fold (2.33-fold) augmentation in invasive capacities of TCTP overexpressing cells (Figure 3a). Since the invasive ability following EMT, correlated with increased activity in the matrix.
metalloproteinases (MMPs), that degrade components of the basement membrane, we investigated whether TCTP activates MMPs. The expression levels of MMPs in both TCTP overexpressing and TCTP-depleted LLC-PK1 cell lysates were compared with that in control cells, using gelatinase specific antibodies to MMP-2 and MMP-9. We found that the expression level of MMP-9 increased in TCTP overexpressing cells, while that of MMP-2 was unchanged (Figure 3b). Conversely, the expression level of MMP-9 decreased following TCTP depletion (Figure 3c). Furthermore, by immunoblotting, we detected secreted MMP-9 and not MMP-2, in the conditioned media of these cells (Figure 3d). Also, gelatin zymographic study demonstrated that TCTP activates MMP-9, concomitantly with the expression of MMP-9 (Figure 3e). Taken together, these results indicate that TCTP facilitates cell invasion through activation of MMP-9.

**TCTP promotes cell migration via mTORC2/Akt/GSK3β/β-catenin pathway.** EMT is regulated by ERK, Ras/MAPK, PI3K/Akt, Smads, RhoB and β-catenin pathways among others. In our previous studies, we examined the activation of Akt by overexpression of TCTP, focusing on Akt signaling and found that phosphorylation of Akt at Ser473 residue significantly increased in TCTP overexpressing cells, with no change in the phosphorylation of Akt at Thr308 (Figure 4a). Full activation of Akt is known to be achieved by PDK-1-dependent phosphorylation at Thr308 and mTORC2-dependent phosphorylation at Ser473. We found, as expected, that PI3K which is upstream of PDK-1, is not activated by TCTP overexpression. Since GSK3β is well-known as downstream target of Akt, we examined whether overexpression of TCTP alters the activity of GSK3β. As shown in Figure 4a, overexpression of TCTP increased the phosphorylation of GSK3β (Ser9) and deactivates it while total GSK3β levels remained unchanged, indicating the absence of GSK3β activation. Phosphorylation and functional inactivation of GSK3β stabilize cytoplasmic β-catenin thereby inducing nuclear translocation of β-catenin. Nuclear accumulation of β-catenin is another characteristic change during EMT. Based on these results, we hypothesized that TCTP activates β-catenin signaling pathways by inducing nuclear translocation of β-catenin. To test this hypothesis, we performed subcellular fractionation and evaluated nuclear translocation of β-catenin following TCTP overexpression. Figure 4b shows nuclear accumulation of β-catenin in TCTP overexpressing cells. Immunofluorescence microscopy also showed translocation of β-catenin from membrane to nucleus following overexpression of TCTP (Figure 4c). Moreover, phospho-Akt (Ser473) is known to be a key downstream molecule in mTOR complex 2 (mTORC2). Therefore, we investigated whether mTORC2 is involved in TCTP-mediated cell migration using several signaling molecule inhibitors: PI3K/Akt/mTOR kinase inhibitor, LY294002; mTORC1 inhibitor, rapamycin; and mTOR kinase inhibitor, PP242, which acts on both mTORC1 and mTORC2. Treatment with LY294002 and PP242 suppressed the migration of TCTP overexpressing LLC-PK1 cells.
cells (Figure 4d). In contrast, inhibition of mTORC1 by rapamycin did not affect TCTP induced effects on cell migration. Thus, activation of mTORC2 seems to be involved in TCTP induced cell migration. Overall, these findings suggest that overexpression of TCTP enhances cell motility through mTORC2/Akt/GSK3β/β-catenin pathways and that TCTP plays a role in EMT through these signaling pathways.

Downregulating TCTP inhibits mesenchymal-like changes and suppresses pulmonary metastasis of mouse melanoma cells. Having established that TCTP is a positive regulator of EMT process and knockdown of TCTP inhibits EMT transition in vitro, using LLC-PK1 cells, and that EMT is a critical regulator of cancer metastasis, we investigated whether downregulation of TCTP represses development of mesenchymal-like features and decreases the metastatic potential of cancer cells in vivo. To this end, we infected stable mouse melanoma B16F10 cells with TCTP specific shRNA expressing lentivirus or control and obtained two distinct clones in which TCTP was significantly downregulated. We examined morphological changes in the stable cell lines by phase contrast microscopy. We found that lack of TCTP led to close cell-cell contacts, which indicates diminished mesenchymal changes. Also, diminished spike-like structures were observed in TCTP downregulated B16F10 cells (Figure 5a). Moreover, mesenchymal markers such as N-cadherin, α-SMA, and fibronectin were significantly reduced in TCTP downregulated B16F10 cells (Figure 5b). When we examined MMP expressions in stable cell lysates, MMP-9 expression significantly diminished in TCTP downregulated stable cells whereas MMP-2 levels did not change, consistent with results depicted in Figure 3 (Figure 5c). Immunostaining showed reduced N-cadherin in TCTP downregulated B16F10 cells (Figure 5d). In addition, significant changes were observed in the morphology of TCTP downregulated B16F10 cells compared to control cells, following F-actin staining with phalloidin. We noticed that B16F10-shCont cells exhibited elongated fibroblast-like morphology whereas B16F10-shTCTP cells showed flattened cell shapes (Figure 5e). As shown in Figure 5f, knockdown of TCTP decreased the nuclear accumulation of β-catenin. Since cancer metastasis is a complex multi-step process involving both migration and invasion, we measured the migration and invasion capacities of B16F10-shCont and B16F10-shTCTP #1 cells by transwell migration (Figure 5g) and invasion assays (Figure 5h), respectively. Both the migration and invasion of TCTP downregulated B16F10 cells were significantly reduced compared to those of control B16F10 cells. To understand the role of TCTP in metastatic potential, we also performed in vivo pulmonary metastasis assay. We injected both B16F10-shCont and B16F10-shTCTP #1 cells intravenously into the tail veins of male BALB C nude mice and monitored metastasis in lungs. We observed significant metastasis after 11 days of intravenous injection, of B16F10-shCont cells. The numbers and areas of metastatic colonies on the surface of lungs were significantly less in mice injected with B16F10-shTCTP cells, than in those injected with B16F10-shCont cells (Figure 5i). These findings clearly demonstrate that downregulation of TCTP inhibits pulmonary metastasis of melanoma cells by reducing the invasiveness and migration of melanoma cells.
Figure 5 | Silencing TCTP promotes MET process and suppresses pulmonary metastasis of mouse melanoma cell line. (a) Phase-contrast microscopic images of B16F10 cells infected with lentiviral control shRNA (shCont) or shRNA vector targeting TCTP (shTCTP #1 and #2). Images were taken with ×100 magnification. Scale bar 200 μm. (b) Expression levels of mesenchymal markers were determined by immunoblotting. GAPDH was used as a loading control. (c) The expression levels of MMP-2 and MMP-9 in cell lysates were examined by immunoblotting. (d, e) Fluorescence microscopic staining of N-cadherin (d) and F-actin (e) were performed with B16F10-shCont, shTCTP #1 and #2 (red). Nuclear DNA was stained with DAPI (blue). (f) The nuclear translocation of β-catenin was evaluated by immunoblotting after subcellular fractionation. GAPDH was used for the cytosolic fraction, and lamin A/C was used for the nuclear fraction. (g, h) B16F10-shCont, B16F10-shTCTP #1 and #2 cells were subjected to the transwell migration (g) and transwell invasion (h) assay. Experiments were done in triplicate and representative images were taken with ×100 magnification using. Quantification was carried by counting the number of migratory cells that had infiltrated the filter. Values are means ± S. E. M. *P<0.05, **P<0.01, ***P<0.001. (i) Effect of TCTP depletion on the pulmonary metastasis was determined. Both B16F10-shCont and shTCTP #1 cells were injected intravenously into the tail vein of male BALB C/nude mice with 1 × 10^6 cells (n=6/group). After 11 days, the lungs were extirpated and areas of nodules were calculated. Three representatives per each set are shown. Normal lung indicates control injected with PBS.
Discussion

The present study is the first demonstration that TCTP is a positive regulator of EMT. We showed that TCTP overexpression in well polarized epithelial LLC-PK1 cells enhanced cell motility and invasivity via EMT process, evidenced by the expression of EMT-related markers and morphological changes. This study also demonstrated that TCTP-induced phosphorylation at Akt at Ser473 by activation of mTORC2, and nuclear translocation of β-catenin by inhibiting GSK3β activity. We also showed that downregulation of TCTP inhibits EMT transition in both renal epithelial cells and melanoma cells and suppresses pulmonary metastasis of melanoma.

Reorganization of cytoskeleton and concomitant alteration of cell morphology are essential for cancer cell motility and invasivity and are thus regarded as typical phenomena during EMT process43. Several observations clearly indicate that TCTP is associated with cytoskeletal modification. In xenopus XL2 cells, TCTP was shown to colocalize with F-actin at the leading edge of lamellipodia-like structure and regulate cell shape in a cytoskeleton-dependent manner46. It was also demonstrated that cofilin, an actin binding protein, promotes metastasis33,34 and that TCTP influences F-actin cytoskeleton modification by competing with cofilin at the cofilin-binding site of actin46. In this context, we stained F-actin cytoskeleton with phalloidin and observed that overexpression of TCTP in LLC-PK1 cells led to rearrangement of actin cytoskeleton and formation of stress fibers (Figure 1e). Stress fibers, induced by RhoA, connected to focal adhesions, have been shown to play a critical role in cell motility regulation33,34. Kloc et al observed the positive correlation with expression level of TCTP and RhoA in ovarian cancer cell line47. Thus, RhoA may be a downstream agent responsible for TCTP induced stress fiber formation. Further studies need to be performed to elucidate the detailed mechanism for TCTP-induced stress fiber formation.

The mammalian target of rapamycin (mTOR) which plays pivotal roles in cell growth, proliferation, survival and migration5,36, exists in two distinct multiprotein complexes, rapamycin sensitive mTOR complex 1 and rapamycin insensitive mTOR complex 2 (mTORC2). mTORC1 plays critical roles in protein synthesis and cell growth by phosphorylation of translational regulators such as ribosomal S6 kinase1 (S6K1) and 4E-BP137. Whether TCTP regulates mTORC1 signaling in cell growth, proliferation and protein synthesis is being debated38-40. Studies in Drosophila revealed that TDC2 directly interacts with Drhēb GTPase, a positive regulator of mTORC1, and acts as its guanine nucleotide exchange factor (GEF)41. mTORC2 regulates actin cytoskeleton rearrangement thereby promoting cell migration42,43. We observed previously42 as well as in the current study, elevation of Akt phosphorylation at Ser473 residue in TCTP overexpressing cells (Figure 4a). Ser 473 is a key downstream residue in mTORC2. Our data indicate that TCTP affects mTORC2 signaling, which this study, using pharmacological inhibitors, showed, is involved in TCTP induced cell migration (Figure 4d). Thus, TCTP seems to affect and also use both mTORC1 and mTORC2 pathways; it regulates cell cycle and metabolisms by activating mTORC1 and alters cytoskeletal organization and motility through mTORC2.

Loss of E-cadherin is a hallmark of EMT process and is strongly associated with poor clinical outcome44,45 in cancer. We found that ectopic expression of TCTP reduced E-cadherin expression and redirected its localization from membrane to cytosol (Figure 1b, d). One principal mechanism which reduces E-cadherin expression involves transcriptional repressors. Therefore, we examined whether TCTP is associated with E-cadherin transcriptional repressors. We found that TCTP elevated ZEB1, Slug, and twist, transcriptional repressors of E-cadherin (Figure 1c). The exact mechanism of how TCTP upregulates these transcriptional repressors has not been elucidated yet. Several studies demonstrated that β-catenin/TCF4 binds directly to the ZEB1 and Slug promoter and activates their transcription36,46. We observed nuclear accumulation of β-catenin in TCTP overexpressing LLC-PK1 cells using subcellular fractionation and immunofluorescence techniques (Figure 4b, c), and diminished nuclear β-catenin in TCTP downregulated melanoma cells (Figure 5f). Based on these findings, TCTP might regulate the expression of E-cadherin transcriptional repressors through activation of β-catenin signaling. However, additional studies using β-catenin specific shRNA/siRNA are needed to clarify the role of β-catenin in TCTP-induced EMT process. Also, understanding of TCTP’s role in the mTORC2/Akt/GSK3β axis is needed to establish the specific TCTP signaling in invasion and metastasis.

Tumor invasion and metastasis are multistep processes involving proteolysis of extracellular matrix (ECM) and destruction of basement membranes as initial events in the metastatic cascade47. Matrix metalloproteinases (MMPs), are a family of zinc-dependent endopeptidases that plays an important role in proteolysis of ECM48, MMP-2 and MMP-9 playing especially important roles in tumor invasion and metastasis49,50. Our study shows that ectopic expression of TCTP in LLC-PK1 cells significantly enhanced cell invasion (Figure 3a). Also, the expression of MMP-9 increased in TCTP overexpressing cells (Figure 3b) and the activity of secreted MMP-9 was concomitantly elevated (Figure 3d, e). In a previous study of MMP expression levels in TCTP overexpressing MCF10A cells, we found that only MMP-3 and MMP-13 were upregulated while MMP-2 and MMP-9 levels did not change49. The difference in the cell line used may explain the observed variation.

The role of TCTP in metastasis is less well characterized than in tumorigenesis. A previous study showed that knocking down of TCTP in colon cancer cells inhibited liver metastasis7. We confirmed this effect of TCTP knockdown in the metastasis of melanoma. TCTP downregulated B16F10 cells also exhibited significantly suppressed pulmonary metastasis (Figure 5i). In addition, we showed that TCTP downregulated B16F10 cells markedly reduced mesenchymal markers such as N-cadherin, α-SMA, and fibronectin (Figure 5b). This study is the first to report that knocking down TCTP, and the resultant inhibition of mesenchymal-like changes is a rational approach to inhibiting cancer cell migration and invasion, involved in metastasis. Furthermore, metastasis, which is a multistep process that involves proliferation, migration, and invasion, the proliferative effect by TCTP might play a role in metastasis, at least in part. A unified view of TCTP’s regulation in signaling pathways (e.g. Src, EGF, mTORC2/Akt etc.) and in proliferation and invasion is necessary to propose TCTP as a target for cancer metastasis. A fuller understanding of TCTP’s regulatory role in proliferation and invasion is needed before one can propose TCTP as a target for cancer metastasis.

Methods

Cells and Cell culture. Porcine renal proximal tubule LLC-PK1 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA), and were maintained in Medium 199 supplemented with 5% fetal bovine serum at 37°C and 5% CO2. Mouse melanoma B16F10 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C and 5% CO2.

Sources of antibodies and other reagents. Antibodies against E-cadherin and N-cadherin were obtained from BD Bioscience (NJ, USA); ZEB1/TCF8, MMP-2, MMP-9, phospho-Akt (Ser473), phospho-Akt (Thr308), Akt, phospho-GSK3β (Ser), GSK3β, phospho-Pi3K, Pi3K p85α, β-catenin, β-actin and lamin A/C antibodies were from Cell Signaling Technology (MA, USA); fibronectin and α-smooth muscle actin (SMA) antibodies were from Sigma-Aldrich Biotechnology (LP, USA); vimentin, green fluorescent protein (GFP), Twist, and Slug antibodies were from Santa Cruz Biotechnology (CA, USA); and TCTP antibodies were obtained from LabFrontier (Seoul, Korea). 2- (4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), rapamycin, and PP242 were obtained from Sigma-Aldrich Biotechnology (LP, USA).

Overexpression of TCTP by adenoviral infection and transient transfections with TCTP shRNA. Cells were infected to overexpress TCTP as described previously with minor modification49. Adenovirus vectors expressing the transgene TCTP, containing green fluorescent protein (Ad-TCTP-GFP) or empty vectors (Ad-Vector) were used to infect the LLC-PK1 cells. When multiplicity of infection (MOI) was 10, LLC-PK1 cells were co-cultured with Ad-TCTP-GFP or Ad- GFP at 37°C, in a humidified atmosphere with 5% CO2 for 2 h. Construct for intracellular synthesis of TCTP siRNA vector (pSUPER-shTCTP) was generated as previously described49.
Transfection of TCTP shRNA was performed by using WelFect-EXTM PLUS transfection reagent (WeGENE, Korea). The level of suppression of TCTP gene was determined by immunoblotting.

Generation of stable cell lines by lentiviral transduction. Lentiviruses were generated as described[55] with minor modification. Briefly, TCTP shRNA cloned P.LK.O, pHSV-G, and A.B were cotransfected in HEK293FT cells with Lipofectamine 2000 (Invitrogen, CA, USA). Viral particles were harvested at 48 and 72 hr, and infected into cells in the presence of 10 μg/ml polybrene. Infected cells were selected with puromycin (0.8 μg/ml).

Immunoblotting. Immunoblotting were performed as described previously with minor modification[55]. Briefly, cells were infected and incubated in the serum free medium for 72 hr, and infected into cells in the presence of 10 μM Tris–Cl (pH 7.4), 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM sodium chloride (NaCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM sodium orthovanadate (Na3VO4) and 1 mM sodium fluoride (NaF) with protease inhibitor cocktails (Roche Molecular Biochemicals) and phosphatase inhibitor cocktails (Sigma-Aldrich Bioreagents, CA, USA). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane.

Preparation of conditioned medium. Proteins were precipitated from equal-volume aliquots of supernatant using 15% ice-cold trichloroacetic acid (TCA). The precipitates were washed twice with 100% acetic acid, air-dried, dissolved in sample buffer. Samples were detected using immunoblotting method.

Gelatin Zymography. Gelatin zymography was performed as described previously with minor modification[55]. Briefly, cells were infected and incubated in the serum free medium for 72 min at 37°C to remove cellular debris. Conditioned medium was then concentrated with Amicon Ultra-4 filter (30 K) (Millipore, USA) at 7,500 g for 30 min at 4°C. Samples were electrophoresed under nonreducing conditions onto 10% SDS-PAGE gels normalized with 0.1% gelatin. The gels were washed with 2.5% Triton X-100, and incubated at 37°C for 24 h in incubation buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM CaCl2, 50 mM NaCl, and 0.05% Brij 35. The gel was stained with Coomassie blue stain (0.5% Coomassie blue R-250, 40% methanol, and 10% acetic acid), followed by destaining (40% methanol, 10% acetic acid). The image was taken with the LAS-3000 (Fujifilm, Japan).

Wound-healing assay. Wound healing assay was performed as described previously with minor modification[55]. Briefly, LLC-PK1 cells were grown to confluence in a 6-well plate and were infected with Ad-GFP or Ad-TCTP-GFP virus. After 24 hours, cells were wounded using 1 ml sterile pipette tip. The wounded area was photographed at each time courses for 48 hours. The wound healing capacities were measured using GraphPad Prism 5 software (GraphPad Software Inc., CA, USA).

Transwell migration/invasion assay. Migration assays were performed using uncoated cell culture inserts with 8 μm pores (Corning Life Sciences, NY, USA) according to manufacturer’s instructions. Briefly, LLC-PK1 cells were infected with Ad-GFP or Ad-TCTP-GFP virus; or were transfected with pSUPER or pSUPER-uncoated cell culture inserts with 8 μm pores. The inserts were sham-wounded and suspended in PBS. Cells (1 × 105 in 100 μl) were injected intravenously into the tail vein of BALB/c nude mice (Jung-Ang Lab, Animal, Inc., Seoul, Korea). After 11 days of intravenous injection, the lung was extirpated and the areas of black spherical B16F10 colonies were analyzed using Image software (NIH, USA). All animal studies were conducted in accordance with IACUC guidelines and were approved by IACUC committee at Ewha Womans University (Approval ID: 2013-01-114).

Statistical analysis. Data are presented as means and their standard errors. Data were analyzed using GraphPad Prisms 5 software (GraphPad Software Inc., CA, USA). Statistical significance was determined using Student’s t-test.

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Author contributions
S.Y.B. designed and performed all experiments and wrote manuscript. H.J.K. and K.J.L. conducted experimental metastasis assay as well as data analysis. K.L. participated in the conception and design of the study and the writing of the manuscript.

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