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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/GSK3 β / 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.

Translationally controlled tumor protein (TCTP) is a highly conserved multifunctional protein present in all eukaryotes, across animal and plant kingdoms. TCTP regulates numerous fundamental processes by interacting with many cellular proteins. Since its discovery in ascites tumor cells, TCTP has been implicated in tumorigenesis and cancer progression. Several studies revealed that TCTP acts as a cell survival protein modulating apoptosis. TCTP also regulates cell cycle, interacting with microtubules¹. Depletion of TCTP by shRNA in colon cancer cell lines significantly reduced cell migration, invasion and hepatic metastasis². However, the mechanisms by which TCTP contributes to cancer metastasis are not fully understood.

Following our finding that TCTP interacts with the third cytoplasmic domain of Na,K-ATPase α subunit and inhibits the pump activity³, inhibition of Na,K-ATPase has been implicated in pathologic states including hypertension, cataract, and tumorigenesis⁴⁻⁶. Moreover, Na,K-ATPase subunits have been suggested as markers of epithelial to mesenchymal transition (EMT)⁷. Na,K-ATPase expression was found reduced during TGF- β 1 mediated EMT. These findings together, suggest a possible association of TCTP and EMT.

EMT is a pivotal biological process that allows a well-polarized epithelial cell, which is immotile which normally interacts with basement membrane, to undergo multiple biochemical changes to mesenchymal cell phenotypes, including enhanced migratory capacity, invasiveness, elevated resistance to apoptosis and greatly increased production of ECM components^{8,9}. During EMT, epithelial cells change from cobble stone-like morphology and acquire scattered, fibroblast-like morphology that characterizes mesenchymal cells, along with altered cell adhesion molecules, enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, greatly increased production of extracellular matrix (ECM) components^{8,9} and invasiveness derived via extracellular matrix degradation. EMT harmonizes with the reverse process, known as mesenchymal-epithelial transition (MET) has been shown to play important roles in developmental process and tissue repair^{10,11}. Aberrant regulation of EMT results in pathological processes such as fibrosis, tumor invasiveness, and metastasis, the process by which cancer cells leave the primary tumor environment and migrate to distant sites^{12,13}.

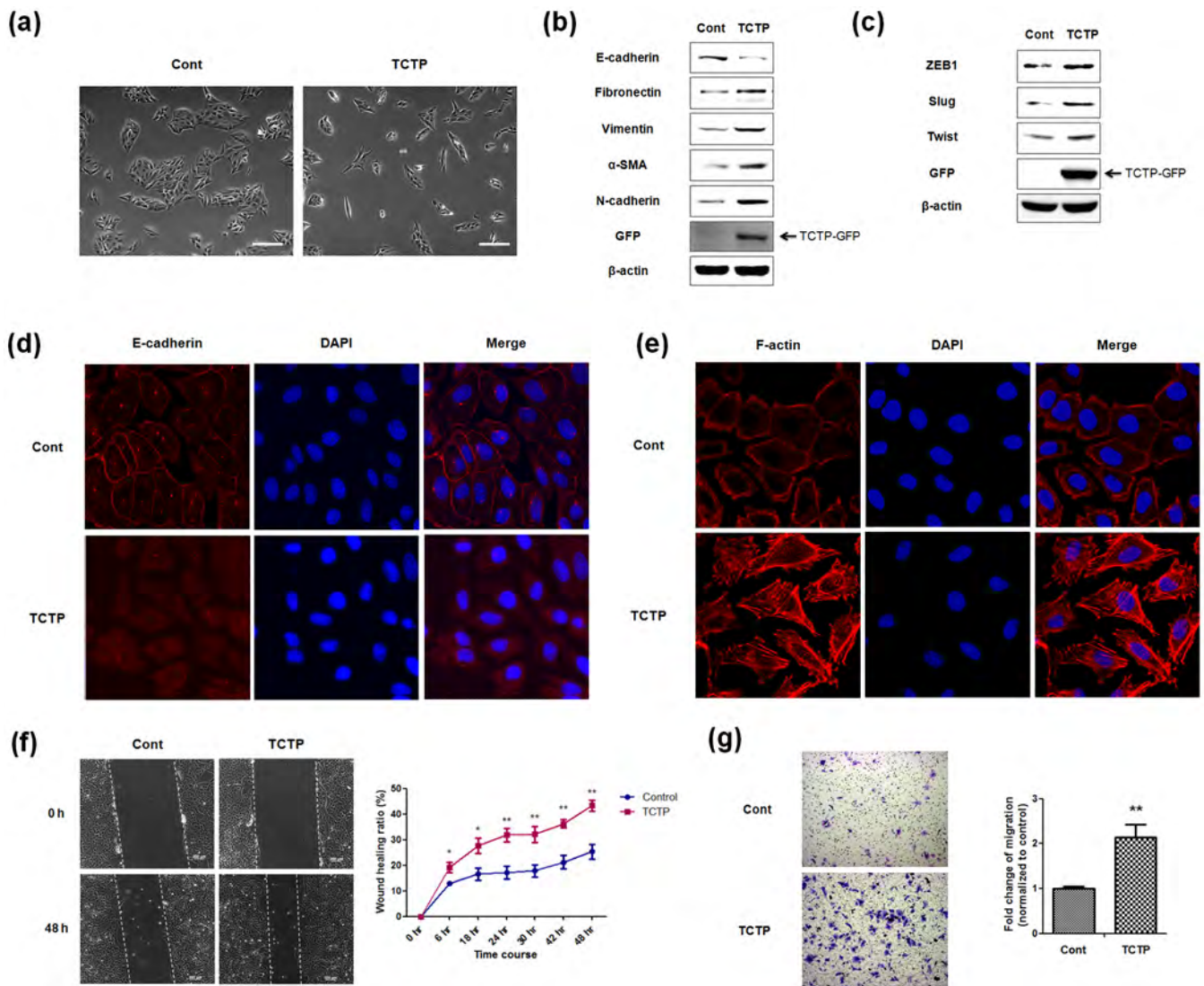


Figure 1 | Ectopic overexpression of TCTP promotes EMT and enhances cell migration. (a) Phase-contrast microscopic images of LLC-PK1 cells infected with control Ad-GFP virus (Cont) or Ad-TCTP-GFP virus (TCTP). Images were taken with $\times 100$ magnification. scale bar 200 μm (b) Expression levels of epithelial marker as well as mesenchymal markers were examined by immunoblotting of control and TCTP overexpressed cells. (c) Expression levels of transcription repressors of E-cadherin were examined by immunoblotting. β -actin was used as a loading control. (d, e) Fluorescence microscopic staining of E-cadherin (d) and F-actin (e) were performed with control and TCTP overexpressed cell (red). Nuclear DNA was stained with DAPI (blue). (f) Wound healing assays were performed with control and TCTP overexpressed cells for 48 hours. Representative images were taken at 0 h and 48 h after wounding, as indicated. Images were taken with $\times 100$ magnification using. Quantification was carried out by measuring the distance migrated compared with the controls. Values are means \pm S. E. M. of three experiments. *P < 0.05, **P < 0.01 (g) Transwell migration assays were performed with LLC-PK1 cells infected with adeno-GFP virus or adeno-TCTP-GFP virus over 18 h. Experiments were done in triplicate and representative images were taken with $\times 100$ magnification using. Quantification was carried by counting the number of migratory cells that had infiltrated the filter. Values are means \pm S. E. M. **P < 0.01.

The reported reduction in Na,K-ATPase expression during TGF- β 1 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 cell², and human glioma¹⁸. 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,

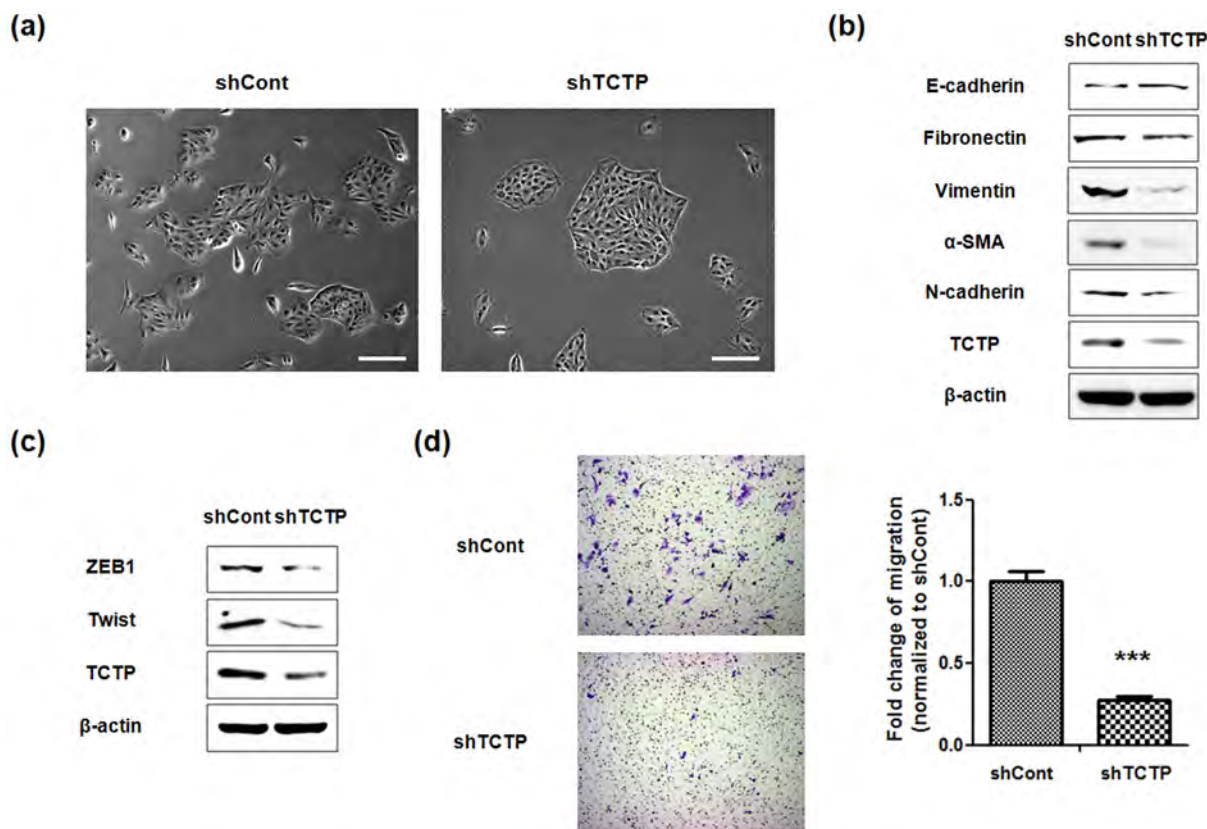


Figure 2 | Knockdown of TCTP reverses EMT phenotypes and suppresses cell migration. (a) Phase-contrast microscopic images of LLC-PK1 cells transfected with control shRNA (shCont) or TCTP shRNA (shTCTP). Images were taken with $\times 100$ magnification. scale bar 200 μm (b) EMT phenotypic markers including E-cadherin and mesenchymal markers were examined by immunoblotting in both LLC-PK1 shCont and shTCTP cells. β -actin was used as a loading control. (c) Expression levels of transcription repressors of E-cadherin in shCont and shTCTP cells were examined by immunoblotting. β -actin was used as a loading control. (d) Transwell migration assays were performed with shCont and shTCTP cells. Experiments were done in triplicate and representative images were taken with $\times 100$ magnification using. Quantification was carried by counting the number of migratory cells that had infiltrated the filter. Values are means \pm S. E. M. *** $P < 0.001$.

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 assay 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

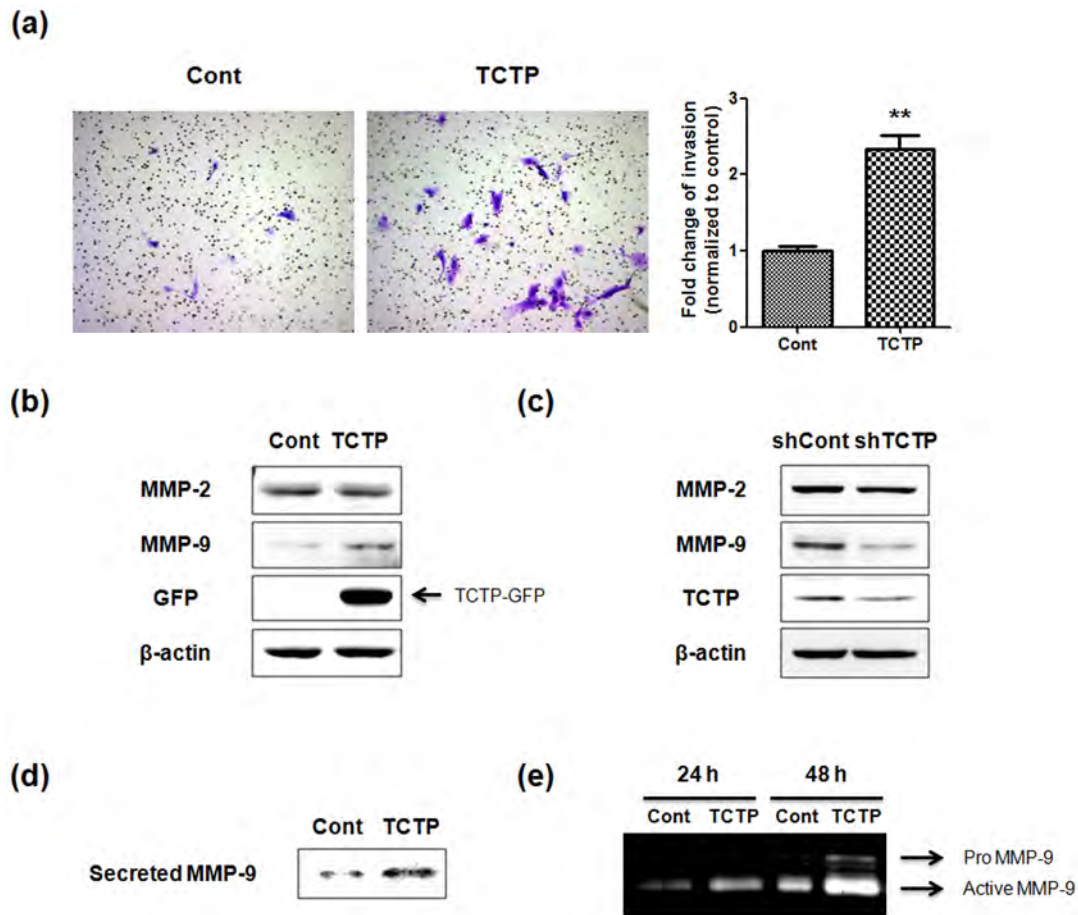


Figure 3 | TCTP enhances cell invasion through MMP-9 activation. (a) Transwell invasion assay was performed with LLC-PK1 cells infected with adeno-GFP virus or adeno-TCTP-GFP virus over 24 hr. Experiments were done in triplicate and representative images were taken with $\times 100$ magnification using. Quantification was carried by counting the number of migratory cells that had infiltrated the filter. Values are means \pm S. E. M. ****** $P < 0.01$. (b, c) The expression levels of MMP-2 and MMP-9 in cell lysates were examined by immunoblotting. (d) Secreted MMP-9 was detected in conditioned media of control and TCTP overexpressed cells by immunoblotting. (e) Conditioned media were collected at indicated time course from adenoviral infection. Gelatinolytic activity of pro and active MMP-9 were visible as a clear area on the gel.

metalloproteinases (MMPs), that degrade components of the basement membrane²⁴, 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/GSK3 β / β -catenin pathway. EMT is regulated by ERK, Ras/MAPK, PI3K/Akt, Smads, RhoB and β -catenin pathways among others⁸. In our previous studies, we examined the activation of Akt by overexpression of TCTP^{6,25}, 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^{26,27}. We found, as expected, that PI3K

which is upstream of PDK-1, is not activated by TCTP overexpression. Since GSK3 β is well-known as downstream target of Akt²⁸, we examined whether overexpression of TCTP alters the activity of GSK3 β . As shown in Figure 4a, overexpression of TCTP increased the phosphorylation of GSK3 β (Ser⁹) and deactivates it while total GSK3 β levels remained unchanged, indicating the absence of GSK3 β activation. Phosphorylation and functional inactivation of GSK3 β stabilize cytoplasmic β -catenin thereby inducing nuclear translocation of β -catenin. Nuclear accumulation of β -catenin is another characteristic change during EMT²⁹. 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

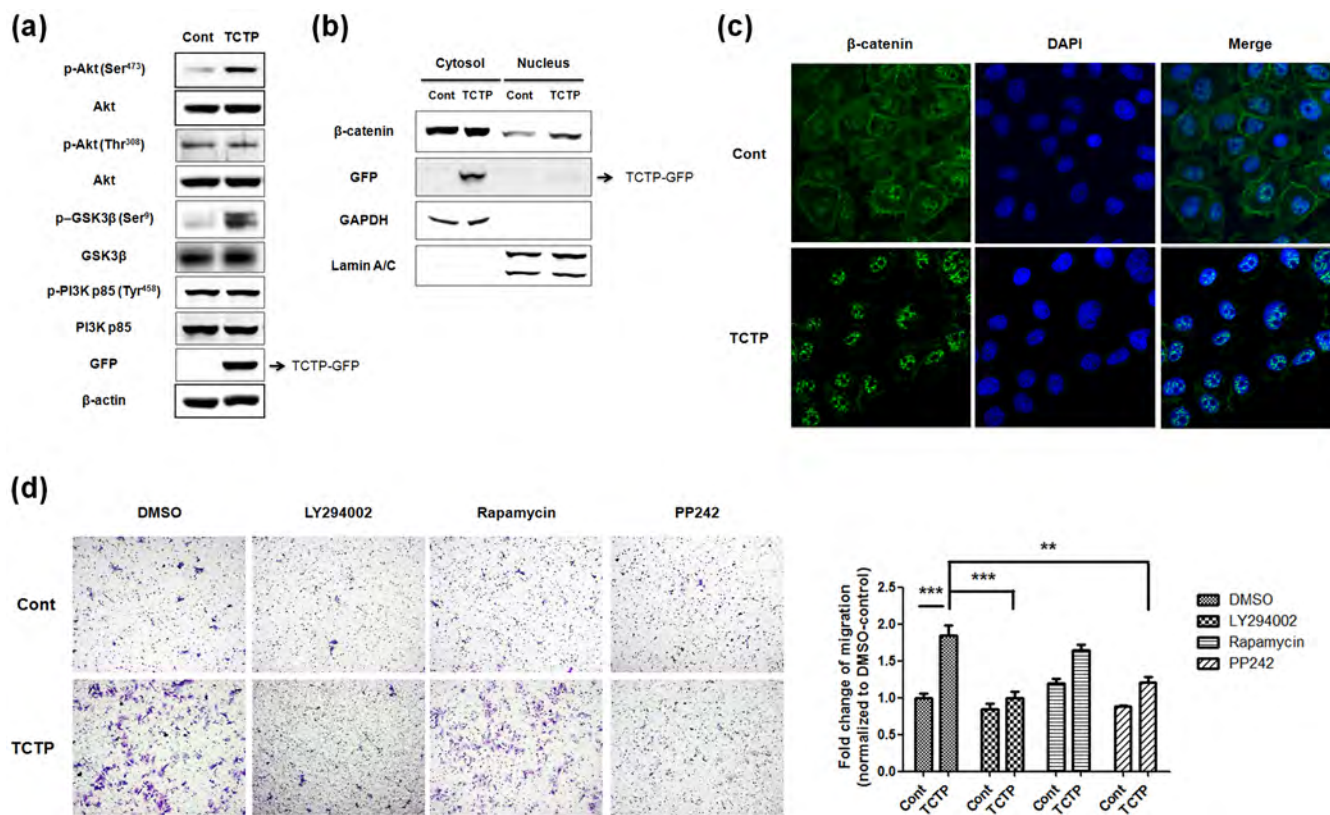


Figure 4 | TCTP promotes cell migration through mTORC2/Akt/GSK3β/β-catenin pathway. (a) Phosphorylation states of Akt (Ser⁴⁷³) and GSK3β (Ser⁹) in control and TCTP overexpressed cells were examined by immunoblotting. (b) The nuclear translocation of β-catenin was evaluated by immunoblotting after subcellular fractionation. The purity of each fraction was monitored by the respective markers, GAPDH for the cytosolic fraction, and lamin A/C for the nuclear fraction. (c) Fluorescent microscopic visualization of β-catenin (green) in control and TCTP overexpressed cells. Nuclear DNA was stained with DAPI (blue). (d) Both control and TCTP overexpressed LLC-PK1 cells treated with 10 μM of LY294002, 100 nM of rapamycin, and 5 μM of PP242 for 18 h were subjected to transwell migration 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 < 0.01, ***P < 0.001.

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 metastasis²³, 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^{8,23}, 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.

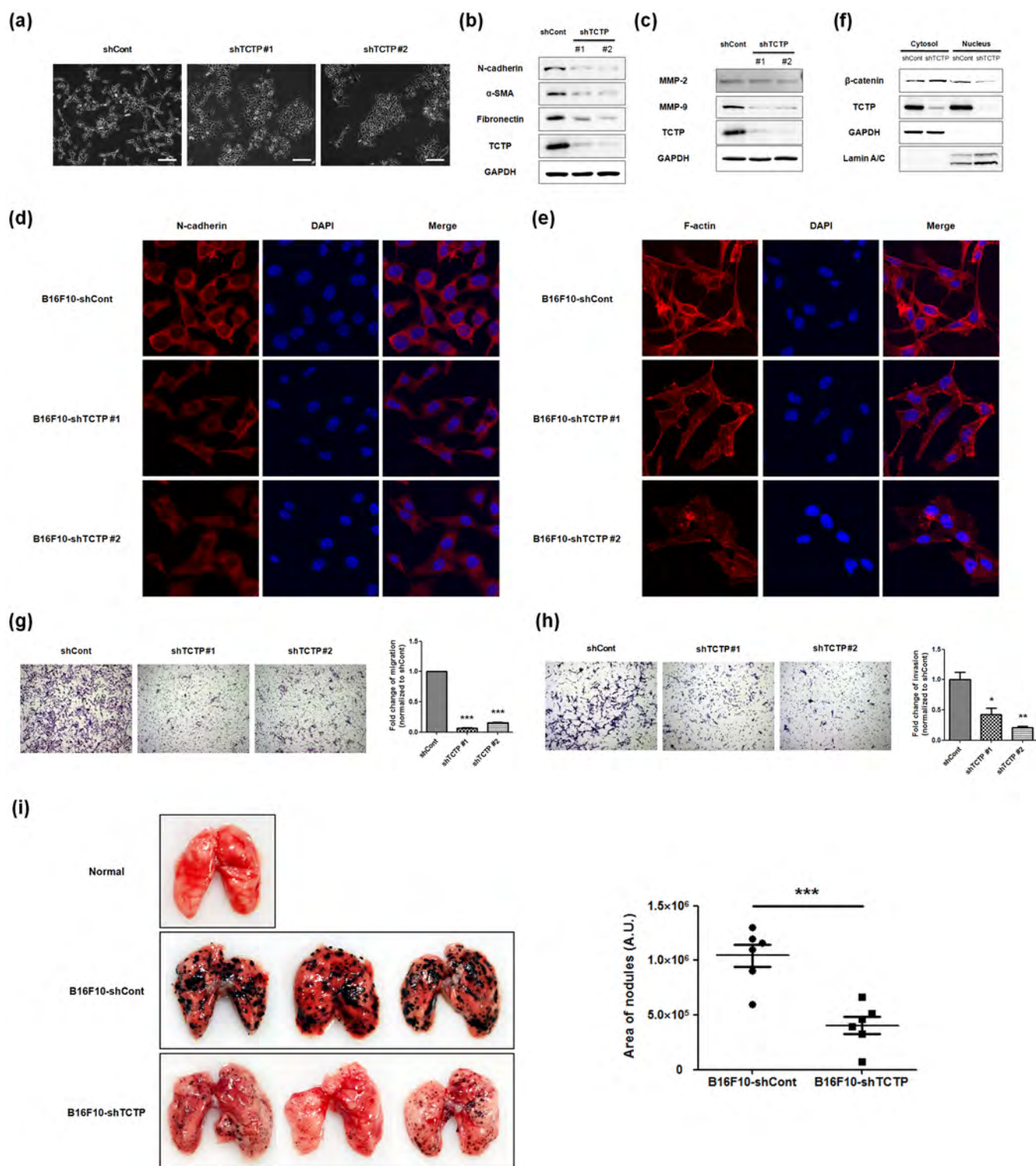


Figure 5 | Silencing TCTP promotes MET process and suppresses pulmonary metastasis of mouse melanoma cell line. (a) Phase-contrast microscopic images of B16F10 cells infected with lentiviral control shRNA (shCont) or shRNA vector targeting TCTP (shTCTP #1 and #2). Images were taken with $\times 100$ magnification. scale bar $200 \mu\text{m}$ (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 $\times 100$ magnification using. Quantification was carried by counting the number of migratory cells that had infiltrated the filter. Values are means \pm 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 ($n = 6/\text{group}$). 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 GSK3 β 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 process²³. 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 manner³⁰. It was also demonstrated that cofilin, an actin binding protein, promotes metastasis³¹ and that TCTP influences F-actin cytoskeleton modification by competing with cofilin at the cofilin-binding site of actin³². 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 regulation^{33,34}. Kloc *et al* observed the positive correlation with expression level of TCTP and RhoA in ovarian cancer cell line¹⁷. 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 migration^{35,36}, 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 debated^{38–40}. Studies in *Drosophila* revealed that dTCTP directly interacts with dRheb GTPase, a positive regulator of mTORC1, and acts as its guanine nucleotide exchange factor (GEF)⁴¹. mTORC2 regulates actin cytoskeleton rearrangement thereby promoting cell migration^{36,42}. We observed previously^{6,25} as well as in the current study, elevation of Akt phosphorylation at Ser⁴⁷³ residue in TCTP overexpressing cells (Figure 4a). Ser⁴⁷³ 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 outcome^{43,44} 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 transcriptions^{45,46}. 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/GSK3 β 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 metastasis^{49,50}. 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 change⁶. 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 metastasis². 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 multi-step 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, EGFR, 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-GSK3 β (Ser⁹), GSK3 β , phospho-P13K, P13K p85 α , 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 modification⁶. 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 described⁵¹.



Transfection of TCTP shRNA was performed by using WelFect-EX™ PLUS transfection reagent (WelGENE, Korea). The level of suppression of TCTP gene was determined by immunoblotting.

Generation of stable cell lines by lentiviral transduction. Lentiviruses were generated as described⁵² with minor modification. Briefly, TCTP shRNA cloned PLKO.1, pVSV-G, and Δ8.9 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 modification⁵³. Briefly, cell extracts were prepared in modified RIPA buffer [50 mM 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 (Na₂VO₄) and 1 mM sodium fluoride (NaF)] with protease inhibitor cocktails (Roche Molecular Biochemicals) and phosphatase inhibitor cocktails (Sigma-Aldrich Biotechnology, LP, 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% acetone, air-dried, dissolved in sample buffer. Samples were detected using immunoblotting method.

Gelatin Zymography. Gelatin zymography was performed as described previously with minor modification⁵⁴. Briefly, cells were infected and incubated in the serum free media for indicated time. Cell supernatants were collected and centrifuged at 12,000 rpm for 10 min at 4°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 polymerized with 1 mg/ml 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 modification⁵⁵. 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 calculated by measuring the distance of the migrating edge. Mean values were obtained from at least 3 separate experiments.

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-shTCTP. After 24 hours, Cells were harvested with Trypsin/EDTA and resuspended in serum-free medium at a density of 5.0 × 10⁵ cells/ml. B16F10 stable cells were resuspended in serum-free medium at a density of 1.0 × 10⁶ cells/ml. 100 μl of cells were seeded into the upper chamber. Lower chambers were filled with 650 μl medium containing 10% FBS as a chemoattractant. After 18 hours cells were fixed and permeabilized with 100% methanol and stained with 0.5% crystal violet/20% methanol. The non-migrating cells on the upper surface of the filter were removed by cotton swab. The number of migratory cells was measured by counting at × 100 magnifications using a microscope. Invasion assays were performed using the BD BioCoat™ BD Matrigel™ Invasion Chamber (BD Bioscience, NJ, USA) according to the manufacturer's instructions. Briefly, infected LLC-PK1 cells were resuspended in serum-free medium at a density of 1.0 × 10⁶ cells/ml and B16F10 stable cells were resuspended in serum-free medium at a density of 2.0 × 10⁵ cells/ml. 500 μl of cells were seeded into the upper chamber and lower chambers were filled with 750 μl medium containing 10% FBS as a chemoattractant. After 24 hours, the transwell invasion assay was performed with the same method as transwell migration assay.

Immunofluorescence confocal microscopy. Confocal microscopy was performed as described previously with minor modification⁶. Briefly, cells were grown on glass cover slips to 70% confluence and were infected with 10 MOI Ad GFP or TCTP-GFP virus. Cells were incubated for 24 hours and then fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. For staining E-cadherin, cells were fixed with 100% methanol for 15 minutes at -20°C. After washing with PBS, the cells were permeabilized in 0.2% Triton X-100 in PBS and blocked in 1% bovine serum albumin. Cells were then stained with mouse monoclonal anti-E-cadherin antibody (1:200), anti-N-cadherin antibody (1:200) and rabbit polyclonal anti-β-catenin (1:200) and then probed with Alexa Fluor 488 or 594 conjugated goat anti-mouse IgG (Invitrogen, CA, USA). For F-actin staining, Cells were stained with rhodamine conjugated phalloidin (Molecular Probes, OR, USA). Counterstain with DAPI was performed by mounting in ProLong Gold antifade reagent (Invitrogen, CA, USA). Cells were photographed at × 400 magnifications with a fluorescence confocal microscope

(LSM510, Zeiss, Germany). Cell morphology was examined under an inverted microscope (Zeiss, Germany) at × 400 magnification.

Preparation of Nuclear fraction. Nuclear protein was extracted using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) following the manufacturer's supplied protocol

Experimental metastasis assay. Experimental metastasis assay was performed as described previously⁵⁶ with mild modification. Briefly, B16F10 cells were trypsinized and suspended in PBS. Cells (1 × 10⁶ 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 ImageJ 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 Prism 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.

Additional information

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