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RESEARCH PAPER



# *PCK2* promotes invasion and epithelial-to-mesenchymal transition in triple-negative breast cancer by promoting TGF-β/SMAD3 signaling through inhibiting TRIM67-mediated SMAD3 ubiquitination

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**ABSTRACT**

*PCK2*, which encodes mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M), is upregulated in various cancers. We demonstrated high expression of PEPCK-M in approximately half of triple-negative breast cancers (TNBCs) previously. TNBC is associated with an aggressive phenotype and a high metastasis rate. In this study, we investigated the role of *PCK2* in TNBC. *PCK2* knockdown suppressed proliferation and mTOR signaling in TNBC cells. In addition, cell invasion/migration ability and the expression of epithelial-to-mesenchymal transition (EMT) markers were positively correlated with *PCK2* expression in TNBC cells via regulation of transforming growth factor-β (TGF-β)/SMAD3 signaling. *SMAD3* was positively regulated by *PCK2* in TNBC cells. Knockdown of *SMAD3* in *PCK2*-overexpressing TNBC cells reduced the expression levels of EMT markers, Snail and Slug, and suppressed cell invasion/migration. In addition, *PCK2* knockdown attenuated the stimulatory effect of TGF-β on SMAD3 phosphorylation in TNBC cells. PEPCK-M promotes the protein and mRNA expression of SMAD3 via competitive binding to tripartite motif-containing 67 (TRIM67), an E3 ubiquitin ligase, to reduce SMAD3 ubiquitination, which leads to promoting nuclear translocation of SMAD3 and autoregulation of SMAD3 transcription. Moreover, high *PCK2* mRNA expression was significantly associated with poor survival in TNBC patients. In conclusion, our study revealed for the first time that *PCK2* activates TGF-β/SMAD3 signaling by regulating the expression and phosphorylation of SMAD3 by inhibiting TRIM67-mediated SMAD3 ubiquitination and promoting the stimulatory effect of TGF-β to promote TNBC invasion. The regulatory effect of *PCK2* on mTOR and TGF-β/SMAD3 signaling suggests that *PCK2* is a potential therapeutic target for suppressing TNBC progression.

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## Background

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer-related deaths in women, with an estimated 2.3 million new cases and 0.7 million related deaths in 2020.[1] The prognosis of patients with breast cancer differs according to molecular subtype and stage, with the worst outcomes in patients with triple-negative breast cancer (TNBC) and stage IV disease.[2] TNBC is characterized by the absence of the estrogen receptor (ER) and progesterone receptor (PR) and a lack of human epidermal growth factor 2 (HER2) gene amplification and accounts for approximately 15% of breast cancers. TNBC is associated with an aggressive phenotype and a high rate of metastasis.[3,4] Cancer metastasis often involves the induction of epithelial-to-mesenchymal transition (EMT), which leads to the development of an invasive cell phenotype. Several signaling pathways, such as the Hedgehog, Wnt, Notch, or transforming growth factor (TGF)-β pathways have been reported to participate in EMT induction[5,6] and many targeted agents are under development to treat TNBC.[7] Among these oncogenic signaling pathways, TGF-β signaling confers metastatic potential in breast cancer cells through EMT induction.[8,9]

Recently, alterations in metabolic enzyme expression have been found in various cancers and have been shown to cause metabolic reprogramming and tumor progression.[10,11] Therefore, cancer-specific metabolic enzymes are potential targets for cancer treatment. Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the conversion of oxaloacetate to phosphoenolpyruvate and links the tricarboxylic acid cycle to glycolysis/gluconeogenesis.[12] Cytosolic PEPCK (PEPCK-C)

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and mitochondrial PEPCK (PEPCK-M) are two isoenzymes of PEPCK and are encoded by *PCK1* and *PCK2*, respectively.[12,13] PEPCK-M has been demonstrated to promote cell proliferation and metabolic adaptation in certain types of cancers.[14–16] *PCK2* overexpression has been observed in breast cancer according to The Cancer Genome Atlas (TCGA) analysis.[16] Recently, we found that PEPCK-M is differentially expressed in different subtypes of breast cancer. High expression of PEPCK-M has been identified in 46% of TNBC tumors.[17] In addition, we demonstrated that PEPCK-M promotes the proliferation and cell cycle progression of $ER^+$ breast cancer cells via upregulation of the mTOR pathway.[17] The function of *PCK2* in TNBC is not well understood. In this study, we explored the function of *PCK2* in TNBC and demonstrated that *PCK2* promotes TNBC cell invasion and EMT via the upregulation of TGF-β/SMAD3 signaling through the inhibition of tripartite motif-containing 67 (TRMI67)-mediated SMAD3 ubiquitination and the promotion of SMAD3 transcription autoregulation.

## Materials and methods

### Cell lines, plasmids, and reagents

MDA-MB-231 cells were kindly provided by Dr. Wen-Chun Hung (National Institute of Cancer Research, National Health Research Institutes, Tainan, Taiwan). Hs578T cells were purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). MDA-MB-468 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231 and MDA-MB-468 cells were cultured in Leibovitz's L-15 medium supplemented with 2 mm L-glutamine, 10% fetal bovine serum, and antibiotics. Hs578T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 0.01 mg/mL human insulin, 10% fetal bovine serum, and antibiotics. The incubator was maintained at 37°C with 5% $CO_2$. The shRNA sequences targeting *PCK2*, *SMAD3* and pLKO.1-null-T (vector control) were purchased from the National RNAi Core Facility of Academia Sinica. The plasmids for *PCK2* overexpression and the vector control (EX-Z2611-Lv157, pEZ-Lv157) were obtained from GeneCopoeia (Rockville, MD, USA). Recombinant human TGF-β protein was purchased from PeproTech (Rocky Hill, NJ, USA). MG132 was purchased from MedChemExpress (Monmouth Junction, NJ, USA).

### Establishment of stable cell clones

We used a lentiviral transduction approach to knock down or overexpress *PCK2* and *SMAD3* in cell lines. We followed the instructions provided by the National RNAi Core Facility of Academic Sinica. PEPCK-M and SMAD3 expression in stable cell clones was confirmed using western blotting.

### Cell proliferation assay

MDA-MB-468 ($2 \times 10^4$), MDA-MB-231 ($1 \times 10^4$), and Hs578T ($2 \times 10^4$) cells with or without *PCK2* knockdown or overexpression were seeded in 24-well culture plates. Cell proliferation under each indicated condition was assessed using the methylene blue colorimetric assay. Details of the methylene blue colorimetric assay are described in our previous paper.[18] The cell proliferation rate on Day 0 was set to 1 (100%) as the reference, and the proliferation rate of the cells from Days 1 to 4 was calculated by dividing the absorbance at 595 nm each day by the absorbance at 595 nm on Day 0. Each data point was measured in triplicate, and the results are presented as the mean ± standard error.

### Western blotting

The details of the western blot analysis are described in our previous paper.[19] Antibodies against PEPCK-M (GTX114919) were purchased from GeneTex (Irvine, CA, USA). Antibodies against GAPDH (sc -32,233) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Antibodies against E-cadherin (Cat No. 610182) and N-cadherin (Cat No. 610921) were purchased from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Antibodies against p-SMAD3 (ab52903) were purchased from Abcam (Cambridge, MA, USA). Antibodies against SMAD3 (#9523), Snail (#3879), Slug (#9585), and Vimentin (#5741) were purchased from Cell Signaling Technology (Danvers, MA, USA).

### Preparation of nuclear and cytosolic proteins

Cells were lysed with lysis buffer (10 mm Tris, pH 7.5, 10 mm NaCl, 3 mm $MgCl_2$, 0.05% NP40, 1 mm EGTA) for 10 minutes at 4°C. The lysate was separated into a supernatant and a pellet by centrifugation at 14,000 rpm for 10 minutes at 4°C. The supernatant was collected and centrifuged at 14,000 rpm for 15 min at 4°C to obtain cytosolic protein. The pellet containing nuclei was washed with wash buffer (10 mm PIPES, pH 6.8, 300 mm sucrose, 3 mm $MgCl_2$, 1 mm EGTA, 25 mm NaCl) twice and then resuspended in wash buffer. The resuspended pellet was layered over 1 M sucrose buffer and then centrifuged at 14,000 rpm for 10 min at 4°C to remove nonsedimented cellular debris. The pellet was washed with wash buffer twice, and nuclear extraction buffer (20 mm HEPES, pH 7.9, 300 mm NaCl, 1.5 mm $MgCl_2$, 0.2 mm EDTA) was added. The sample was incubated on ice for 30 min and then centrifuged at 14,000 rpm for 15 min at 4°C to obtain the supernatant, which contained the nuclear protein. Equal amounts of nuclear and cytosolic proteins were fractionated by SDS–PAGE for western blot analysis.

### Immunoprecipitation and ubiquitination assay

Immunoprecipitation was performed as described previously.[20] Cells were lysed with RIPA buffer, and equal amounts (1 mg) of cellular proteins were incubated with anti-PEPCK-M (GTX114919, GeneTex), anti-TRIM67 (24369–-1-AP, Proteintech) or anti-rabbit IgG antibodies (sc-2762, Santa Cruz Biotechnology) overnight at 4°C. The immune complexes were then precipitated with protein A-Sepharose beads at 4°C for an additional 2 hours. The immunocomplexes were then collected and washed with RIPA buffer before immunoblotting. Western blotting was performed by probing

the membranes with anti-PEPCK-M or anti-SMAD3 antibodies to detect the interaction of TRIM67 with PEPCK-M or SMAD3. For the ubiquitination assay, cells were treated with 20 μM MG132 for 4 hours and washed with PBS. Then, RIPA lysis buffer was used to harvest the cells, and the cellular protein was extracted after centrifugation at 13,000 rpm for 15 minutes at 4°C. Equal amounts (1 mg) of cellular proteins were incubated with anti-SMAD3 (#9523) or anti-rabbit IgG (sc-2762, Santa Cruz Biotechnology) antibodies overnight at 4°C, followed by the addition of protein A-Sepharose beads and incubation for 2 hours. The immunocomplexes were washed and eluted for subsequent western blotting to visualize polyubiquitylated protein bands with an anti-ubiquitin antibody (GTX630148, GeneTex).

### Expression array and gene set enrichment analysis (GSEA)

We performed a gene expression microarray analysis of shPCK2-transduced and vector-control-transduced MDA-MB-468 cells. Gene sets or molecular pathways enriched with differentially expressed genes were analyzed by GSEA according to a previously described protocol.[21,22]

### Quantitative RT–PCR

RNA was extracted using TriPure Isolation Reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. RNA was quantified using a NanoDrop 2000 (Thermo Scientific, Wilmington, USA) spectrophotometer. Two micrograms of total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT–PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) with an ABI 7500 Fast system (Applied Biosystems) with the cycle threshold (Ct) determined. Melting curve was performed to test the primer specificity. The concentration of the primers was 300 nM. The mRNA expression level of SMAD3 was normalized with the mRNA expression level of internal control, GAPDH, and calculated with the value of $2^{-\Delta Ct}$ ($\Delta Ct = Ct^{SMAD3}\text{-}Ct^{GAPDH}$). The relative expression level of SMAD3 in each condition relative to control, shCtrl or vector control, was calculated with the value of $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta CT^{test\ sample} - \Delta CT^{control\ sample}$). The sequences of the primers used were as follows: SMAD3-forward, 5'-GCATGGACGCAGGTTCTCC-3'; SMAD3-reverse, 5'-GGCTC-GCAGTAGGTAACTGG-3'; GAPDH-forward, 5'-ACGTGATGCAGAACCACC-TACTG-3'; and GAPDH-reverse, 5'-ACGACGGCTGCAAAAGTGGCG-3'.

### In vitro cell invasion and migration assays

*In vitro* cell invasion and migration assays were performed as described previously.[20] For the invasion assay, MDA-MB-468 ($1.5\times10^4$ cells/well in 100 μl of serum-free medium), MDA-MB-231, and Hs578T ($1\times10^4$ cells/well in 100 μl of serum-free medium) cells with or without *PCK2* knockdown were seeded in the upper compartment of a 24-well Transwell (Corning, NY, USA) with a polycarbonate filter (8 μm pore size) coated with Matrigel (Corning, NY, USA). Medium containing 10% FBS with or without the addition of 5 ng/mL TGF-β to the lower compartment served as a chemoattractant. The procedures for the migration assay were the same as those used for the invasion assay, but the polycarbonate filters were not coated with Matrigel. Noninvasive or nonmigrating cells on the surface of the filter were removed using cotton swabs 16–18 hours later. Invading or migrating cells were stained with 0.5% Coomassie Brilliant Blue solution and counted under a microscope.

### Evaluation of the prognostic value of PCK2 in TNBC patients

Two online databases, the Kaplan – Meier plotter (www.kmplot.com.) and the Breast Cancer Integrative Platform (BCIP) database (http://www.omicsnet.org/bcancer/.), were used to evaluate the prognostic value of *PCK2* in TNBC patients. The correlation of the *PCK2* mRNA expression status with relapse-free survival (RFS), overall survival (OS) and distant metastasis-free survival (DMFS) was assessed in 534, 201 and 424 TNBC patients, respectively, based on the Kaplan – Meier plotter database, an online tool containing gene expression profiles and the survival status of patients from the GEO, EGA and TCGA datasets.[23] The correlation of the *PCK2* mRNA expression status of tumors with RFS, OS and DMFS was analyzed in 24, 49 and 49 TNBC patients, respectively, based on the BCIP database, an integrative platform containing gene expression profiles and clinical information of breast cancer from the METABRIC, TCGA, and GEO datasets.[24]

### Statistical analysis

The Wilcoxon rank-sum test was used to compare the relative migration and invasion rates between TNBC cells under the indicated conditions using SigmaPlot version 14.0 (SYSTAT Software Inc., USA). The threshold for statistical significance was set at $p < .05$. The hazard ratio (HR) and the log-rank $p$ value were calculated automatically via a website tool and used to assess the prognostic value of the *PCK2* mRNA expression status in TNBC.

## Results

### PCK2 promotes cell proliferation and the mTOR pathway in TNBC cells

We evaluated the function of *PCK2* in TNBC cell line proliferation. The proliferation rates of all three TNBC cell lines (MDA-MB-468, Hs578T, and MDA-MB-231) were decreased by *PCK2* knockdown (Figure 1a–c). In contrast, the overexpression of *PCK2* in MDA-MB-231 cells, which have relatively lower expression of PEPCK-M, promoted proliferation compared to that in cells infected with the vector control plasmid (Figure 1d). Since we previously showed that *PCK2* upregulates the mTOR pathway in $ER^+$ breast cancer,[17] we evaluated whether this effect could also be observed in TNBC. We found that the levels of mTOR and its downstream signals (S6K and 4EBP–1) and their phosphorylated forms were reduced in

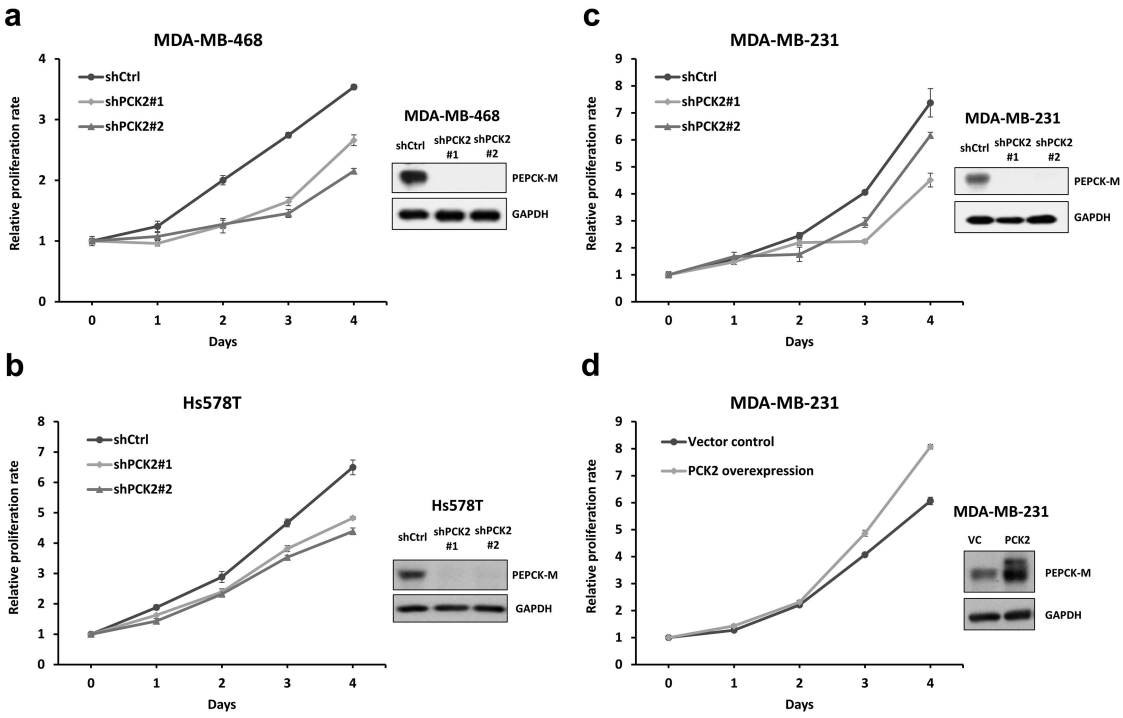


**Figure 1.** The effect of *PCK2* on the proliferation of TNBC cells. (a) The relative proliferation rates of MDA-MB-468 cells with or without knockdown of *PCK2*. The difference on proliferation rate between shCtrl and shPCK2#1: day 1, $p = .029$; day 2, $p = .029$; day 3, $p = .029$; day 4, $p = .029$. The difference of proliferation rate between shCtrl and shPCK2#2: day 1, $p = .057$; day 2, $p = .029$; day 3, $p = .029$; day 4, $p = .029$. (b) The relative proliferation rates of Hs578T cells with or without knockdown of *PCK2*. The difference on proliferation rate between shCtrl and shPCK2#1: day 1, $p = .057$; day 2, $p = .029$; day 3, $p = .029$; day 4, $p = .029$. The difference of proliferation rate between shCtrl and shPCK2#2: day 1, $p = .029$; day 2, $p = .029$; day 3, $p = .029$; day 4, $p = .029$. (c) The relative proliferation rates of MDA-MB-231 cells with or without knockdown of *PCK2*. The difference on proliferation rate between shCtrl and shPCK2#1: day 1, $p = .686$; day 2, $p = .200$; day 3, $p = .029$; day 4, $p = .029$. The difference of proliferation rate between shCtrl and shPCK2#2: day 1, $p = .486$; day 2, $p = .343$; day 3, $p = .029$; day 4, $p = .029$. (d) The relative proliferation rates of MDA-MB-231 cells with or without overexpression of *PCK2*. The difference on proliferation rate between vector control and *PCK2* overexpression: day 1, $p = .200$; day 2, $p = .686$; day 3, $p = .029$; day 4, $p = .029$. Each proliferation rate was replicated 4 times with 4 wells of cells for each condition. The data were shown as mean ± standard error (error bar). Wilcoxon rank-sum test was used for statistical analysis on the difference of proliferation rate.

TNBC cell lines with *PCK2* knockdown (Supplementary Figure S1A). In contrast, mTOR pathway signaling was promoted by *PCK2* overexpression in TNBC cell lines (Supplementary Figure S1B). These results are consistent with those observed in ER$^+$ breast cancer cells.[17]

### PCK2 promotes the invasiveness of TNBC cells

To determine whether *PCK2* expression is correlated with cell invasion and EMT, we next performed invasion and migration assays to evaluate the effects of *PCK2* on invasion and EMT in these cell lines. The invasion assay was performed using transwell units with polycarbonate filters coated with matrigel on the upper surface. The migration assay was performed using transwell units with polycarbonate filters not coated with matrigel on the upper surface. The invaded and migrated cells were counted. The invasion and migration abilities were calculated by the cell count in each condition (shPCK2 or *PCK2* overexpression) divided the cell count in the control condition (shCtrl or VC). The invasion and migration abilities of all three TNBC cell lines were significantly attenuated by *PCK2* knockdown but promoted by *PCK2* overexpression (Figure 2). The expression levels of the EMT markers N-cadherin and/or Vimentin were decreased by the knockdown of *PCK2* but increased by the overexpression of *PCK2* in the three TNBC cell lines (Figure 2). In contrast, the expression level of the mesenchymal-to-epithelial transition (MET) marker E-cadherin was increased by *PCK2* knockdown but decreased by *PCK2* overexpression in MDA-MB-468 cells (Figure 2a). These results indicate that *PCK2* promotes the invasion and EMT of TNBC cells.

### PCK2 promotes TNBC cell invasion and EMT via the regulation of TGF-β/SMAD3 signaling

To understand the signaling pathways regulated by *PCK2* in TNBC cells, we performed gene expression microarray analysis to obtain differential gene expression profiles between MDA-MB-468 cells transduced with shPCK2 and those transduced with the vector control. GSEA revealed that the TGF-β signaling gene set was negatively enriched (down-regulated) in MDA-MB-468 cells with *PCK2* knockdown compared to cells transduced with the vector control (Figure 3a). TGF-β/SMAD3 signaling plays a key role in regulating EMT and cancer metastasis.[25] Decreased expression of *SMAD3*, a crucial regulator of TGF-β signaling, was found in this gene set. To validate the results of the array data, we performed qualitative and quantitative RT–PCR and western blotting for SMAD3 in three TNBC cell lines with or without *PCK2* knockdown or overexpression. The knockdown of *PCK2* reduced the mRNA (Figure 3b) and

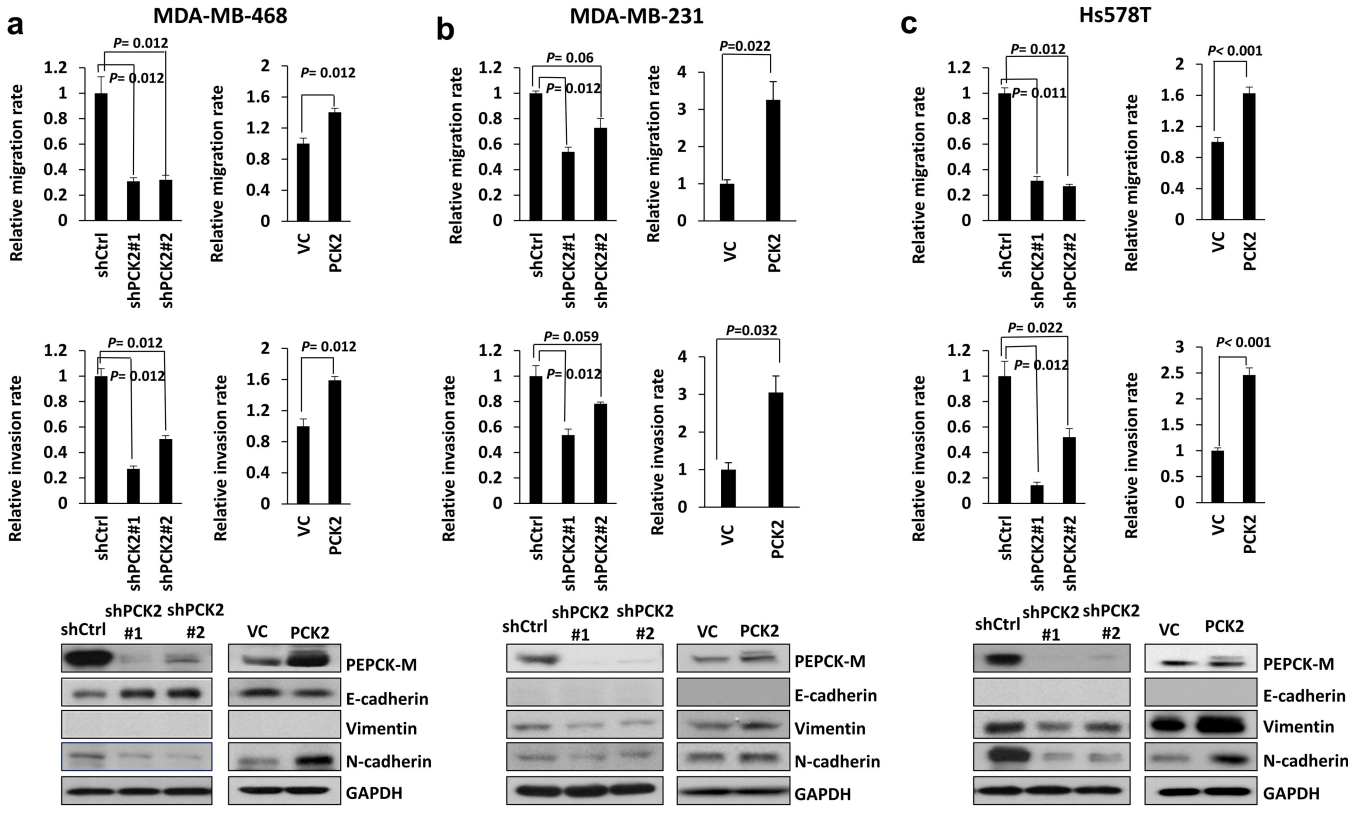


**Figure 2.** The effect of *PCK2* on cell invasion and the expression of EMT markers of TNBC cells. (a) The relative migration/invasion abilities, expression of EMT markers (N-cadherin and Vimentin) and expression of MET marker (E-cadherin) in MDA-MB-468 cells with or without knockdown or overexpression of *PCK2*. (b) The relative migration/invasion abilities, expression of EMT markers (N-cadherin and Vimentin) and expression of MET marker (E-cadherin) in MDA-MB-231 cells with or without knockdown or overexpression of *PCK2*. (c) The relative migration/invasion abilities, expression of EMT markers (N-cadherin and Vimentin) and expression of MET marker (E-cadherin) in Hs578T cells with or without knockdown or overexpression of *PCK2*. Each invasion and migration rate was replicated 5 times with 5 wells of cells for each condition. The data were shown as mean ± standard error (error bar). Wilcoxon rank-sum test was used for statistical analysis on the difference of invasion and migration rate.

protein (Figure 3c) expression levels of SMAD3 in all three TNBC cell lines. In contrast, overexpression of *PCK2* increased the mRNA (Figure 3b) and protein (Figure 3c) levels of SMAD3 in all three TNBC cell lines. These results indicated that *PCK2* upregulates SMAD3 in TNBC cells at the transcriptional level.

Furthermore, we aimed to determine whether *PCK2* promotes cell invasion and EMT in TNBC through the regulation of TGF-β/SMAD3 signaling. We assessed the levels of the phosphorylated form of SMAD3 and downstream targets of TGF-β/SMAD3 signaling, Snail and Slug, in three TNBC cell lines with or without *PCK2* knockdown or overexpression. Snail and Slug are transcription factors that contribute to EMT.[26] *PCK2* knockdown reduced the expression of phosphorylated SMAD3, Snail, and Slug in all three TNBC cell lines (Figure 4a). In contrast, *PCK2* overexpression increased the expression of phosphorylated SMAD3, Snail, and Slug in these cell lines (Figure 4a). To confirm the association of invasion and the EMT phenotype with *PCK2* and TGF-β/SMAD3 signaling, we evaluated the expression levels of TGF-β/SMAD3 effector downstream targets and EMT markers and performed invasion and migration assays in TNBC cells with *PCK2* overexpression and *SMAD3* knockdown. The increased expression of phosphorylated SMAD3, Snail, Slug, and EMT markers (N-cadherin and/or Vimentin) in TNBC cell lines overexpressing *PCK2* was attenuated by *SMAD3* knockdown (Figure 4b). The promotion of TNBC cell invasion and migration induced by *PCK2* overexpression was attenuated by *SMAD3* knockdown (Figure 4c). Taken together, these results demonstrate that *PCK2* promotes the invasion and EMT of TNBC cells via upregulation of the TGF-β/SMAD3 signaling pathway.

## *PCK2 promotes TNBC cell invasiveness by regulating the stimulatory effect of TGF-β on SMAD3 phosphorylation*

To understand the effect of *PCK2* on the TGF-β signaling pathway, we added TGF-β or vehicle (control) to the culture medium of TNBC cells with or without *PCK2* knockdown and assessed the phosphorylation status of SMAD3 and the invasion/migration ability of the cells. The addition of TGF-β significantly promoted the phosphorylation of SMAD3 (Figure 5a) and augmented the invasion and migration abilities of TNBC cells transduced with the vector control (shCtrl) (Figure 5b). However, the stimulatory effect of TGF-β on SMAD3 phosphorylation (Figure 5a) and invasion/migration (Figure 5b) was not observed in TNBC cells with *PCK2* knockdown. These results demonstrated that *PCK2* regulates the stimulatory effect of TGF-β on TGF-β/SMAD3 signaling-associated cell invasiveness in TNBC cells.

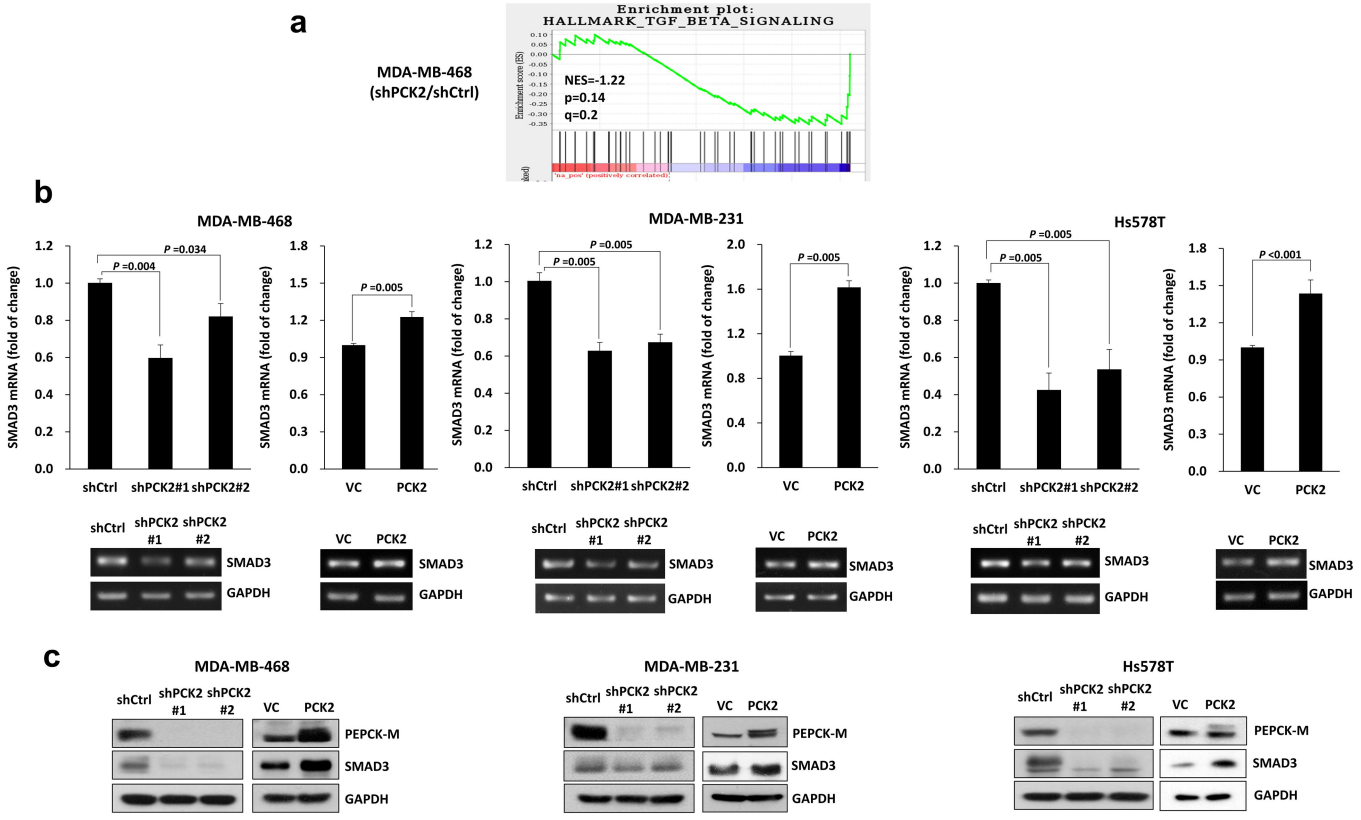


**Figure 3.** The regulation of SMAD3 by *PCK2*. (a) TGF-β signaling pathway gene sets were negatively enriched in MDA-MB-468 cells with *PCK2* knockdown. (b) The mRNA expression levels of *SMAD3* in MDA-MD-468 (left), MDA-MD-231 (middle), and Hs578T (right) cells with or without knockdown or overexpression of *PCK2*. Each mRNA expression was replicated 6 times with 6 samples for each condition. The data were shown as mean ± standard error (error bar). Wilcoxon rank-sum test was used for statistical analysis on the difference of mRNA expression. (c) The protein expression level of SMAD3 in MDA-MD-468 (left), MDA-MD-231 (middle), and Hs578T (right) cells with or without knockdown or overexpression of *PCK2*.

### *PCK2 promotes SMAD3 expression through protein–protein interactions with TRIM67 and the regulation of TRIM67-mediated ubiquitination and proteasomal degradation of SMAD3*

We wanted to investigate how *PCK2* regulates SMAD3 expression. A previous luciferase reporter gene assay revealed that PEPCK-M is a candidate SMAD3-interacting protein.[27] Therefore, we hypothesized that PEPCK-M functions as a nuclear transcription factor or co-factor to upregulate SMAD3 transcription. We extracted nuclear and cytosolic proteins from 3 TNBC cell lines with or without *PCK2* overexpression but did not detect PEPCK-M in the nucleus by western blotting (Figure 6a). The protein expression of SMAD3 in the nucleus was increased in the 3 TNBC cell lines with *PCK2* overexpression compared with that in the control cells (Figure 6a). These results indicated that PEPCK-M is not a nuclear transcription factor or co-factor of SMAD3. Because nuclear SMAD3 protein expression is increased in *PCK2*-overexpressing TNBC cells and because SMAD3 can autoregulate SMAD3 expression,[28] we hypothesized that PEPCK-M interacts with SMAD3 indirectly to regulate SMAD3 expression. We searched for proteins that can interact with both PEPCK-M and SMAD3 by utilizing curated empirical interaction data from BioGRID (https://thebiogrid.org/).[29] We identified 920 SMAD3-interacting proteins and 126 PEPCK-M-interacting proteins. Nine proteins were found to interact with both SMAD3 and PEPCK-M (Figure 6b). Among the nine proteins, we selected TRIM67 as the target because TRIM67 is a class I member of the tripartite motif (TRIM) family of E3 ubiquitin ligases.[30,31] In addition, we found that the ubiquitination level of SMAD3 was reduced in all 3 TNBC cell lines overexpressing *PCK2* (Figure 6c). Furthermore, the reduced protein expression of SMAD3 in TNBC cells with *PCK2* knockdown was partially reversed when the cells were exposed to the proteasome inhibitor MG132 (20 μM) (Figure 6d). These results indicated that the *PCK2*-induced increase in SMAD3 protein expression in TNBC cell lines could occur via inhibition of ubiquitin-mediated SMAD3 degradation. Therefore, we hypothesized that PEPCK-M inhibits TRIM67-mediated SMAD3 ubiquitination by competing with SMAD3 for binding to TRIM67. We performed an immunoprecipitation assay to evaluate whether *PCK2* overexpression disrupted the endogenous interaction between TRIM67 and SMAD3 in TNBC cells. Figure 6e shows the results of coimmunoprecipitation of TRIM67 with PEPCK-M or SMAD3 in all 3 TNBC cell lines with or without *PCK2*

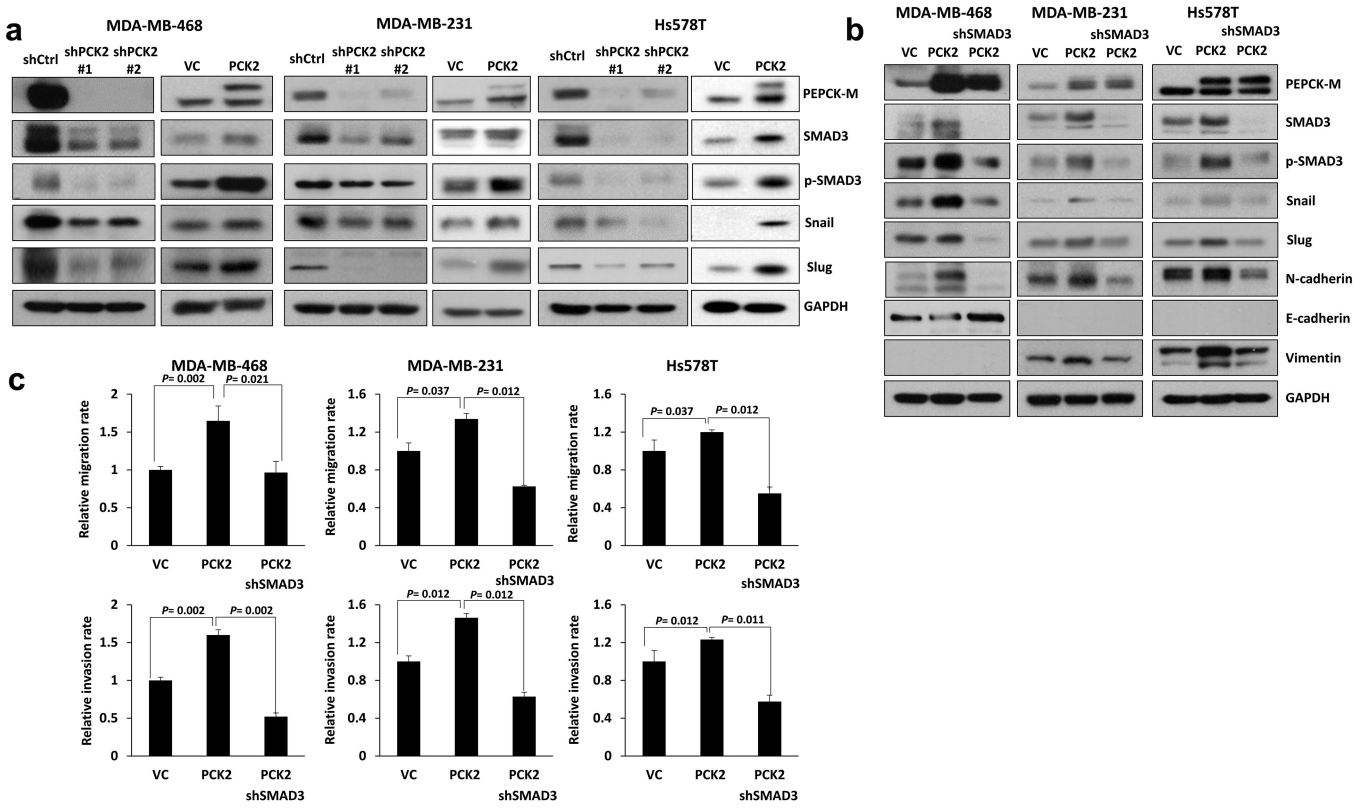


**Figure 4.** The regulatory effect of *PCK2* on tgf-β/SMAD3 signaling and invasiveness of TNBC cells. (a) The protein expression levels of SMAD3, phosphorylated SMAD3, and the targets of tgf-β/SMAD3, Snail and slug, in MDA-MB-468 (left), MDA-MB-231 (middle), and Hs578T (right) cells with or without knockdown or overexpression of *PCK2*. (b) The protein expression levels of SMAD3, phosphorylated SMAD3, Snail, slug, N-cadherin, Vimentin, and E-cadherin in *PCK2*-overexpressing MDA-MB-468 (left), MDA-MB-231 (middle), and Hs578T (right) cells with or without SMAD3 knockdown. The cells infected with vector control were used as controls. (c) The relative invasion and migration abilities of *PCK2*-overexpressing MDA-MB-468 (left), MDA-MB-231 (middle), and Hs578T (right) cells with or without SMAD3 knockdown. The cells infected with vector control were used as controls. Each invasion and migration rate was replicated 5 times with 5 wells of cells for each condition. The data were shown as mean ± standard error (error bar). Wilcoxon rank-sum test was used for statistical analysis on the difference of invasion and migration rate.

overexpression. The overexpression of *PCK2* suppressed the colocalization of endogenous TRIM67 with SMAD3 in all 3 TNBC cell lines (Figure 6e). Moreover, increased SMAD3 nuclear translocation was detected in TNBC cells overexpressing *PCK2* (Figure 6a). Increased SMAD3 nuclear translocation may auto-regulate SMAD3 transcription (Figure 3b). Taken together, these results suggest that PEPCK-M protects SMAD3 against ubiquitination and promotes the nuclear translocation of SMAD3 by competing with SMAD3 for binding to TRIM67.

### *High PCK2 expression is correlated with a poor prognosis in TNBC patients*

To evaluate the prognostic value of *PCK2* in TNBC patients, we performed a bioinformatics analysis to examine the association between *PCK2* expression and survival in TNBC patients. Kaplan-Meier Plotter database analysis revealed that high *PCK2* mRNA expression was significantly associated with shorter RFS (HR = 1.5, log-rank $p = .016$), OS (HR = 2.03, log-rank $p = .065$) and DMFS (HR = 1.46, log-rank $p = .037$) (Figure 7a–c). The results from the BCIP database revealed that a high expression level of *PCK2* was correlated with a poorer prognosis, as indicated by a shorter RFS (HR = 3.63, log-rank $p = .046$), OS (HR = 4.64, log-rank $p = .017$) and DMFS (HR = 6.35, log-rank $p = .006$) in TNBC patients (Figure 7d–f). Collectively, the above results indicated that *PCK2* is a marker of a poor prognosis in TNBC patients.

## Discussion

In this study, we showed that *PCK2* promotes the proliferation and invasion of TNBC cells via the promotion of the mTOR and TGF-β/SMAD3 signaling pathways. We found that PEPCK-M competitively binds to TRIM67, which prevents the SMAD3-TRIM67 interaction and inhibits TRIM67-mediated SMAD3 ubiquitination and proteasomal degradation.

The role of *PCK2* in cancer development has mainly been revealed in the context of promoting cell proliferation.[14,16–18,32] *PCK2* has been shown to be upregulated in response to a glucose-deprived tumor microenvironment to support the growth of non-small cell lung cancer (NSCLC).[16] Inhibition of *PCK2* using siRNA/shRNA or pharmacological inhibitors can efficiently suppress tumor growth in various cancers.[14,17,18,32] Recently, a compound, 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoate, has been identified from in silico structural screen of 7 million compounds to exert inhibitory effect on PCK2 enzyme activity and proliferation of TNBC cell lines *in vitro*.[33] *PCK2* upregulates the mTOR pathway and promotes cell proliferation in pancreatic neuroendocrine tumors and $ER^+$ breast cancer.[17,18] *PCK2* is critical for metabolic switching and

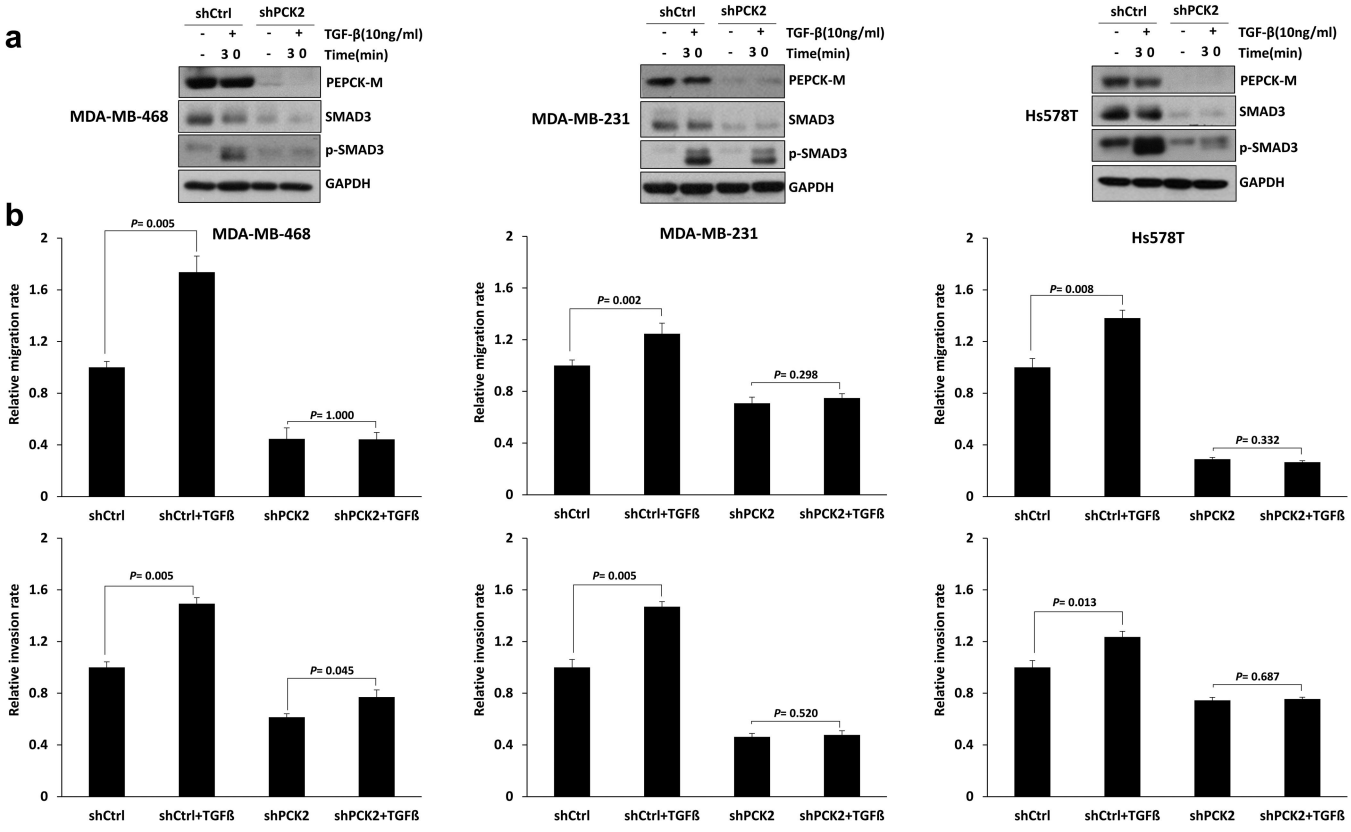


**Figure 5.** The regulatory effect of *PCK2* on the stimulatory effect of tgf-β on SMAD3 phosphorylation and cell invasiveness in TNBC. (a) The protein expression levels of SMAD3 and phosphorylated SMAD3 in MDA-MB-468 (left), MDA-MB-231 (middle), and Hs578T (right) cells with or without knockdown of *PCK2* stimulated with tgf-β. Cells not stimulated with tgf-β, vehicle (water), were used as controls. (b) The relative migration and invasion abilities of MDA-MB-468 (left), MDA-MB-231 (middle), and Hs578T (right) cells with or without knockdown of *PCK2* stimulated with tgf-β. Cells not stimulated with tgf-β, vehicle (water), were used as controls. Each invasion and migration rate was replicated 6 times with 6 wells of cells for each condition. The data were shown as mean ± standard error (error bar). Wilcoxon rank-sum test was used for statistical analysis on the difference of invasion and migration rate.

maintenance of tumor-initiating cells in prostate cancer.[34] In our current study, the activation of the mTOR pathway and promotion of cell proliferation by *PCK2* in TNBC were consistent with the results of our recent study on ER$^+$ breast cancer.[17] In addition, we showed that *PCK2* transcriptionally upregulates SMAD3, which leads to the upregulation of the downstream targets Snail and Slug in TGF-β/SMAD3 signaling and the promotion of EMT in TNBC cells. The expression and prognostic significance of *PCK2* have recently been demonstrated in pancancer analysis through bioinformatic tools.[35] However, *PCK2* has been shown to predict a good prognosis or a poor prognosis in different cancer types.[31] The prognostic role of *PCK2* in TNBC has not been reported. According to our analysis of two databases, a high *PCK2* expression level is associated with a poor prognosis in TNBC patients, which is consistent with the effect of *PCK2* on promoting the proliferation and invasiveness of TNBC cell lines in our current study.

TGF-β signaling has been reported to exert tumor-suppressive effects by inhibiting cell cycle progression and promoting apoptosis. However, TGF-β also exerts a tumor-promoting effect by increasing tumor invasiveness and metastasis.[36,37] The TGF-β signaling pathway is well known for its function as an EMT inducer. Upon activation of the canonical pathway, SMAD2 and SMAD3 are phosphorylated by TGF-β receptors to form a complex with SMAD4. The SMAD complex transcriptionally activates EMT drivers to trigger cell invasion and metastatic potential.[25] Loss of SMAD3 results in TGF-β signaling impairment, suggesting that SMAD3 is a key mediator of this pathway.[38] In this study, using an RNA microarray, we identified a TGF-β signaling gene set that was negatively enriched in TNBC cells with *PCK2* knockdown and confirmed that *PCK2* affects invasion/migration and EMT by promoting TGF-β/SMAD3 signaling in TNBC cells.

The regulation of SMAD3 by mechanisms such as transcriptional or posttranslational modifications and the effects of SMAD3 on TGF-β signaling-associated EMT and cancer metastasis have been thoroughly investigated. Tong et al. demonstrated that myocardin directly interacts with SMAD3 and stabilizes the SMAD3/SMAD4 complex to facilitate TGF-β-induced EMT and metastasis in NSCLC.[39] Acetylation of SMAD3 by lysine acetyltransferase 6A (KAT6A) promotes SMAD3 association with the oncogenic chromatin modifier tripartite motif-containing 24 (TRIM24) and increases SMAD3 activation, which increases the metastatic potential of TNBC.[40] SMAD3

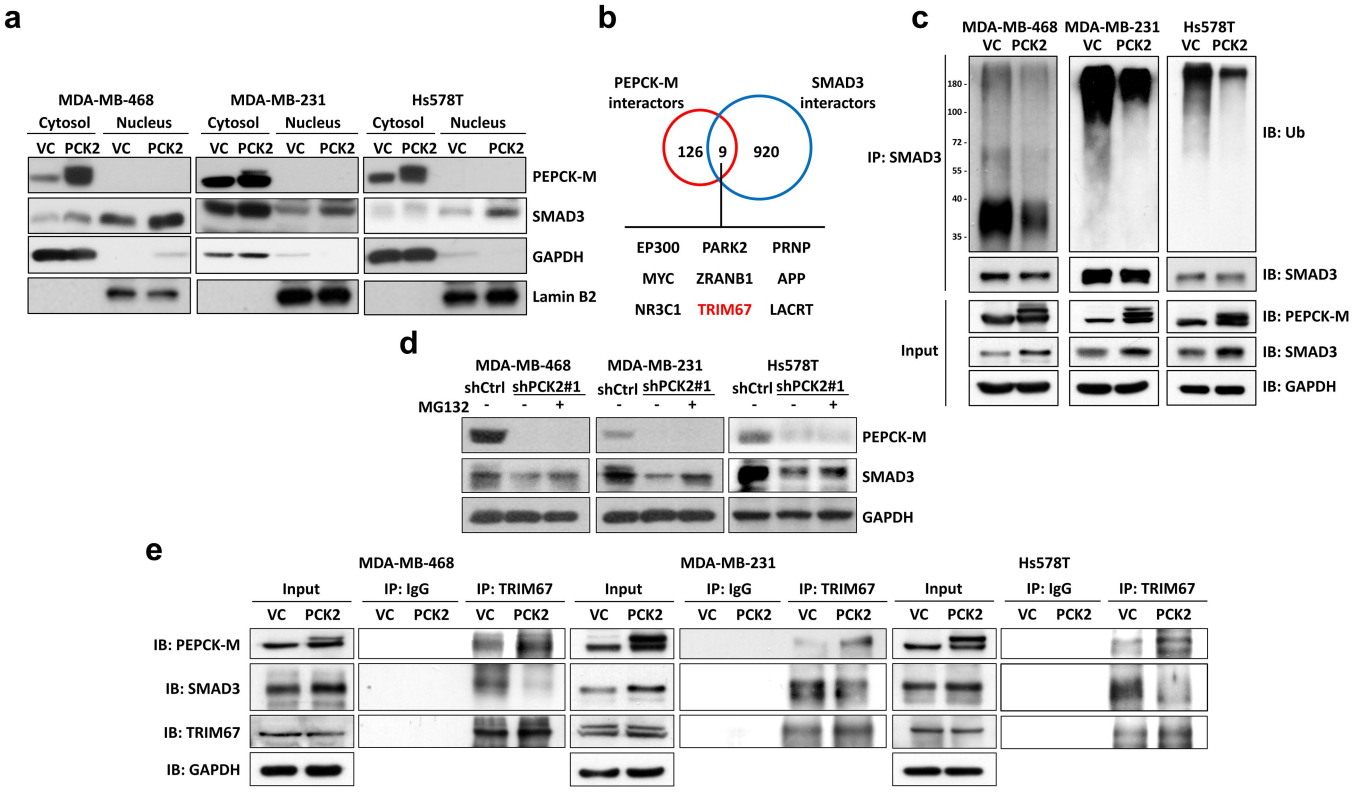


**Figure 6.** *PCK2* regulates SMAD3 expression through protein–protein interactions with TRIM67 and the regulation of TRIM67-mediated ubiquitination and proteasomal degradation of SMAD3. (a) Western blot analysis of the cytosolic and nuclear expression of pepck-M and SMAD3 in MDA-MB-468 (left), MDA-MB-231 (middle), and Hs578T (right) cells with or without *PCK2* overexpression. GAPDH and Lamin B2 were used as loading controls for the proteins present in the cytosolic and nuclear fractions, respectively. (b) Protein–protein interactions were compiled from the BioGRID database for human pepck-M and SMAD3. A total of 126 and 920 proteins were found to interact with PEPCK-M (red circle) and SMAD3 (blue circle), respectively. Nine of these proteins can interact with both pepck-M and SMAD3. (c) Immunoprecipitation analysis of SMAD3 and ubiquitin in MDA-MB-468 (left), MDA-MB-231 (middle), and Hs578T (right) cells with or without *PCK2* overexpression. (d) SMAD3 protein expression in MDA-MB-468 (left), MDA-MB-231 (middle), and Hs578T (right) cells with or without *PCK2* knockdown and with or without MG132 treatment. (e) Immunoprecipitation analysis of the PEPCK-M-TRIM67 and SMAD3-TRIM67 interaction in MDA-MB-468 (left), MDA-MB-231 (middle), and Hs578T (right) cells with or without *PCK2* overexpression.

methylation mediated by EZH2 promotes SMAD3 phosphorylation by TGF-β receptors and is correlated with hyperactivation of TGF-β/SMAD3 signaling and promotion of EMT and metastasis in breast cancer.[41] Transcriptional upregulation of SMAD3 has also been reported to be an important mechanism for the activation of TGF-β signaling in several studies. The transcription of *SMAD3* has been shown to be increased by homeobox A1 and leads to the promotion of cell proliferation and metastasis in bladder cancer.[42] miR-135-5p has been shown to negatively regulate the mRNA and protein expression of SMAD3 and inhibit TGF-β-mediated EMT and cell invasion by suppressing the TGF-β/SMAD pathway.[43] ALKBH5, an RNA demethylase, has also been shown to negatively regulate TGF-β/SMAD signaling by removing the $m^6A$ modification of *TGFβR2*, *SMAD3* and *SMAD6* mRNA in NSCLC.[44] In the present study, we demonstrated that *PCK2* transcriptionally upregulates *SMAD3* and promotes the invasion and EMT of TNBC cells. The enhanced invasion/migration and EMT activities of TNBC cells overexpressing *PCK2* were suppressed by *SMAD3* knockdown. These results indicate that SMAD3 plays a crucial role in the progression of TNBC via *PCK2*. Moreover, we elucidated a novel mechanism underlying the effects of *PCK2* on the regulation of SMAD3 expression through TRIM67-mediated SMAD3 ubiquitination. The TRIM family plays an important role in a variety of cellular signaling transductions and biological processes as well as in cancer development, progression, and drug resistance and has oncogenic or tumor-suppressive functions in different cancer types.[30] Recently, TRIM67 was identified as a tumor suppressor in colorectal cancer that binds directly to the p53 protein and protects it from MDM2-mediated ubiquitination.[45] In contrast, TRIM67 has been shown to act as a tumor promoter to induce Notch signaling in NSCLC through increasing the ubiquitination of delta-like noncanonical Notch ligand 1 (DLK1) via its RING domain.[31] In the present study, we found that PEPCK-M directly binds to TRIM67 to prevent SMAD3 from interacting with TRIM67, resulting in the inhibition of TRIM67-mediated ubiquitination and proteasomal degradation of SMAD3. In addition, increased nuclear translocation of SMAD3 may autoregulate the transcription of SMAD3.[28] Our results demonstrate a novel mechanism by

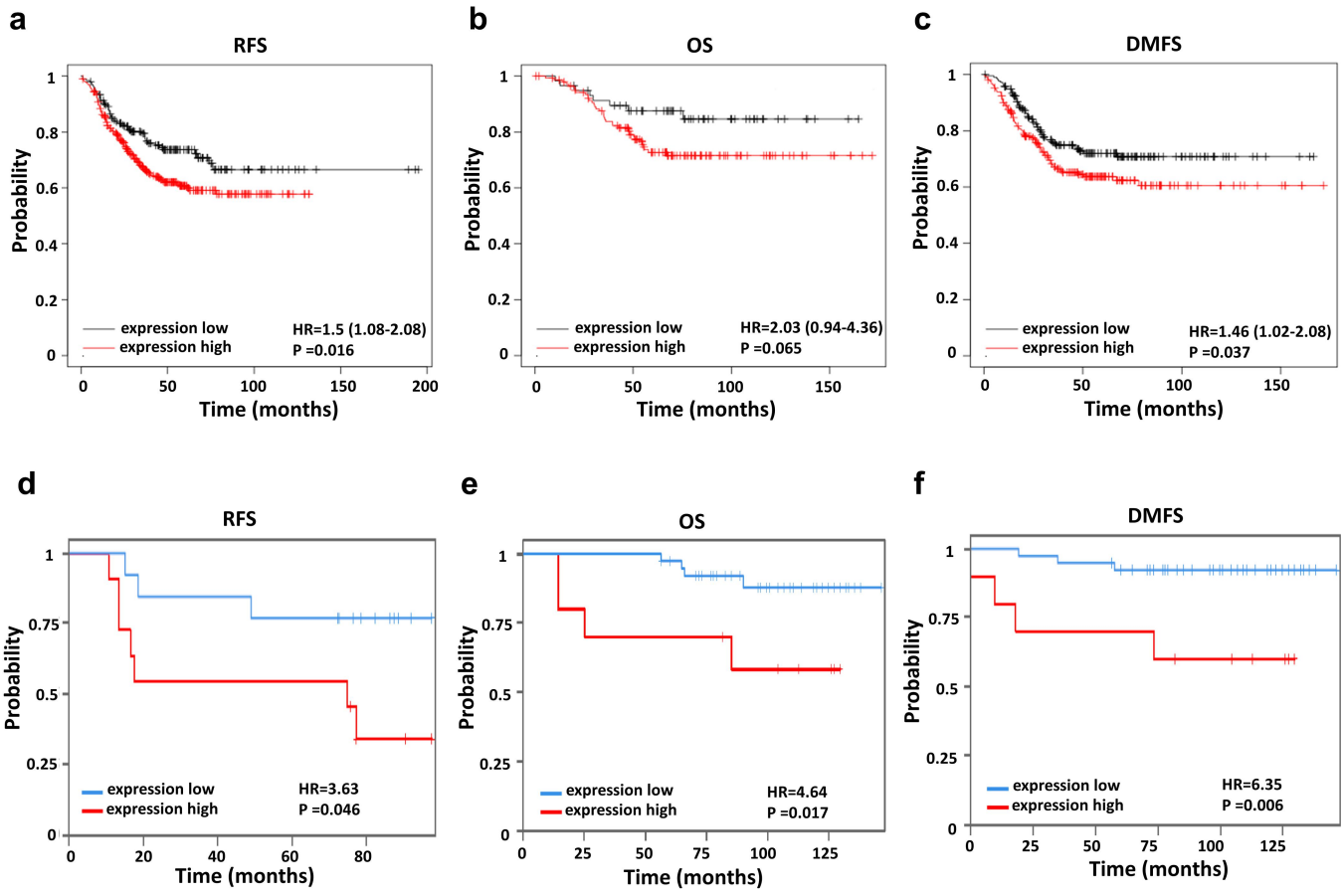


**Figure 7.** Association of the *PCK2* mRNA expression level with the survival of TNBC patients. (a) Recurrence-free survival (RFS) analysis for TNBC patients with high or low *PCK2* mRNA expression levels via the Kaplan–Meier plotter database (www.kmplot.com.). (b) Overall survival (OS) analysis for TNBC patients with high or low *PCK2* mRNA expression levels via the Kaplan–Meier plotter database. (c) Distant metastasis-free survival (DMFS) analysis of TNBC patients with high or low *PCK2* mRNA expression levels via the Kaplan–Meier plotter database. (d) RFS analysis of TNBC patients with high or low *PCK2* mRNA expression levels via the BCIP database (http://www.omicsnet.org/bcancer/.). (e) OS analysis of TNBC patients with high or low *PCK2* mRNA expression levels via the BCIP database. (f) DMFS analysis of TNBC patients with high or low *PCK2* mRNA expression levels via the BCIP database.

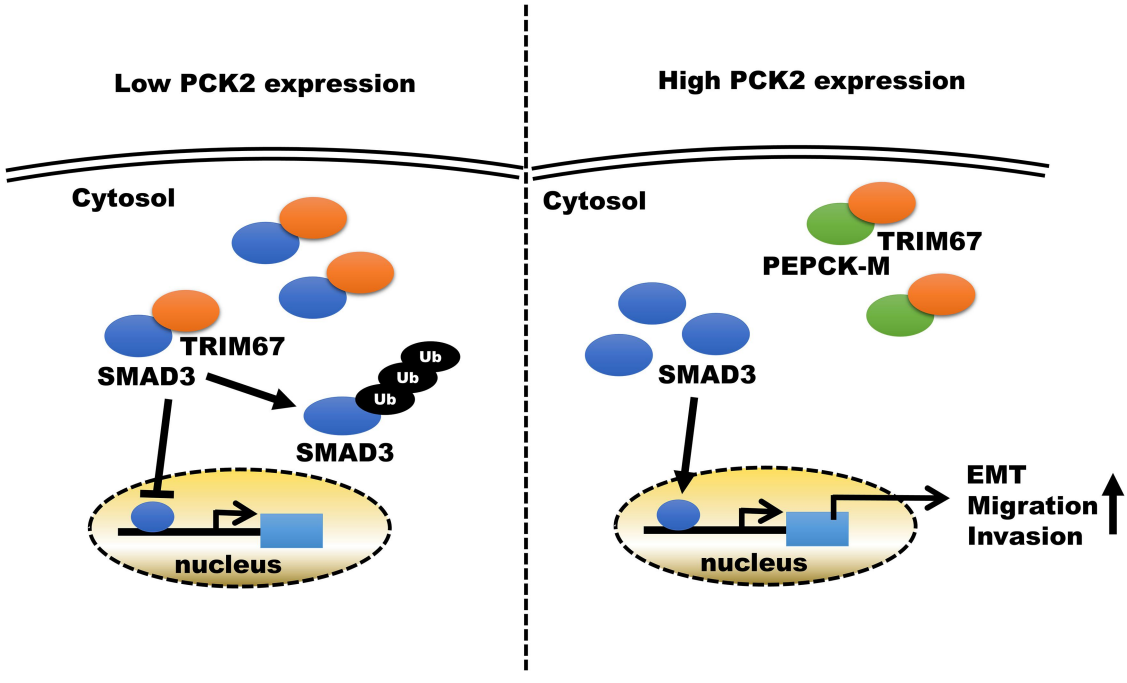


**Figure 8.** The putative model by which *PCK2* regulates SMAD3 expression and invasion and EMT in TNBC. When *PCK2* expression is low in TNBC, TRIM67 interacts with SMAD3 and causes SMAD3 ubiquitination and proteasomal degradation, resulting in suppression of the autoregulation of SMAD3 transcription. When *PCK2* expression is high in TNBC, the encoded protein PEPCK-M competitively binds to TRIM67. The SMAD3–TRIM67 interaction is disrupted, which protects SMAD3 against TRIM67-mediated ubiquitination and promotes the nuclear translocation of SMAD3. Enhanced transcription of SMAD3 promotes the transcription of emt-related molecules and the invasion of TNBC cells.

which *PCK2* increases the protein and mRNA expression of SMAD3 in TNBC. This putative model is shown in Figure 8. Furthermore, we showed that the knockdown of *PCK2* diminished the stimulatory effect of TGF-β on SMAD3 phosphorylation and EMT in TNBC cells. This result suggested that *PCK2* regulates cell invasion and EMT not only by upregulating SMAD3 expression but also by sensitizing cells to TGF-β stimulation of TGF-β/SMAD3 signaling. Although recent studies have linked *PCK2* to cell invasion and EMT, its regulatory mechanism is not well understood.[46–48] In our study, we found that *PCK2* promotes the invasion and EMT of TNBC cells via the activation of TGF-β/SMAD3 signaling. However, this has not been reported previously. The limitation of this study is lack of clinical sample to demonstrate the correlation of PEPCK-M with SMAD3

## Conclusions

*PCK2* promotes the proliferation and invasion of TNBC cells via the activation of the mTOR and TGF-β/SMAD3 signaling pathways. We discovered a novel connection between *PCK2*, a metabolic enzyme, and the TGF-β/SMAD3 pathway, an EMT signaling pathway. We identified PEPCK-M as a positive regulator of TGF-β/SMAD3 activation via the inhibition of TRIM67-mediated SMAD3 degradation. Our findings suggest that *PCK2* is a potential therapeutic target for the inhibition of TGF-β/SMAD3 signaling-dependent cancer metastasis.

## List of abbreviations

| | |
|---|---|
| TNBC | Triple-negative breast cancer |
| ER | Estrogen receptor |
| PR | Progesterone receptor |
| HER2 | Human epidermal growth factor 2 |
| EMT | Epithelial-to-mesenchymal transition |
| MET | mesenchymal-to-epithelial transition |
| TGF-β | transforming growth factor-β |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PEPCK-C | cytosolic phosphoenolpyruvate carboxykinase |
| PEPCK-M | mitochondrial phosphoenolpyruvate carboxykinase |
| NSCLC | Non-small-cell lung cancer |
| KAT6A | Lysine acetyltransferase 6A |
| TRIM67 | Tripartite motif-containing 67 |

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## Authors' contributions

T.C., W.F., and H.T. wrote the main manuscript text. T.C., and K. H. prepared Figures 1–5 and supplementary Figure S1. W.F. and H. L. prepared Figures 6–8. S.J. prepared Figure 3. H.H., P.C., S.J., W. H., T.C., W.F., and H.T. performed data analysis. T.C. and W. F. designed the experiments. H.H. was involved in proposing this study. H.T. proposed, initiated and supervised the study. All authors reviewed and approved the final manuscript.

## Availability of data and materials

All data generated or analyzed during the current study are included in this published article and its supplementary information files.

## Ethics approval and consent to participate

The study was approved by the Institutional Review Board of the National Health Research Institutes (NHRI) (EC1071201-E) and was performed in accordance with the guidelines and regulations of the NHRI.

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