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ORIGINAL PAPER

CLIP170 inhibits the metastasis and EMT of papillary thyroid cancer through the TGF-β pathway

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Abstract

Metastasis poses a significant challenge in combating tumors. Even in papillary thyroid cancer (PTC), which typically exhibits a favorable prognosis, high recurrence rates are attributed to metastasis. Cytoplasmic linker protein 170 (CLIP170) functions as a classical microtubule plus-end tracking protein (+TIP) and has shown close association with cell migration. Nevertheless, the specific impact of CLIP170 on PTC cells remains to be elucidated. Our analysis of the GEO and TCGA databases unveiled an association between CLIP170 and the progression of PTC. To explore the impact of CLIP170 on PTC cells, we conducted various assays. We evaluated its effects through CCK-8, wound healing assay, and transwell assay after knocking down CLIP170. Additionally, the influence of CLIP170 on the cellular actin structure was examined via immunofluorescence; we further investigated the molecular expressions of epithelial-mesenchymal transition (EMT) and the transforming growth factor-β (TGF-β) signaling pathways through Western blotting and RT-qPCR. These findings were substantiated through an in vivo nude mouse model of lung metastasis. We observed a decreased expression of CLIP170 in PTC in contrast to normal thyroid tissue. Functionally, the knockdown of CLIP170 ($CLIP170^{KD}$) notably enhanced the metastatic potential and EMT of PTC cells, both in vitro and in vivo. Mechanistically, $CLIP170^{KD}$ triggered the activation of the TGF-β pathway, subsequently promoting tumor cell migration, invasion, and EMT. Remarkably, the TGF-β inhibitor LY2157299 effectively countered TGF-β activity and significantly reversed tumor metastasis and EMT induced by CLIP170 knockdown. In summary, these findings collectively propose CLIP170 as a promising therapeutic target to mitigate metastatic tendencies in PTC.

Keywords CLIP170 · Papillary thyroid cancer · Metastasis · EMT · TGF-β pathway

Introduction

Thyroid cancer is a malignant tumor of the endocrine system. Papillary thyroid carcinoma (PTC) emerges as one of its most widespread forms and explains the continuously increasing incidence of thyroid cancer [1, 2]. Despite not being a primary cause of mortality in PTC patients, there exists a high recurrence rate post-treatment, significantly impacting patients' overall survival [3, 4]. Noteworthy, the risk of recurrence is strongly associated with tumor cell metastasis [5, 6]. Thus, the pursuit of molecular targets capable of impeding metastasis has become imperative in addressing advanced stages of PTC.

Epithelial-mesenchymal transition (EMT) plays a crucial role in endowing tumor cells with metastatic potential [7]. Epithelial-mesenchymal transition cells undergo cytoskeletal rearrangement. After the epithelial cell junctions are disrupted, epithelial actin undergoes structural remodeling and transfers from the cortex to the leading edge of cells, forming lamellipodia, filopodia, and invaginating cells, which will facilitate cell migration [8, 9]. The TGF-β signaling pathway stands as a key driver of EMT across various cancer cell types, including its association with EMT and

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radioiodine tolerance in PTC [10]. Activation of TGF-β may thus contribute to increased migratory capacity in PTC cells.

CLIP170, known for its role as a +TIP protein binding and stabilizing growing microtubule plus ends [11], has garnered attention for its regulatory influence on cellular lamellipodia formation [12, 13]. This underscores its potential significance in tumor cell metastasis. While studies have extensively explored CLIP170's impact on breast and pancreatic cancer progression [12, 14], its effect on PTC cell metastasis remains uncharted territory.

Our previous investigation involving 34 families encompassing 77 PTC patients revealed mutations in the CLIP170 gene within two families [15]; examination of TCGA data showcased a prevalence of copy number deletion variants over gains in PTC, alongside decreased CLIP170 expression in tumors compared to para-neoplastic tissues, implying a potential role for the CLIP170 gene as an anti-oncogene in PTC. Crucially, CLIP170 knockdown ($CLIP170^{KD}$) notably enhanced cell migration, invasion, and EMT in PTC cells, while not impacting cell proliferation. These effects were mediated via TGF-β signaling activation, which could be partially reversed by specific TGF-β inhibitors, highlighting the contribution of $CLIP170^{KD}$-mediated TGF-β activation to PTC cell metastasis and EMT.

In conclusion, this study underscores the potential significance of $CLIP170^{KD}$-induced TGF-β activation in driving metastasis and EMT in PTC cells.

Materials and methods

Cell culture and transfection

All cells and vector (pLKO.1) used in this study were obtained from the Institute of Pathology, West China Hospital, Sichuan University. The TPC-1 and BCPAP cells were grown in RPMI1640 medium (Gibco) and the HEK293T cells were grown in DMEM medium (Gibco). All media contained 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 ug/ml streptomycin. Both cells were cultured at 37 °C in an incubator with 5% CO_2, 95% O_2. Plasmid DNA transfections were executed using Lipofectamine 8000 (Beyotime Biotechnology) following standard protocols in accordance with the manufacturer's guidelines. All shRNA sequences utilized for knockdown were referenced from the validated knockdown efficiency sequences on the official website of Sigma, and the nucleic acid fragments were synthesized from Tsingke Biological Technology Co., Ltd. Primer information is given in Supplementary Table 1.

Wound healing assay, transwell migration assay, and CCK-8 assay

Wound healing experiments: A 200 ul sterile pipette tip was utilized to create a scratch when cells reached 100% confluence in a six-well plate. Afterward, cells were washed three times with PBS. The incubation persisted in the incubator after supplementing with 2 ml of medium/well, with observations and image acquisitions at 0 h and 24 h, respectively. Transwell assay: a 200 ul 2×10^5/well (migration) and 3×10^5/well (invasion) cell was incubated into the transwell chamber, while 600 ul of fresh medium containing 10% FBS was added to the lower chamber for up to 48 h. Each well was then washed three times with PBS. Cells were fixed using 1 ml of methanol for 20 min and washed three times with PBS. Finally stained with crystal violet and counted. Cell Counting Kit-8: 5×10^3/well cells were incubated in 96-well plates, with each well containing 90 µl of medium and 10 µl of CCK8 (Beyotime, China) reagent for 2 h. The absorbance values of OD450 were measured using the multilabel plate reader at 24 h, 48 h, 72 h, and 96 h, respectively.

Enzyme-linked immunosorbent assay (ELISA)

The Human TGF-β1 ELISA kit was purchased from ABclonal (RK0055). Assays were performed according to the manufacturer's instructions.

Real-time PCR

Total cellular RNA was extracted using Trizol (Vazyme). Reverse transcription was performed according to the instructions of ABscript III RT Master Mix (ABclonal). Real-time qPCR was performed based on instructions for Genious 2X SYBR Green Fast qPCR Mix (ABclonal), according to the following procedure: pre-denaturation at 95 °C for 3 min, followed by 40 cycles of reactions at 95 °C for 5 s and 60 °C for 30 s. The reference gene GAPDH was employed for normalization; primer information is given in Supplementary Table 2.

Protein extraction and Western blotting

The total cellular protein was extracted using RIPA lysate (Biosharp) according to standard procedures. Denatured protein lysates were separated by electrophoresis and transferred to PVDF membranes. PVDF membranes were blocked in TBST supplemented with 0.1% Tween 20 and 5% skim milk and then incubated overnight at 4 °C with the corresponding primary antibody. Subsequently, it was incubated with the corresponding secondary antibodies followed

by three washes with TBST. Proteins were visualized with ECL chemiluminescent solution (Biosharp) on an automated chemiluminescent imager (Tanon, 5200 Multi, China) and quantified using image j software. Information on the antibodies used is available in Supplementary Table 3.

Immunohistochemistry (IHC) staining

IHC was performed on lung metastasis model samples to determine the expression profiles of target genes. Briefly, tissue paraffin sections (4 μm) were subjected to heat-induced epitope retrieval using ethylene diamine tetraacetic acid (EDTA) (AR0023; Boster, Wuhan), and then the sections were treated with blocking buffer. The primary antibody was incubated overnight at 4 °C with the prepared sections for further immunohistochemistry kit (SA1020; Boster, Wuhan) for staining.

Immunofluorescence

Cells were inoculated in appropriate amounts on glass coverslip. Before staining, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. 0.1% Triton X-100/PBS was applied for 5 min at room temperature, followed by blocking with 10% goat serum for 1 h. Incubate overnight at 4 °C with the corresponding primary antibody. The slides were then stained with the corresponding fluorescent secondary antibody, closed with fluorescence quenching blocking solution (containing DAPI), and observed with laser confocal microscopy.

Animals experiment

Female mice aged 4–5 weeks were purchased from Jiangsu Collective Pharmachem Biotechnology Co., Ltd. and housed in an SPF-grade laboratory animal center. TPC-1 cells stably expressing shCLIP170 (3 mice) and control TPC-1 cells (3 mice) were injected into the tail vein of nude mice at 1×10^6 cells/100 μl to establish the metastasis model. At 8 weeks post-injection, lungs were collected, washed with PBS, and fixed in 4% paraformaldehyde. The fixed lung tissues were embedded in paraffin, sectioned, stained with HE, observed with light microscopy, and images were captured. All animal experiments were conducted following guidelines approved by the Animal Ethics and Use Committee of the Second Hospital of Lanzhou University.

Gene expression data analysis

Normalized gene expression data for papillary thyroid cancer were downloaded from TCGA and GEO databases. In total, RNA-sequencing data for 510 papillary thyroid cancer samples and 653 normal thyroid samples were analyzed.

Statistics

Analysis and plotting of experimental data were completed using GraphPad Prism9. All data are expressed as mean ± SD, and statistical significance was determined by unpaired Student's *t* test. $P < 0.05$ was considered a statistically significant.

Results

Low expression of CLIP170 in papillary thyroid cancer

We conducted a comprehensive analysis of CLIP170 copy number variations (CNV) within tumors using the TCGA database to assess the potential correlation between CLIP170 expression levels and tumor progression. Among the 26 tumors examined for CLIP170 CNV (Fig. 1A), we observed a notably higher incidence of CLIP170 CNV Losses compared to CNV Gains (5.00% vs. 3.31%). This disparity suggests a potential association between CLIP170 deletion and tumor progression. Subsequently, we delved deeper into examining the correlation between CLIP170 and PTC progression. We investigated the expression differences of CLIP170 in cancer and para-neoplastic tissues by GEO and TCGA datasets. In four GEO datasets with a total of 108 paired samples, our analysis indicated consistently lower CLIP170 expression levels in PTC samples compared to adjacent para-cancerous tissues (Fig. 1B–E); similarly, within the TCGA dataset, there was a significant reduction in CLIP170 gene expression in PTC samples compared to non-cancerous tissues (Fig. 1F). These findings collectively suggest a potential correlation between diminished CLIP170 expression and the progression of PTC.

CLIP170 is essential for the metastasis of PTC cells

To investigate the potential role of the CLIP170 gene in PTC cells lines, we constructed stable knockdown of CLIP170 by infecting cells with lentivirus harboring the shCLIP170 in TPC-1 and BCPAP cells. Subsequently, we validated the efficiency of CLIP170 knockdown in RNA and protein levels. The results showed that CLIP170 was significantly decreased at RNA and protein levels (Fig. 2A and B). Then, we sought to investigate the biofunction of CLIP170 on PTC cells. However, CCK8 assay showed that $CLIP170^{KD}$ had no significant effect on the proliferation of PTC cells (Fig. 2C and D). Surprisingly, wound healing assay and transwell assay indicated that $CLIP170^{KD}$ significantly facilitated the migration and invasion ability of PTC cells (Fig. 2E–G).

In addition, we established a model of cell metastasis in nude mice via tail vein injection, aiming to investigate

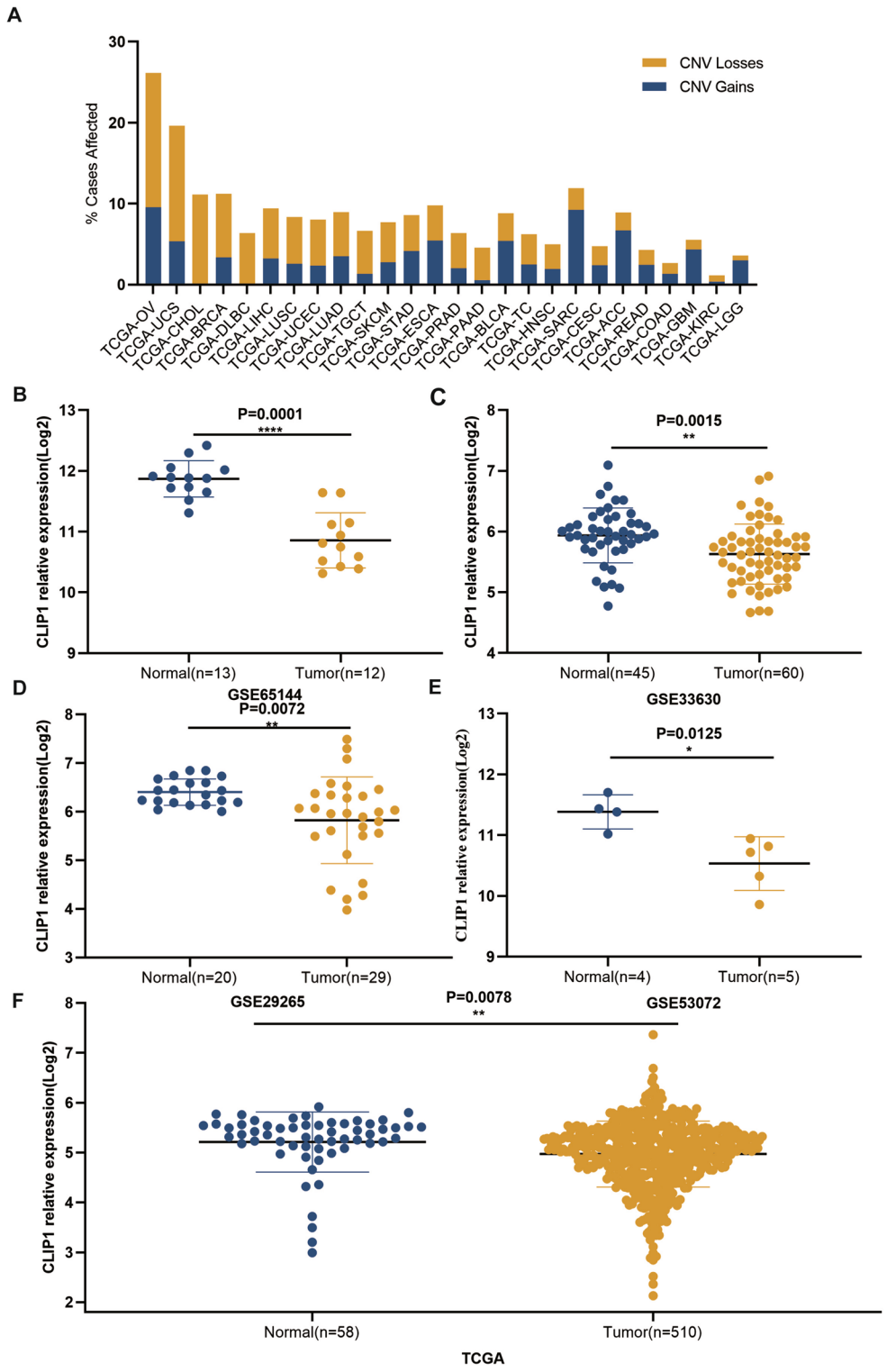

Fig. 1 CLIP170 expression in thyroid cancer. **A** Copy number variants of CLIP170 in various tumors form TCGA database. **B**–**E** CLIP170 expression difference from GEO database including GSE29265, GSE33630, GSE53072, GSE65144. **F** CLIP170 expression difference from TCGA database

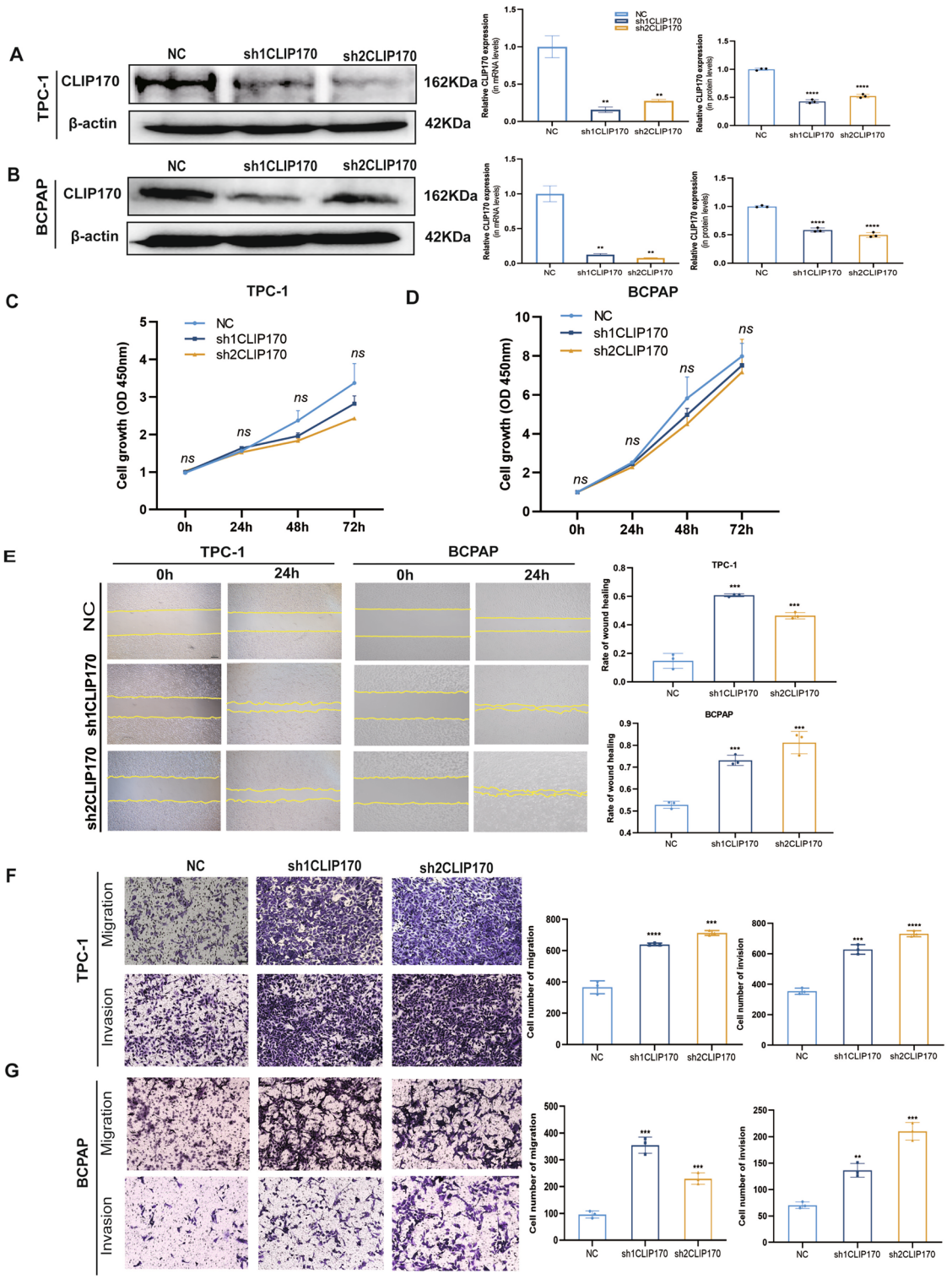

Fig. 2 CLIP170 was involved in PTC cell metastasis in vitro. **A** and **B** Knockdown efficiency in protein and RNA levels. **C** and **D** The CCK-8 assays assessing the proliferation capacity after CLIP170 knock down. **E** The wound healing was used to assess migration capacity after CLIP170 knock down. **F** and **G** The transwell assay was used to assess migration and invasion after CLIP170 knock down. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$

whether CLIP170^{KD} still promotes the metastasis of PTC cells in vivo. We confirmed that the lungs of nude mice of the CLIP170^{KD} exhibited significant differences compared to control mice (Fig. 3A). HE staining showed that the lung tissues of all the mice of NC had no obvious abnormalities, while the lungs of CLIP170^{KD} nude mice formed metastatic foci of PTC cells (Fig. 3B). In addition, we performed immunohistochemical staining of lung tissues from the shCLIP170 and the NC to compare the levels of CLIP170 and TGF-β1/2. We found that CLIP170 was decreased in the shCLIP70 compared to the NC (Fig. 3C); however, TGF-β1/2 showed an opposite trend. In conclusion, the results mentioned above confirm that CLIP170^{KD} enhances the cell metastasis of PTC cells both in vivo and in vitro, which is essential for the malignant development of the tumor.

CLIP170 was involved in cell EMT in PTC cells

The capability of tumor cells to metastasis is highly correlated with EMT. Based on the above results, we speculated that CLIP170^{KD} might promote EMT in PTC cells. To validate this speculation, we detected changes in EMT-related markers. As shown in Fig. 4A–D, the expression of E-cadherin decreased significantly, while the expression of N-cadherin and Vimentin increased remarkably in both CLIP170^{KD} PTC cells. This result implies that CLIP170^{KD} promotes EMT in PTC cells.

EMT is mediated by actin cytoskeleton remodeling. Therefore, we subsequently sought to investigate whether CLIP170^{KD} drives EMT by regulating the distribution of major component of the cytoskeleton (F-actin) and the formation of cell lamellipodium. We found that F-actin accumulated significantly in the cortical region of the CLIP170^{KD} TPC-1cells and formed outward protrusions (Fig. 4E). However, this protrusion has not yet formed a lamellipodia, and we speculate that this cellular state is likely to be in a transitional state before the formation of lamellipodia. This suggests that CLIP170^{KD} facilitates the promotion of F-actin assembly and protrusion formation.

CLIP170 knockdown activated the TGF-β pathway in PTC cells

To explore the mechanism that CLIP170^{KD} leads to tumor cells metastasis and EMT, we investigated the status of signaling pathways that are closely associated with metastasis and EMT. TGF-β is currently one of the most closely associated pathways with metastasis and EMT. Hence, we detected the levels of TGFβ1 in cultures of PTC cells by EILSA. Our results showed that TGFβ1 was significantly higher in CLIP170^{KD}cells than in NC (Fig. 5A and B). This indicates that CLIP170^{KD} promotes the expression of TGFβ1 in PTC cells, suggesting that CLIP170^{KD} may activate the TGF-β signaling pathway. To verify the activation of the TGF-β signaling pathway, we examined the primary target genes downstream of the TGF-β signaling pathway, and protein expression of the key signaling molecules in the TGF-β signaling pathway. Our results demonstrated that the expression levels of the downstream target genes ANGPT, TAGLN, CYR61, and CTGF were also significantly increased (Fig. 5C and D), and the phosphorylation level of Smad2/3 and Erk1/2 increased significantly in CLIP170^{KD} PTC cells (Supplementary Fig. 1A and B). All these results suggest that CLIP170^{KD} activates the TGF-β pathway in PTC cells.

TGF-β inhibitor reverse metastasis and EMT caused by CLIP170 knockdown

Based on the essential role of TGF-β in metastasis and EMT, we hypothesized that the metastasis and EMT of PTC cells are also mediated by the activation of the TGF-β pathway. To verify this hypothesis, we used a TGF-β pathway inhibitor (LY2157299) to investigate the functional dependence of TGF-β. Our results revealed a dose-dependent decrease in the expression level of phosphorylated Smad2/3 in PTC cells upon incubation with LY2157299 (5–20 uM) for 48 h (Supplementary Fig. 2A). We chose 5 uM as the optimal dosage with statistical significance in the following experiments. As shown in Fig. 6A and B, the phosphorylation level of Smad2/3 and Erk1/2 was elevated in CLIP170^{KD} cells compared with NC cells, whereas it was decreased after adding the inhibitor LY2157299. Similarly, the expression of the primary downstream target genes of the TGF-β signaling pathway was elevated in the CLIP170^{KD} cells compared to the NC cells; yet it was reduced in CLIP170^{KD} cells in the inhibitor group compared to the solvent group (Fig. 6C and D). The above results strongly indicated that LY2157299 successfully inhibited the activation of the TGF-β signaling pathway induced by CLIP170 knockdown.

We further verified the involvement of the TGF-β signaling pathway in PTC cell metastasis and EMT. The result of transwell assay revealed that the migration and invasion capability of CLIP170^{KD} cells were significantly increased in CLIP170^{KD} cells; however, it diminished after adding LY2157299 (Fig. 6E and F). Similarly, wound healing assay showed that the area of wound healing at 24 h was significantly increased in CLIP170^{KD} cells. Yet it was reduced compared to the solvent group after adding the inhibitor LY2157299 (Supplementary Fig. 2B). Subsequently, we also verified the effect on the EMT of PTC cells after adding LY2157299. The results are also consistent with the above (Fig. 6G and H). In conclusion, all results indicated that CLIP170^{KD} significantly promoted the metastasis and EMT of PTC. Importantly, inhibition of the TGF-β signaling pathway using LY2157299 partially restored the metastasis and EMT of CLIP170^{KD}-induced. These findings

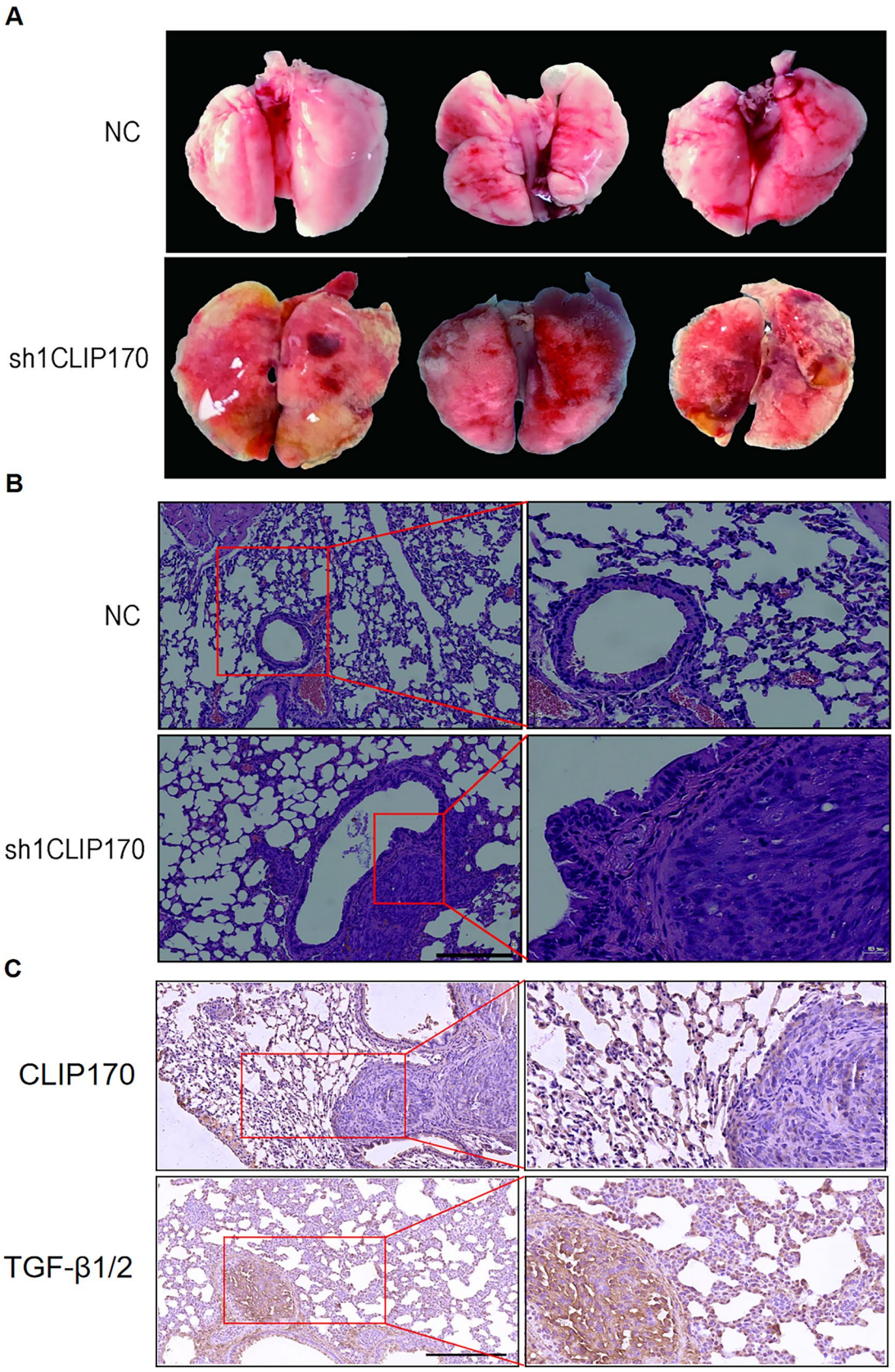

Fig. 3 CLIP170 was involved in PTC cell metastasis in vivo. **A** Metastatic foci of NC and CLIP170^{KD} TPC-1 cells in nude mice lung. **B** HE staining of metastatic foci in nude mice lung tissue. **C** Immunohistochemical staining for CLIP170 and TGF-β1/2 (Scale bar, 200 um)

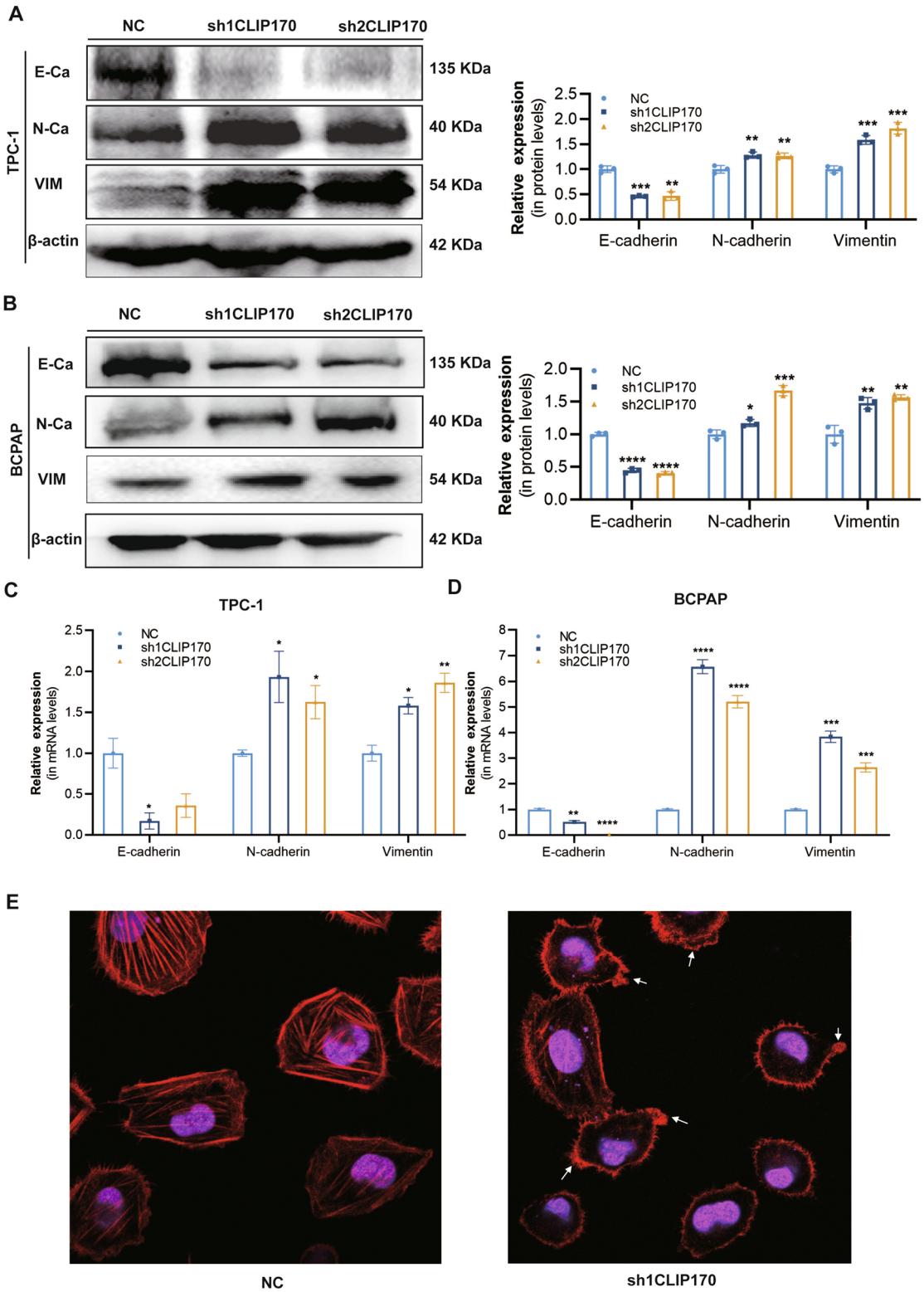

Fig. 4 Knockdown of CLIP170 promotes EMT in PTC cells. **A** and **B** CLIP170^{KD} leads to alteration of EMT marker in protein level. **C** and **D** CLIP170^{KD} leads to alteration of EMT marker in RNA level. **E** Immunofluorescence detection of F-actin after CLIP170 knock down. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$

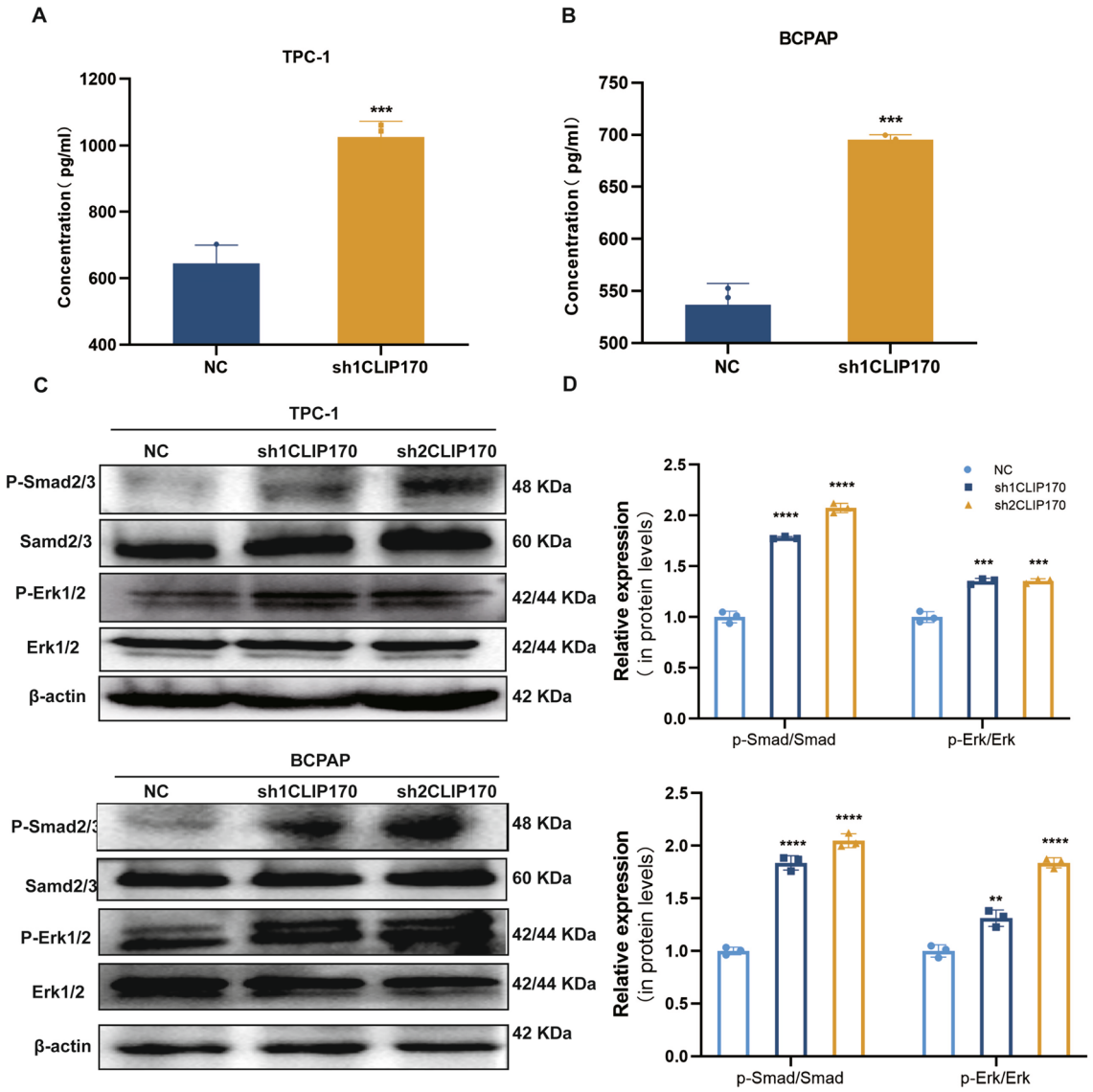

Fig. 5 CLIP170^{KD} regulated TGF-β pathway activation in PTC cells. **A** and **B** EILSA assay to detect the level of TGFβ1 in NC and CLIP170^{KD} PTC cell cultures. **C** and **D** Western blotting of phosphorylation of Smad2/3 and Erk1/2 after CLIP170 knock down. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

underscore the crucial involvement of the TGF-β pathway in CLIP170^{KD}-mediated metastasis and EMT in PTC cells.

Discussion

Thyroid cancer is a malignant neoplasm originating from follicular epithelial cells [16], and PTC is one of them with a relatively favorable prognosis. Although advances in research on studying the pathogenesis of PTC, the high recurrence rate of PTC patients due to metastasis is still a major concern; therefore, it remains urgent to explore the factors associated with tumor cell metastasis.

Previous studies found that CLIP170 regulates the sensitivity of cancer cells to paclitaxel through a microtubule-dependent mechanism [17, 18]. Yet, recent studies highlight its involvement in cell migration and invasion in pancreatic, breast, and endothelial cells [12–14, 19, 20]. Our study aligns with these findings, demonstrating that CLIP170 affected the metastasis of PTC cells in vitro and in vivo.

Epithelial-mesenchymal transition (EMT), pivotal in tumor metastasis, involves cytoskeletal and expression characteristics changes. As the basis of cytoskeletal rearrangement, F-actin plays a critical role in mediating cell migration [21, 22]. It has been shown that CLIP170 promotes the rapid assembly of actin filaments triggered by

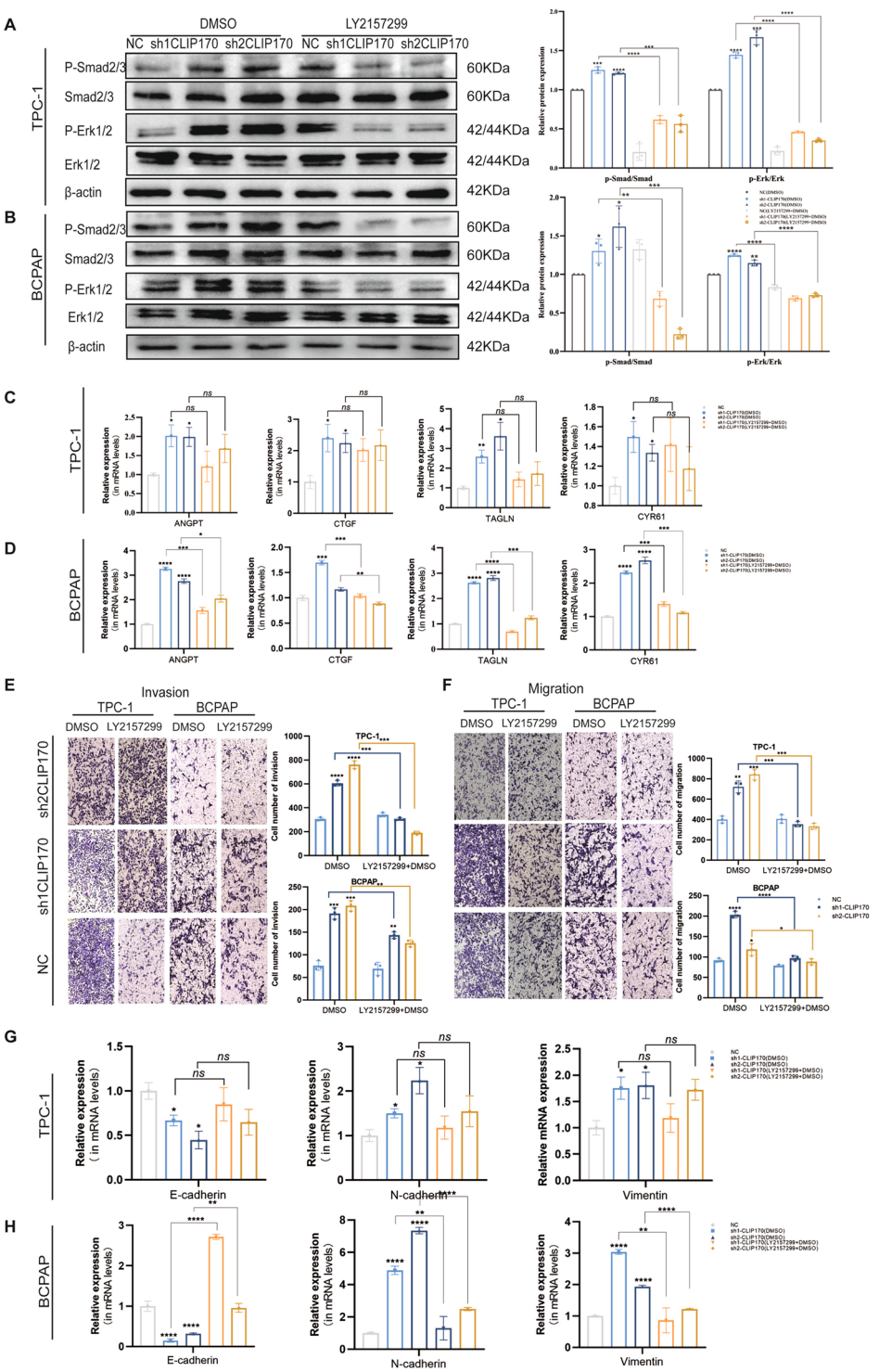

Fig. 6 TGF-β inhibitor rescued the metastasis and EMT induced by CLIP170 knockdown in PTC cells. **A** and **B** Western blotting assay of CLIP170^{KD}-induced phosphorylation of Smad2/3 and Erk1/2 after adding the TGF-β inhibitor. **C** and **D** The mRNA expression of key signaling molecules in the TGF-β signaling pathway after adding TGF-β inhibitor. **E** and **F** Transwell assays after adding TGF-β inhibitor. **G** and **H** The expression of EMT marker genes was observed by RT-qPCR after adding the TGF-β inhibitor. $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$

the plus-end of growing microtubules in vitro, thereby directly linking microtubules to actin dynamics [23]. Surprisingly, our study found that the knockdown of CLIP170 facilitates the accumulation of F-actin in the cell cortex and the formation of outwardly directed actin protrusions. In other words, CLIP170 is present in PTC as a negative regulator of lamellipodia, which is contradictory to previous reports. While most +TIPs typically promote cell migration, it was still found that some +TIPs inhibit the degradation of the extracellular matrix or prevent the formation of invadopodia instead [12, 24]. These studies provide us with an alternative line of thought. In addition, although this actin protrusion in our study has not yet formed lamellipodia, it may be an intermediate state

for lamellipodia formation. We speculate that CLIP170 may synergize with other +TIPs on the formation of cell membrane pseudopods, which is still under investigation.

Given TGF-β's role in inducing EMT, we hypothesized its involvement in CLIP170^{KD}-induced EMT. Our results confirm this hypothesis, with TGF-β pathway inhibition restoring phenotypes induced by CLIP170^{KD}. In addition, the activation of TGF-β is known to promote the reorganization of the actin cytoskeleton [25, 26]. Therefore, this also explains the formation of actin protrusion promoted by CLIP170^{KD} and corroborates the role of CLIP170 on PTC cells metastasis.

In conclusion, our study demonstrated that in PTC cells, TGF-β was activated when CLIP170 was knocked down, which promotes cell metastasis and EMT. However, it is unclear whether TGF-β activation is involved in the rearrangement of the actin backbone and formation of lamellar pseudopods in PTC cells and whether other +TIPs are involved in CLIP170-promoted lamellipodia formation. However, we demonstrated the significance of CLIP170-TGF-β axis on PTC cells metastasis, offering a crucial avenue for future research.

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Author contributions BM and YX carried out the experiments and writing-original draft; HG and YY contributed toward study design and data analysis; and CY and YP contributed toward data analysis, interpretation, and revision of manuscript. All authors have reviewed the final version of the manuscript and approved it for publication.

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Data availability All data generated or analyzed in this study are included in this published article.

Declarations

Conflict of interest There is no conflict of interest between all authors.

Ethical approval Ethical approval was obtained from the Ethical Committee of Lanzhou University the Second Hospital (22021A-544).

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