Research Paper

Up-regulation of PCOLCE by TWIST1 promotes metastasis in Osteosarcoma

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Abstract

Procollagen C-proteinase enhancer protein (PCOLCE) was originally identified as an enhancer to facilitate the catalysis of procollagens by BMP1. PCOLCE participates in the reconstitution of extracellular and corneal repair. The elevation of PCOLCE in blood indicates that breast cancer has metastasized into the bones. However, direct research on PCOLCE has not been reported.

Methods: ECM candidates were identified by RNA-seq analysis from 4 normal and 16 osteosarcoma tissues. The *in vitro* migration and invasion abilities of osteosarcoma cells were determined by a Transwell assay. A spontaneous metastatic osteosarcoma model was established to assess osteosarcoma metastasis *in vivo*. The N-linked glycosylated amino acids were identified by PNGase F treatment combined with Western blotting. The mechanism of TWIST1 regulating PCOLCE transcription was elucidated by luciferase, qPCR and ChIP assays.

Results: PCOLCE was markedly up-regulated in human osteosarcoma tissues compared to its expression in noncancerous adjacent tissues; high *PCOLCE* expression in tissues correlated with a poor patient prognosis, and the knockdown of *PCOLCE* by shRNAs impaired the migration, invasion and lung metastasis of osteosarcoma cells. The overexpression of wild-type PCOLCE, but not its N29Q mutant, promoted migration, invasion and metastasis, indicating that the glycosylation of PCOLCE at Asn29 is necessary for its functions in osteosarcoma. TWIST1, a key transcription factor in metastasis, was also overexpressed in osteosarcoma tissues and positively correlated with either PCOLCE or its potential procollagen substrates, such as COL1A1, COL1A2, COL5A1, COL8A2 and COL10A1.

Conclusion: Our findings are the first to provide evidence that PCOLCE plays a critical role in promoting the lung metastasis of osteosarcoma, and this up-regulation of PCOLCE by TWIST1 may lead to a new therapeutic strategy to treat patients with metastatic osteosarcoma.

Key words: PCOLCE, TWIST1, Osteosarcoma, Metastasis, N-glycosylation

Introduction

Osteosarcoma, the most common primary malignant bone tumor, is composed of malignant osteoblasts that produce immature bone or osteoid tissues [1]. It arises primarily in adolescence and childhood and has a second peak incidence in those over the age of 50 [2]. Despite the improvement of treatment strategies, such as surgery or chemotherapy, the overall 5-year survival is approximately 75% for nonmetastatic osteosarcoma but has remained at 20% for metastatic cases over the

past 30 years [3, 4]. Thus, it is urgent to understand the molecular mechanism of osteosarcoma metastasis.

The extracellular matrix (ECM) is composed of numerous biochemically distinct proteins that participate in various biological processes, such as embryonic development, tissue homeostasis and cell differentiation [5, 6]. Abnormal ECM dynamics also play a critical role in cancer progression and metastasis. For instance, matrix metalloproteinases (MMPs), the main enzymes involved in ECM degradation, are highly expressed in cancer and promote cancer metastasis [7, 8]. Lysyl oxidase (LOX) crosslinks collagens and elastins in the extracellular matrix and promotes bone metastasis in hypoxic cancers by inducing premetastatic bone lesions [9]. Collagens, the most abundant components of the ECM, are excessively deposited in the extracellular matrix to form the tumor microenvironment and to modulate cancer cell polarity and migration [9]. The down-regulation of BMP-1 (bone morphogenetic protein-1), which processes fibrillar collagen types I-III, leads to the suppression of TGFβ in non-small cell lung cancer cells [10].

Procollagen C-proteinase enhancer protein (PCOLCE) is a secreted glycoprotein that enhances the activity of procollagen C-proteinases to participate in ECM reconstruction [11-13]. PCOLCE binds to the C-propeptide of procollagen III and heparin sulfate through its CUB and NTR domains, respectively, leading to the enhancement of BMP-1 activity and collagen precursor maturation [14, 15]. It has been reported that the dysregulation of PCOLCE is involved in numerous diseases. For instance, the expression of PCOLCE positively correlates with muscle and liver fibrosis [16]. A PCOLCE deficiency contributes to deficient corneal repair [17]. Mutated PABPN1 binds to PCOLCE and entraps it within the nuclear compartment, leading to oculopharyngeal muscular dystrophy [18]. However, the role of PCOLCE in cancer remains to be explored. In the present study, we found that PCOLCE was highly expressed in osteosarcoma and may play an important role in promoting the lung metastasis of osteosarcoma.

Materials and methods

RNA preparation, RNA-seq and analysis

The total RNA from osteosarcoma tissues was extracted using the TRIzol Reagent (Invitrogen). The cDNA was synthesized with mRNA enriched by Oligo(dT) beads. Libraries for next-generation sequencing were constructed by blunting the ends of the cDNA; this was followed by A-tailing, ligation to sequencing adapters, and PCR with indexed

sequencing primers. RNA-seq was carried out using Illumina HiSeq™ 2500 by Gene Denovo Biotechnology Co. (Guangzhou, China), and 10 Gb of the RNA-seq data of every sample are available from the GEO database (GEO: GSE00000). The low-quality reads were trimmed by Trimmomatic (Version 0.32)[19], and the clean reads were aligned to the human genome (GRCh38) using Tophat2 (Version 2.0.13)[20]. The expression levels of the genes were quantified by Cuffquant in the Cufflinks (Version 2.2.1)[21] suite. All the processes were processed by Gene Denovo Co. (Guangzhou, China).

Clinical samples

Paracancerous tissues and tumor samples were obtained from recruited osteosarcoma patients. The patients were diagnosed according to their clinicopathologic characteristics at the Department of Musculoskeletal Oncology at The First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China). No patient had received radiotherapy and/or chemotherapy prior to surgery. The samples were immediately frozen in liquid nitrogen for RNA extraction and qRT-PCR. The clinical information of 20 patients, whose samples were collected for RNA-seq, are listed as Supplementary Table S1 (sheet 3), whereas the clinical information of 68 patients, whose samples were collected for qRT-PCR, are listed as Supplementary Table S2.

Cell culture and transfection

U2OS and HOS were obtained from American Type Culture Collection. U2OS/MTX300 is methotrexate-resistant and is derived from U2OS. All cell lines were cultured in 10% fetal bovine serum containing DMEM (Dulbecco's Modified Eagle Medium) at 37°C and 5% CO_2. Stable cell lines were cultured in complete medium supplemented with 0.5 µg/mL puromycin. The transfection experiments were performed according to the manufacturer's instructions included with Lipofectamine 2000™ (Invitrogen).

His-tagged protein purification

Stable cell lines expressing PCOLCE-His or N29Q-His were lysed by cell lysis buffer (phosphate 20 mM, NaCl 0.5 M, imidazole 20 mM, Triton X-100 2%, pH 7.4) at 4°C for 1 h, and the lysate was centrifuged to remove the cell debris before it was transferred to HiTrap (GE, 17040501). After washing with 5 ml binding buffer (phosphate 20 mM, NaCl 0.5 M, and imidazole 20 mM, pH 7.4), the protein was eluted with 1 ml elution buffer (phosphate 20 mM, NaCl 0.5 M, and imidazole 500 mM, pH 7.4). The elution product was diluted by adding 14 ml of PBS before it was concentrated to a 0.5 ml product by 10

KD Amicon Ultra-4 (Millipore). All buffer used in this process was supplemented with a protease inhibitor cocktail (Sigma).

Construct and plasmids

PCOLCE and N29Q CDSs were amplified in a thermal cycler using PrimeSTAR (TaKaRa, Japan) and were then infused with psin-EF1, which is used for producing lentiviruses, using the Clone Express II One Step Cloning Kit (Vazyme Biotech). The primers used for cloning *PCOLCE* and the N29Q mutant are as follows:

psin-PCOLCE-F: 5′-ATT GGG ATC CCC GGA CGG CCA CCA TGC TGC CTG CAG CCA CA-3′;

psin-PCOLCE-R: 5′-GCA TGC GGA TCA CTA GTG TCA GTC CTG GGA CGC AGC A-3′;

N29QF: 5′-AGA CCC CCC AAT ACA CCA GAC CCG TGT T-3′;

N29QR: 5′-TGT ATT GGG GGG TCT GGC CCT GGG CAA-3′.

The *PCOLCE* CDS was amplified from cDNA derived from U2OS, and the N29Q mutant was generated by an overlap-extension strategy.

pLKO.1-puro was inserted with double-stranded oligonucleotides, which generates shRNA in the cell line, and the sequences are as follows:

PCOLCE-sh#1-s: 5′-CCG GTG AAG AAA GGA GTC AGT TAT CCT CGA GGA TAA CTG ACT CCT TTC TTC ATT TTT-3′;

PCOLCE-sh#1-as: 5′-AAT TAA AAA TGA AGA AAG GAG TCA GTT ATC CTC GAG GAT AAC TGA CTC CTT TCT TCA-3′;

PCOLCE-sh#2-s: 5′-CCG GCG CTG ACC TTC GAG AAG TTT GCT CGA GCA AAC TTC TCG AAG GTC AGC GTT TTT-3′;

PCOLCE-sh#2-as: 5′-AAT TAA AAA CGC TGA CCT TCG AGA AGT TTG CTC GAG CAA ACT TCT CGA AGG TCA GCG-3′.

RNA extraction and qRT-PCR

The total RNA was extracted using the RNAprep Pure Cell/Bacteria Kit (Tiangen). The total RNA (500 ng) was reverse-transcribed using a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme). qRT-PCR was performed using the ChamQ Universal SYBR qPCR Master Mix (Vazyme) on LightCycler 480 (Roche). All qRT-PCR samples were repeated at least 3 times. The primer sequences are as follows:

qGAPDH-F: 5′-GGA GCG AGA TCC CTC CAA AAT-3′;

qGAPDH-R: 5′-GGC TGT TGT CAT ACT TCT CAT GG-3′;

qPCOLCE-F: 5′-GTG CGG AGG GGA TGT GAA G-3′;

qPCOLCE-R: 5′-CGA AGA CTC GGA ATG

AGA GGG-3′;

qTWIST1-F: 5′-GTC CGC AGT CTT ACG AGG AG-3′;

qTWIST1-R: 5′-GCT TGA GGG TCT GAA TCT TGC T-3′;

qCOL1A1-F: 5′-GAG GGC CAA GAC GAA GAC ATC-3′;

qCOL1A1-R: 5′-CAG ATC ACG TCA TCG CAC AAC-3′;

qCOL1A2-F: 5′-GGC CCT CAA GGT TTC CAA GG-3′;

qCOL1A2-R: 5′-CAC CCT GTG GTC AAA CAA CTC-3′;

qRT-col5A1-F: 5′-TAC AAC GAG CAG GGT ATC CAG-3′;

qRT-col5A1-R: 5′-ACT TGC CAT CTG ACA GGT TGA-3′;

qRT-col8A2-F: 5′-GCT GGC TTA GGC AAA CCT G-3′;

qRT-col8A2-R: 5′-GGA CTC CCA CAC CGT CTA CT-3′;

qRT-col10A1-F: 5′-ATG CTG CCA CAA ATA CCC TTT-3′;

qRT-col10A1-R: 5′-GGT AGT GGG CCT TTT ATG CCT-3′.

Dual-Luciferase assay

293T cells were plated in 24-well plates and were transiently transfected with 200 ng of PCOLCE promoter-containing pGL-3 plasmids together with pTK-cLuc as the normalization control. 48 h later, the luciferase activity was measured for 3 independent experiments using the Dual-Luciferase Assay Kit (Promega).

Assessment of cell migration and invasion

Cells were seeded into Boyden chambers containing 24-well Transwell plates with a pore size of 8 μm (BD Bioscience). The upper chamber was either left uncoated for the migration assay or precoated with 50 μl 1:8 diluted Matrigel (BD Bioscience) for the invasion assay. U2OS (5×10^4), U2OS/MTX300 (5×10^4) and HOS (3×10^4) cells were seeded into the upper chamber. The upper chamber was filled with 200 μL serum-free specified medium, whereas the lower chamber was filled with complete medium containing 10% FBS. Following incubation for 12 h (U2OS, HOS) and 24 h (U2OS/MTX300), the cells that had invaded into the lower chamber were fixed with 4% paraformaldehyde and were stained with crystal violet for 1 h at room temperature. The cells in the upper chamber were removed by a cotton swab, and the remaining cells were counted in 5 randomly selected microscopic fields. All experiments were performed in triplicate.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was performed using a ChIP Kit according to the manufacturer's instructions [22]. Briefly, each cell line was seeded into a 15 cm plate. The cells were fixed by adding 10% of the volume of the growth medium. The fixation was stopped by adding 5% of the volume of the stop solution to the existing culture medium. The cells were scratched, collected and lysed to obtain the nuclear pellet. The nuclear pellet was resuspended and sonicated. Five micrograms of antibodies together with protein A/G agarose beads was incubated overnight at 4°C. The bound DNA-protein complexes were eluted after a series of washes. Purified DNA was resuspended in TE buffer for qRT-PCR.

The PCR primer sequences are as follows:

CHIP-PCOLCE-EBX1-F1: 5'-AAC AAG GAC ACT CCC TTC AT-3';

CHIP-PCOLCE-EBX2-R1: 5'-TAG AGG CAG GGT TTC AGC AT-3'.

Western Blotting

Western blotting was performed as previously described [22]. The cells were collected and lysed in RIPA buffer (150 mM NaCl, 0.5% EDTA, 50 mM Tris, 0.5% NP40) and centrifuged for 20 min at 14,000 x g and 4°C. Before being loaded onto the polyacrylamide gel, the protein lysate or concentrated. cell culture supernatant was denatured at 105°C for 10 min in loading buffer (Tris-HCl pH 6.8 60 mM; SDS 2%; bromophenol blue 0.1%; glycerol 25%) supplemented with or without 14.4 mM beta-mercaptoethanol for a reduced or nonreduced reaction, respectively. A total of 20 μg of harvested protein was loaded and separated on a 10% SDS-polyacrylamide gradient gel. The proteins were then transferred onto polyvinylidene difluoride membranes and were blocked with 5% fat-free milk for 2 h at room temperature. The membranes were incubated with the primary antibody and the horseradish peroxidase-conjugated secondary antibody; then, the proteins were detected using an ECL chemiluminescence system (Pierce). The primary antibodies used were as follows: the antibody against GAPDH (13937-1-AP) was from Proteintech, and the antibody against HA (3724) was obtained from Cell Signaling Technology.

PNGase F Treatment

In this study, PNGase F was purchased from NEB corporation and was used to remove almost all N-linked oligosaccharides from the glycoproteins. The cell lysates were prepared as described in the Western blotting process. According to the PNGase F denaturing protocol, 5 μg of cell lysate was mixed with 1 μl of glycoprotein denaturing buffer (10X) and H_2O to make a 10 μl total reaction volume. The mixture was then incubated at 100°C for 10 min and was then chilled on ice for 10 sec. Then, 2 μl GlycoBuffer 2 (10X), 2 μl 10% NP-40, 6 μl H_2O, and 1 μl PNGase F was added into the mixture and incubated at 37°C for 1 h.

Supernatant concentration

The cells were cultured in 15 cm dishes with complete media. The media were removed and replaced by 25 ml of DMEM without antibiotics or FBS. After 48 h, the cell supernatant was loaded onto an Ultracel-10 centrifugal Filter Unit for centrifugation at 4000 rpm at 4°C for 1 h. The concentrated supernatants were lysed in reduced and nonreduced reactions for subsequent Western blotting.

Animal experiments

All animal experiments were performed according to the protocols approved by the Research Animal Resource Center of Sun Yat-Sen University and complied with the guidelines. The animal ethics number of this project is GZR2015-137. All animals were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. For the spontaneous metastasis osteosarcoma model *in vivo*, U2OS/MTX300 cells stably expressing luciferase, including the vector, PCOLCE, N29Q, shNC, PCOLCE-sh#1, and PCOLCE-sh#2, were used as described. Six mice were injected separately with 1×10^6 cells in each group. When the tumor reached 1.5 cm in diameter, the mice were injected with a luciferase substrate, and the IVIS Lumina Imaging System (Xenogen) was used to capture the luciferase signal by taking photographs. The mice were then sacrificed, and the lungs were fixed in formalin (4 μmol/L) and stained with hematoxylin and eosin (H&E).

Statistical analysis

The SPSS software was used for statistical analysis. The statistical significance was tested by Student's t-test or a chi-square test. The curve of correlation between *PCOLCE* expression level and the overall survival was plotted by the Kaplan-Meier method. Differences were considered statistically significant when p values were <0.05.

Results

PCOLCE is up-regulated in osteosarcoma tissues

By analyzing our RNA-seq data from 4 noncancerous adjacent tissues and 16 osteosarcoma

tissues (**Fig. S1**), we found that 169 genes encoding ECM proteins were overexpressed in osteosarcoma tissues compared to the expression in noncancerous adjacent tissues (**sheets 1-2 of Table S1**). Notably, *PCOLCE*, a procollagen C-proteinase enhancer protein for stimulating procollagen maturation, was one of the most up-regulated genes in osteosarcoma (**Fig. 1A**). Interestingly, data from CCLE (https://portals.broadinstitute.org/ccle/page?gene= PCOLCE) also indicated that the *PCOLCE* mRNA was dramatically elevated in osteosarcoma cell lines, such as U2OS and HOS cells (**Fig. 1B**). The up-regulation of *PCOLCE* was further confirmed by qRT-PCR using 68 pairs of human osteosarcoma tissues and their adjacent nontumor tissues (**Fig. 1C**), whose clinical information are listed as Supplementary **Table S2**. More importantly, the osteosarcoma patients with high levels of *PCOLCE* had poorer total survivals compare those with low levels of *PCOLCE* (**Fig. 1D**). The results indicate that PCOLCE is up-regulated in osteosarcoma tissues and may be involved in osteosarcoma progression.

Knockdown of *PCOLCE* by shRNA inhibits the lung metastasis of osteosarcoma

To determine the function of PCOLCE in osteosarcoma, we generated two independent short hairpin RNAs against *PCOLCE*, which could efficiently reduce the endogenous expression of *PCOLCE* in U2OS, U2OS/MTX300 and HOS cells (**Fig. S2A-C**). The knockdown of *PCOLCE* significantly inhibited cell migration and invasion *in vitro* (**Fig. 2A and 2B, S3A and S3B**), but did not affect cell viability (**Fig. S2D and S2E**). More importantly, in the orthotopic osteosarcoma metastasis mouse model using U2OS/MTX300-Luc cells with the stable knockdown of *PCOLCE*, only one or two out of five mice in the PCOLCE-shRNAs groups, while four out of five mice in the control group had luminescent signals within the lungs (**Fig. 2C**). Consistently, the mice bearing PCOLCE-sh#1 or PCOLCE-sh#2 cells had significant decreases in lung metastatic burden compared to those bearing control cells (**Fig. 2D and 2E**).

PCOLCE promotes osteosarcoma cell migration and invasion

The stable overexpression of PCOLCE in U2OS, U2OS/MTX300 and HOS cells was also generated (**Fig. S4A-C**). Cell migration and invasion, but not cell viability, were dramatically promoted in these stable cells compared to those in cells stably overexpressing the empty vector (**Fig. 3A and 3B, S4D-G**). Furthermore, we purified His-tagged PCOLCE
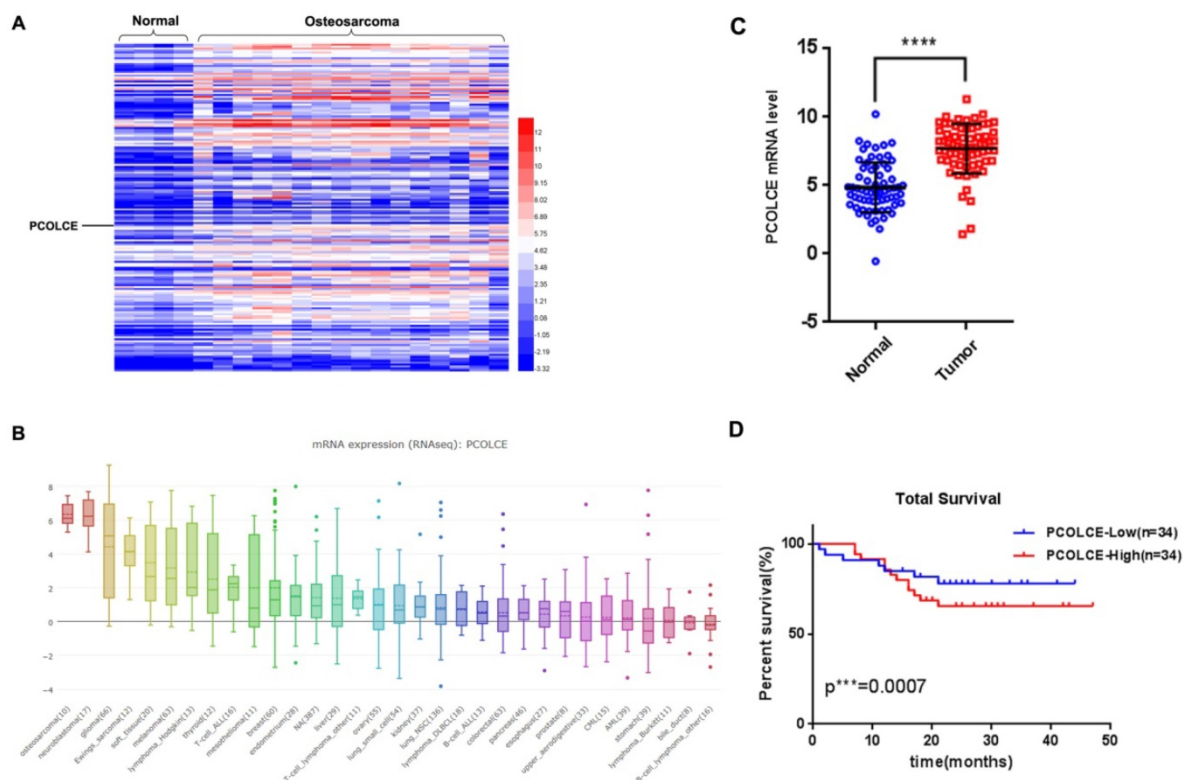

Figure 1. *PCOLCE* is up-regulated in osteosarcoma tissues. (**A**) The expression profiles of extracellular matrix proteins in 4 nontumor adjacent tissues and 16 osteosarcoma tissues are presented by a heatmap. (**B**) The *PCOLCE* mRNA levels in different cancer type cell lines from the CCLE database. (**C**) The mRNA levels of *PCOLCE* in the indicated tissues were analyzed by qRT-PCR (mean ± SD. ****p < 0.0001). (D). The total survival curves were generated based on the *PCOLCE* mRNA levels from 68 osteosarcoma tissues. The actuarial probabilities were calculated using the Kaplan-Meier method. The patients with high levels of *PCOLCE* had poorer total survivals.

recombinant protein from HEK293T cells transiently expressing His-tagged PCOLCE (**Fig. S4H**) to test whether this recombinant protein could promote the migration of osteosarcoma cells. As shown in **Fig. 3C and 3D**, cell migration was significantly promoted in U2OS cells treated with the purified His-tagged PCOLCE recombinant protein in a concentration-dependent manner compared to the cell migration of the control cells. The results suggest that PCOLCE may play a critical role in promoting osteosarcoma metastasis.

The N-glycosylation of PCOLCE is essential for its enhancement of metastasis

Glycosylation modifications are crucial for the ECM-mediated malignant phenotype during cancer [23], and PCOLCE may have N-linked oligosaccharides modified with sialic residues in human serum [24]; we explored which residue of PCOLCE could be glycosylated and the significance of this event. Two putative N-glycosylation sites of PCOLCE were predicted using the N-X-S/T consensus sequence [25] (**Fig. 4A**). Therefore, we generated two PCOLCE mutants by individually changing each Asn (N) into Gln (Q). As shown in **Fig. 4B**, the molecular weight of the N29Q mutant, but not that of the N431Q mutant, was slightly lower than its wild-type counterpart, whereas their molecular weights became identical under PNGase F treatment. As PNGase F specifically removes the N-linked glycosylation, the band shifts of both wild-type and its N431Q mutant, but not of its N29Q mutant, were observed after PNGase F treatment, indicating that PCOLCE has the N-linked glycosylation at N29. Notably, the protein level of the PCOLCE-N29Q mutant was markedly reduced compared to that of its
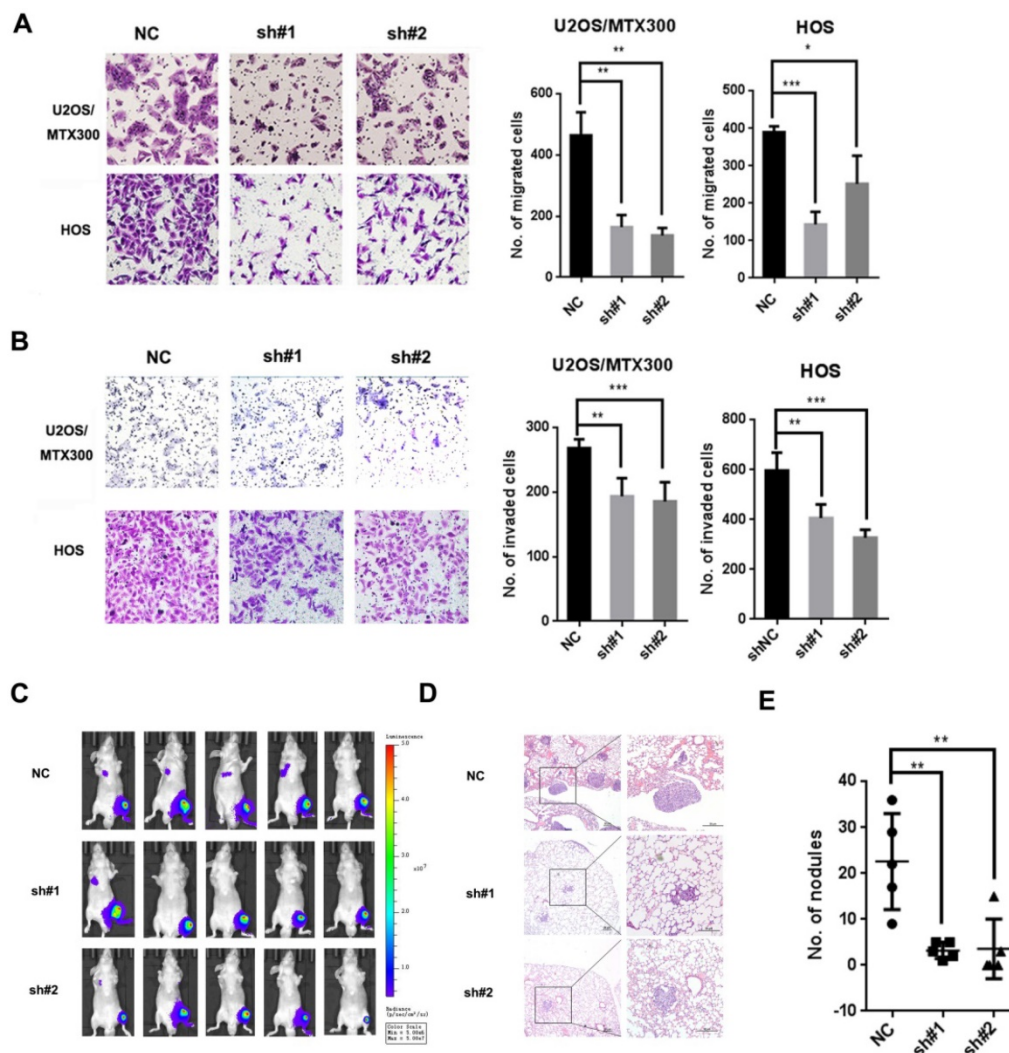

Figure 2. Down-regulation of *PCOLCE* by shRNAs suppresses osteosarcoma metastasis. (A, B). Cell migration **(A)** and invasion **(B)** assays were performed and quantified in the indicated stable cells as described in the Materials and Methods. Representative images and quantification analyses are shown (mean ± SD, n=3, *$p<0.05$, **$p<0.01$, ***$p<0.001$). **(C-E).** The orthotopic osteosarcoma metastasis model *in vivo* using the indicated U2OS/MTX-Luc stable cells, as described in the Materials and Methods section. **(C)** Bioluminescence images for mice in the indicated groups. **(D, E)** Representative images of H&E staining **(D)** and quantification analyses **(E)** of the metastatic lung nodules from the indicated groups (mean ± SD, n=5, **$p<0.01$.

wild-type protein (**Fig. 4B**), indicating that the N-linked glycosylation of PCOLCE at Asn29 may impact its protein stability. Indeed, as shown in **Fig. 4C**, the PCOLCE-N29Q mutant protein had a shorter half-life than that of its wild-type protein. In addition, as shown in **Fig. 4D,** wild-type PCOLCE was detected in both cell lysate and supernatant, while the N29Q mutant was hardly detected in supernatant, indicating that the secretion of the PCOLCE-N29Q mutant into media was much less compared to wild-type PCOLCE. The results suggest that PCOLCE is N-glycosylated at Asn29, which positively regulates its protein stability and its secretion into the ECM.

Next, we asked whether the Asn29 glycosylation of PCOLCE is required for its functions in osteosarcoma. As shown in **Fig. 4E-4H** and **Fig. S5A-C**, the enhancement of cell migration and metastasis induced by wild-type PCOLCE was not observed in the N29Q mutant both *in vitro* and *in vivo*. This finding indicates that the glycosylation of

PCOLCE at Asn29 is necessary for its promotion of osteosarcoma metastasis.

PCOLCE is transcriptionally up-regulated by TWIST1.

Since PCOLCE has been reported to be directly regulated by TWIST1 in mouse embryonic tissues[26], and two putative E-box binding sites for TWIST1, -542~-533 and -408~-399, were predicted in the *PCOLCE* promoter using JASPAR [27] as shown in **Fig. 5A**. In addition, the expression levels were positively correlated between *TWIST1* and *PCOLCE* in osteosarcoma tissues (**Fig. 5B**). Therefore, we speculated that TWIST1 could transcriptionally up-regulate *PCOLCE* in osteosarcoma cells. This was the case, as the mRNA levels of *PCOLCE* were significantly increased in both U2OS and HOS cells stably overexpressing TWIST1 compared to those stably expressing the empty vector (**Fig. 5C**). To test whether *PCOLCE* is a direct target of TWIST1, the
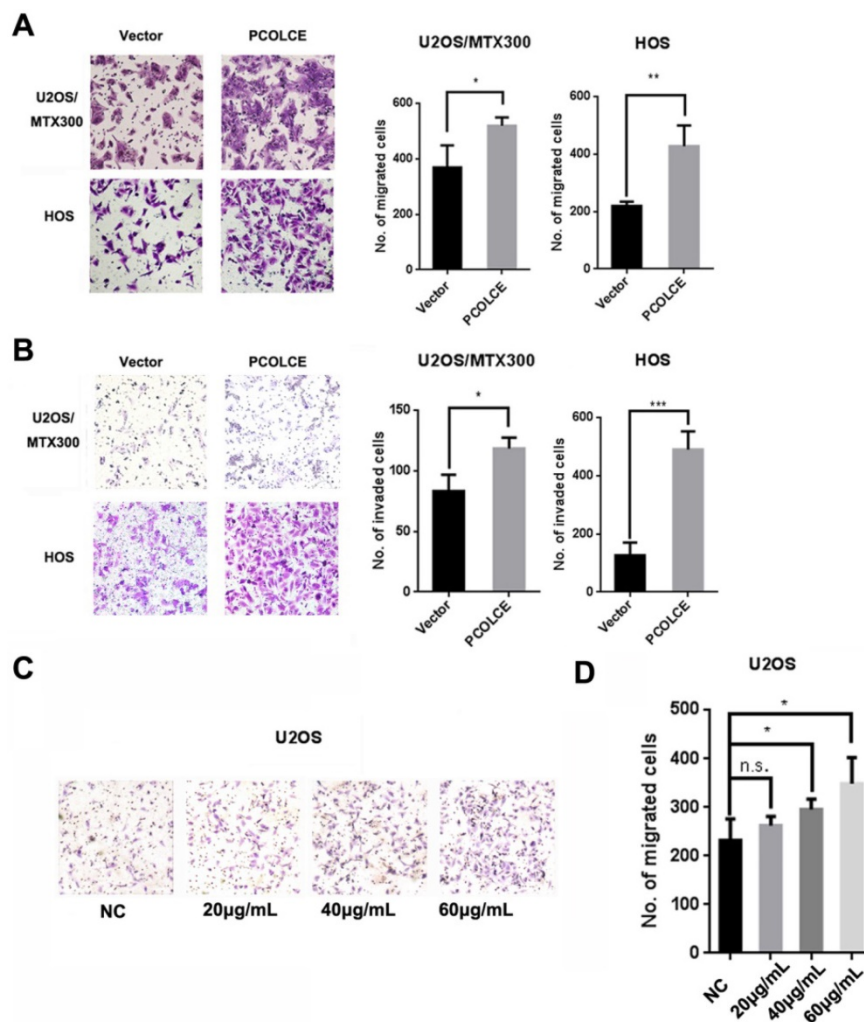

Figure 3. PCOLCE promotes the migration and invasion of osteosarcoma cells. (**A, B**). Cell migration (**A**) and invasion (**B**) assays were performed and quantified in the indicated stable cells as described in the Materials and Methods section. Representative images and quantification analyses are shown (mean ± SD, n=3, *$p<0.05$, **$p<0.01$). (**C, D**) U2OS cells were treated with the indicated concentrations of PCOLCE-His tag protein from (**Fig. S4H**) for 24 h and were then subjected to the migration assay. Representative images and quantification analyses are shown (mean ± SD, n=3, *$p<0.05$).

depletion of a single or double E-box in the dual-luciferase reporter of the *PCOLCE* promoter was generated. As shown in **Fig. 5D**, E-box1, but not E-box2, was crucial for the up-regulation of the *PCOLCE* promoter by TWIST1. Indeed, the association of TWIST1 with the *PCOLCE* promoter

was further verified by a chromatin immunoprecipitation (ChIP) assay (**Fig. 5E**). Taken together, our results indicate that TWIST1 transcriptionally up-regulates *PCOLCE* by directly binding to E-box1 within the *PCOLCE* promoter.
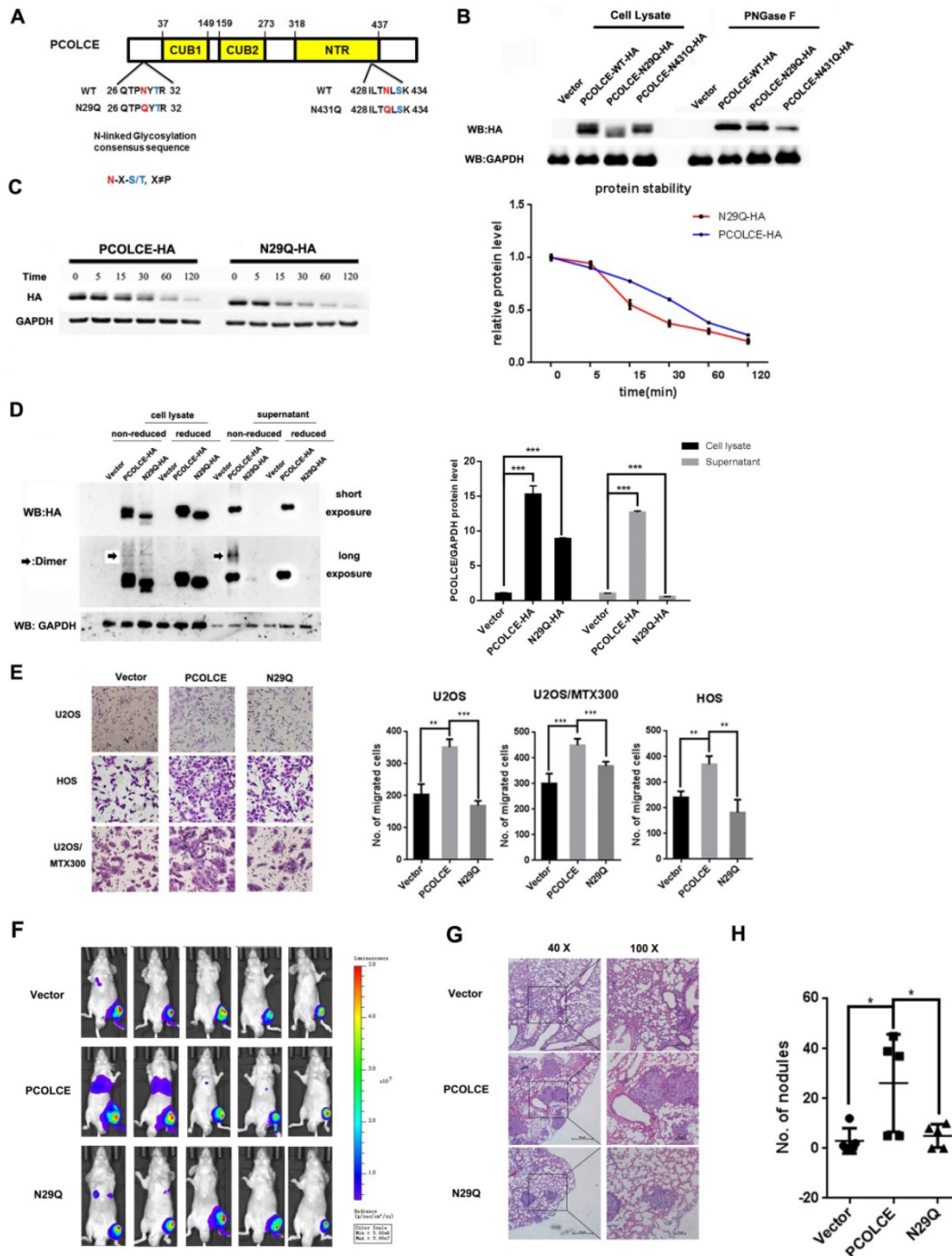

Figure 4. Glycosylation of PCOLCE is essential for its function in osteosarcoma. (**A**) A schematic illustration of the predicted N-linked glycosylation sites in PCOLCE. (**B**) 293T cells were transiently transfected with the indicated plasmids for 48 h and were incubated with or without PNGase F as indicated; then, the cell lysates were harvested and subjected to Western blotting. (**C**) 293T cells were transiently transfected with PCOLCE wild-type or N29Q mutant plasmids for 48 h and were incubated with 20 mg/mL cycloheximide (CHX) for the indicated times. The cell lysates were harvested and analyzed by Western blotting. (**D**) The cell lysates and supernatant with (reduced) or without (nonreduced) β-mercaptoethanol from the indicated cells were analyzed by Western blotting. (**E**) A cell migration assay was performed and quantified in the indicated stable cells as described in the Materials and Methods section. The representative images and quantification analyses were shown (mean ± SD, n=3, **$p<0.01$, ***$p<0.001$). (**F-H**) The orthotopic osteosarcoma metastasis model *in vivo* using the indicated U2OS/MTX-Luc stable cells, as described in the Materials and Methods section. (**F**) Bioluminescence images for mice in the indicated groups. (**G, H**) Representative images of H&E staining (**G**) and quantification analyses (**H**) of the metastatic lung nodules from the indicated groups (mean ± SD, n=5, *$p<0.05$).
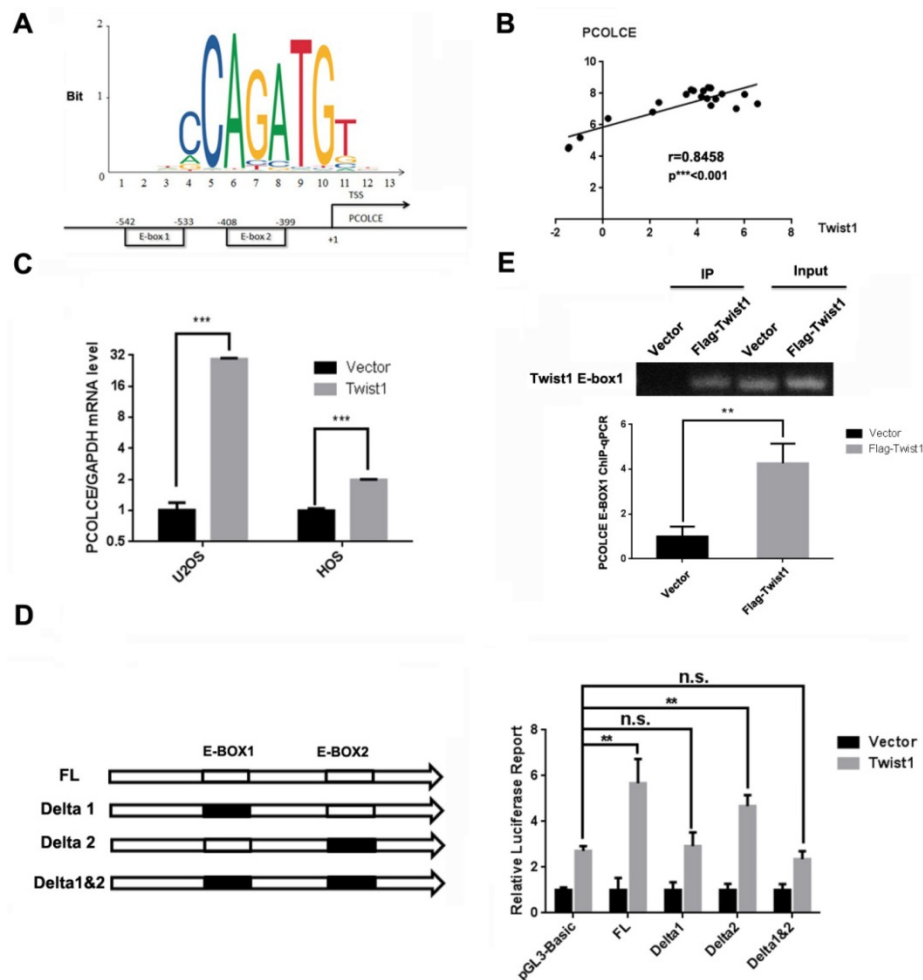
Figure 5. TWIST1 transcriptionally up-regulates *PCOLCE* in osteosarcoma cells. (**A**) A schematic illustration of putative TWIST1 binding sites (E-boxes) in the promoter of *PCOLCE*. (**B**) The correlation between *TWIST1* mRNA levels and *PCOLCE* mRNA levels in the clinical samples (16 osteosarcoma and 4 normal control) shown in **Fig. 1A**. (**C**) The mRNA levels of *PCOLCE* were measured by qRT-PCR in the indicated cells stably transfected with the vector or TWIST1 (mean ± SD, n=3, ***$p<0.001$). (**D**) The ChIP-qPCR assay using an anti-Flag antibody was performed with U2OS cells expressing the vector or Flag-TWIST1 (mean ± SD, n=3, **$p<0.01$). (**E**) HEK293T cells were transfected with the indicated luciferase reporter for 48 h and were then subjected to the luciferase activity assay as described in the Materials and Methods section. (mean ± SD, n=3, **$p<0.01$).

TWIST1 may also up-regulate some procollagens in osteosarcoma tissues

Since PCOLCE promotes the maturation of procollagens, we determined the status of several procollagens in osteosarcoma tissues. As shown in **Fig. 6A-6E**, the mRNA levels of several procollagens, such as *COL1A1, COL1A2, COL5A1, COL8A2 and COL10A,* were positively correlated with *TWIST1* in our RNA-seq data (**Fig. 1A**). Indeed, the mRNA levels of these procollagens were significantly increased in U2OS cells stably expressing TWIST1 compared to those in cells stably expressing the vector (**Fig. 6F**). These results indicate that TWIST1 may also up-regulate some procollagens in osteosarcoma tissues.

Discussion

In this report, we revealed that PCOLCE and some procollagens were up-regulated by TWIST1 in osteosarcoma cell lines and tissues and that the N-glycosylation of PCOLCE at Asn29 was crucial for promoting osteosarcoma metastasis.

PCOLCE specifically enhances the activity of BMP-1, a zinc metalloproteinase that modulates collagen deposition in the extracellular matrix by removing C-propeptides from procollagen I, II and III [11]. Increased collagen deposition is the most well-recognized ECM alteration during cancer progression [28], suggesting that PCOLCE may be involved in cancer metastasis. Recently, Salza et al. reported that PCOLCE might be involved in tumor growth based on the interaction network of PCOLCE [29]. Our results showed that PCOLCE plays a critical role in promoting the metastasis of osteosarcoma, as the overexpression of PCOLCE promotes cell migration, invasion and lung metastasis (**Figs. 3A, 3B, 4E-H, S4D, S4E**) whereas the knockdown of PCOLCE had the opposite effects (**Fig. 2, S3A, S3B**) in osteosarcoma.
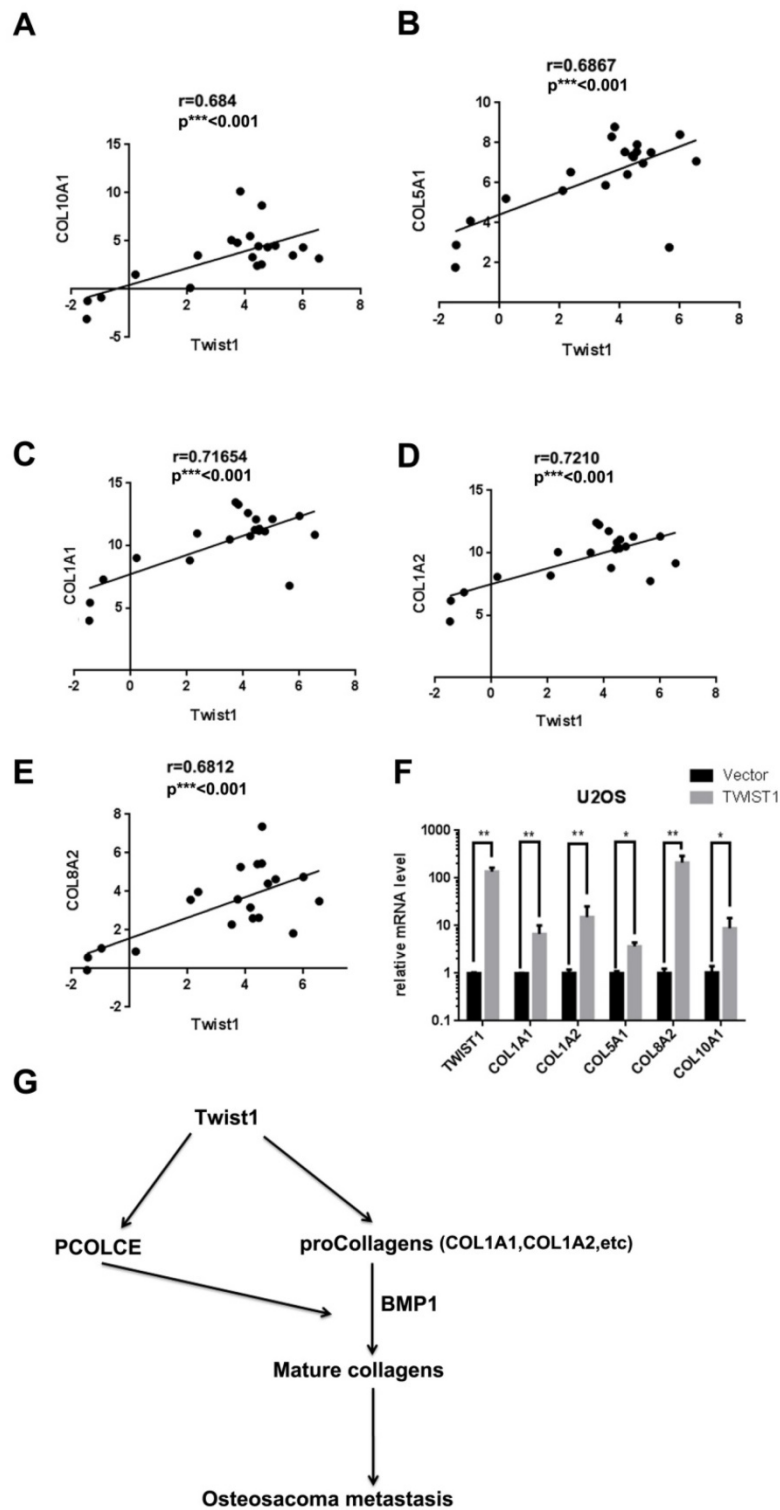
Figure 6. TWIST1 up-regulates Collagen expression. (A-E) The correlations between *TWIST1* mRNA levels and mRNA levels of *COL10A1* (**A**), COL15A1 (**B**), *COL1A2* (**C**), *COL5A1* (**D**) or *COL8A2* (**E**) in the clinical samples (16 osteosarcoma and 4 normal controls) are shown in **Fig. 1A**. (**F**) The mRNA levels of *COL1A1, COL1A2, COL5A1, COL8A2* and *COL10A1* in the U2OS cells with the stable overexpression vector or Flag-TWIST1 were measured by qRT-PCR (mean ± SD, n=3, *$p<0.05$, **$p<0.01$). (**G**) The proposed model for the function and mechanism of PCOLCE in osteosarcoma. TWIST1 may up-regulate both PCOLCE and procollagens, and then PCOLCE helps BMP1 accelerate the maturation of collagen, which in turn promotes osteosarcoma metastasis.

A previous study has indicated that PCOLCE may have N-linked oligosaccharides decorated with sialic residues in human serum [24]. Here, we have demonstrated that PCOLCE is N-glycosylated at Asn29, which is essential for its protein stability, secretion and the enhancement of metastasis in osteosarcoma (**Fig. 4E-H**). Notably, serum PCOLCE may act as a biomarker of diseases, such as muscle

[16], liver [30] and cardiac [31] fibrosis. PCOLCE could be detected in the supernatant of osteosarcoma cells (**Fig. 4D**), and the purified PCOLCE recombinant protein could promote cell migration in U2OS cells (**Figs. 3C-3E**); this indicates that PCOLCE may be a potential noninvasive biomarker to predict metastasis in osteosarcoma patients.

We proved that TWIST1 could up-regulate PCOLCE by directly binding to the E-box1 of the *PCOLCE* promoter (**Fig. 5**). In addition, TWIST1 also increased the mRNA levels of procollagens, such as *COL1A1*, *COL1A2*, *COL5A1*, *COL8A2* and *COL10A* (**Fig. 6A**), which are potential substrates for PCOLCE. This may provide a new explanation that TWIST1 is a key regulator in cancer metastasis in addition to being a key regulator of EMT[32] [33] because the correlations between *TWIST1* mRNA levels and *PCOLCE* mRNA levels were also observed in 18 different cancer types from the ChIPBase v2.0 database [34] (**Supplementary Table 1**).

In summary, we propose that TWIST1 may up-regulate both PCOLCE and procollagens and that PCOLCE helps BMP1 to accelerate the maturation of collagen, which in turn, promotes osteosarcoma metastasis; this is illustrated in **Fig. 6G**.

Supplementary Material

Supplementary figures and table 1.
http://www.thno.org/v09p4342s1.pdf
Supplementary table S1.
http://www.thno.org/v09p4342s2.xlsx
Supplementary table S2.
http://www.thno.org/v09p4342s3.xlsx

Acknowledgments

Competing Interests

The authors have declared that no competing interest exists.

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