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Collagen Type X Alpha 1 (COL10A1) Contributes to Cell Proliferation, Migration, and Invasion by Targeting Prolyl 4-Hydroxylase Beta Polypeptide (P4HB) in Breast Cancer

Authors' Contribution:

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Data Collection B

Statistical Analysis C

Data Interpretation D

Manuscript Preparation E

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Background: Breast cancer, a common malignant tumor, has been considered as the leading cause of cancer-related death in women. Collagen type X alpha 1 (COL10A1) is overexpressed in breast cancer. The current study was designed to determine the functional involvement and regulatory mechanism of COL10A1 on the growth and metastasis of breast cancer.

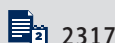
Material/Methods: COL10A1 and Prolyl 4-hydroxylase beta polypeptide (P4HB) expressions in normal tissues and tumor tissues of breast cancer patients were obtained from the GEPIA dataset. COL10A1 and P4HB levels in breast cancer cell lines were detected by real-time quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. Furthermore, the interaction between COL10A1 and P4HB was confirmed by co-immunoprecipitation (Co-IP) assay. Cell Counting Kit-8 (CCK-8) and colony formation assay were applied to evaluate cell proliferation and clone-forming abilities of breast cancer cells. In addition, wound healing assay and transwell assay were performed to measure cell migration and invasion capabilities, respectively, in breast cancer.

Results: The GEPIA dataset presented overexpressed COL10A1 and P4HB in tumor tissues of breast cancer patients. COL10A1 and P4HB expression levels were greatly upregulated in breast cancer cell lines. In addition, COL10A1 could directly interact with P4HB. Functionally, overexpressed COL10A1 boosted the proliferation and metastasis of breast cancer cells and silenced COL10A1 impeded the progression of breast cancer. More importantly, knockdown of P4HB weakened the promoting effects of overexpressed COL10A1 on cell proliferation, migration, and invasion in breast cancer.

Conclusions: COL10A1 promotes the malignant progression of breast cancer by upregulating P4HB expression, indicating that COL10A1 functions as an oncogene in breast cancer.

Keywords: **Breast Neoplasms • Genes, Tumor Suppressor • Tumor Markers, Biological**

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Background

Breast cancer is the most common malignant tumor of women worldwide and seriously threatens women's physical and mental health [1,2]. At present, there is a lack of specific examination methods for breast cancer. Meanwhile, breast cancer is prone to recurrence and metastasis, which brings great difficulties to clinical treatment [3]. Current therapeutics for breast cancer are surgery, radiotherapy and chemotherapy. However, the overall prognosis of breast cancer patients is poor and effective therapy is urgently needed [4]. Breast cancer is classified into 4 subtypes and obvious differences exist in therapeutics and prognosis for different breast cancer subtypes [5]. Identification of breast cancer subtypes prompted the development of novel targeted agents [6]. Furthermore, precision medicine for the therapy of breast cancer significantly improved the survival rate and quality of life of breast cancer patients.

COL10A1 is one of the members of the collagen family and is highly expressed in several tumor types [7]. For instance, COL10A1 expression in gastric tumor tissue is higher than that in adjacent normal tissue and overexpressed COL10A1 is associated with poor survival [8]. COL10A1 knockdown inhibits gastric cancer cell proliferation, migration, and invasion [9]. Interesting, COL10A1 has been confirmed to be overexpressed in different subtypes of breast cancer. Besides, it is documented that higher expression of COL10A1 in breast cancer patients is closely related to worse overall survival (OS), relapse-free survival (RFS), disease-free survival (DFS), and distant metastasis-free survival (DMFS) [10]. Therefore, we investigated COL10A1 as a potential therapeutic target for breast cancer.

P4HB is one of the core genes in the beta subunit of prolyl 4-hydroxylase and belongs to the protein disulfide isomerase (PDI) family [11]. It serves as an endoplasmic reticulum (ER) chaperone to suppress the aggregation of misfolded proteins [12]. Previous studies reported that P4HB is associated with the tumorigenesis and development of multiple tumors. P4HB is highly expressed in colon cancer tissues and P4HB knockdown can enhance colon cancer cell apoptosis [13]. Silenced P4HB significantly suppresses cell viability, invasion and migration abilities of HepG2/ADR cells [14]. Furthermore, clinical data shows that P4HB is highly expressed in kidney renal clear cell carcinoma (KIRC) tissues and is associated with poor OS of KIRC patients [15]. Based on these findings, P4HB may serve as a potential molecular marker in cancer diagnosis and therapy. However, the effects of P4HB on the growth and metastasis of breast cancer have been unclear.

In this study, we examined the relationship between COL10A1 and P4HB, and further investigated their functional roles and molecular mechanisms in the tumorigenesis and progression

of breast cancer. Our study revealed that COL10A1 can act as an oncogene in breast cancer by promoting P4HB, and our results may develop new strategies on targeted therapy for breast cancer.

Material and Methods

Cell Culture and Transfection

Human breast epithelial cells (MCF-10A), luminal-type human breast cancer cell line (MCF-7), triple-negative human breast cancer cell line (MDA-MB-231), and Her2-amplified breast cancer cell line (SK-BR-3) were purchased from American Tissue Culture Collection (ATCC, VA, USA). The Her2-amplified breast cancer cell line (SUM190PT) was purchased from Ningbo Mingzhou Biotechnology Co., Ltd. MCF-10A, MCF-7, MDA-MB-231, and SK-BR-3 were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, UT, USA) and SUM190PT was cultured in RPMI-1640 medium (Hyclone, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone, UT, USA) and 1% penicillin/streptomycin (Gibco, NY, USA) at 37°C in a humidified incubator with 5% CO₂.

The pcDNA-COL10A1, shRNA-COL10A1, si-P4HB, and the negative control were purchased from GenePharma (Shanghai, China) and transfected into cells using Lipofectamine® 3000 (Invitrogen, CA, USA) according to the manufacturer's protocol. Transfection efficiency was verified by RT-qPCR analysis.

Gene Expression Profiling Interactive Analysis (GEPIA)

GEPIA Dataset Analysis (<http://gepia.cancer-pku.cn/>) is a web-based tool, providing data on 9736 tumors and 8587 normal samples from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) projects. GEPIA was applied to analyze the differential expression of COL10A1 and P4HB between normal tissues and tumor tissues in breast cancer patients.

Cell Counting Kit-8 (CCK-8) Assay

CCK-8 assay was performed to evaluate cell proliferation. Briefly, at 48 h after transfection, cells were seeded into 96-well plates at a density of 5×10³ cells/well for 24 h. We then added 10 μl CCK-8 solution (Beyotime, Shanghai, China) to each well and incubated it for 2 h at 37°C. The absorbance at 450 nm of each well was measured using a microplate reader (Bio-Rad, CA, USA).

Colony Formation Assay

For the colony formation assay, transfected MCF-7 and MDA-MB-231 cells (2×10³) were seeded onto 6-cm plates and

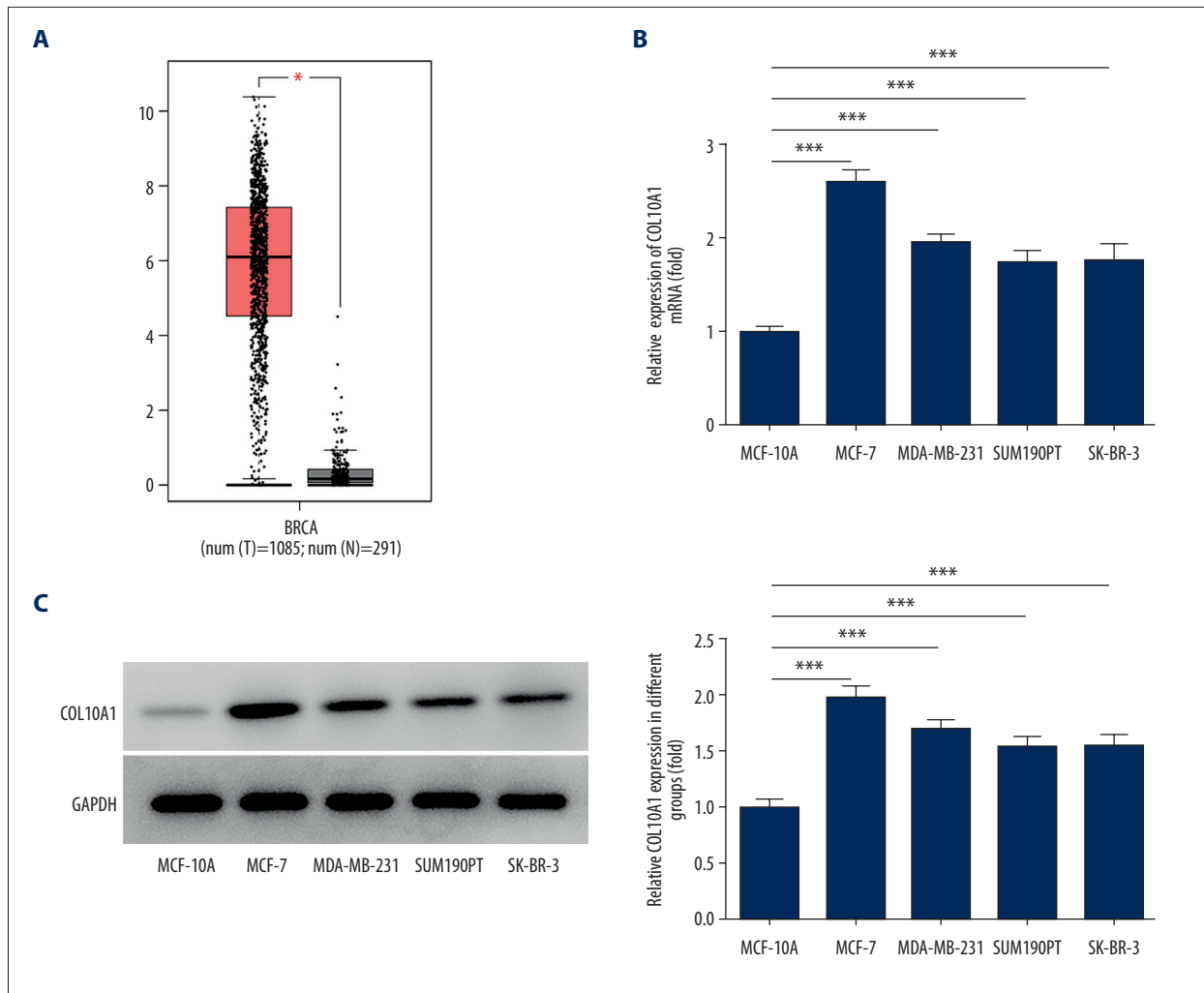


Figure 1. COL10A1 was greatly elevated in breast cancer. **(A)** COL10A1 expression in tumor tissues or normal tissues of breast cancer patients was analyzed based on GEPIA repository (<http://gepia.cancer-pku.cn/>). **(B)** RT-qPCR analysis of COL10A1 expression in human breast epithelial cells (MCF-10A) and breast cancer cell lines (MCF-7, MDA-MB-231, SUM190PT, SK-BR-3). **(C)** Western blot analysis of COL10A1 expression in human breast epithelial cells (MCF-10A) and breast cancer cell lines (MCF-7, MDA-MB-231, SUM190PT, SK-BR-3). *** $P < 0.001$.

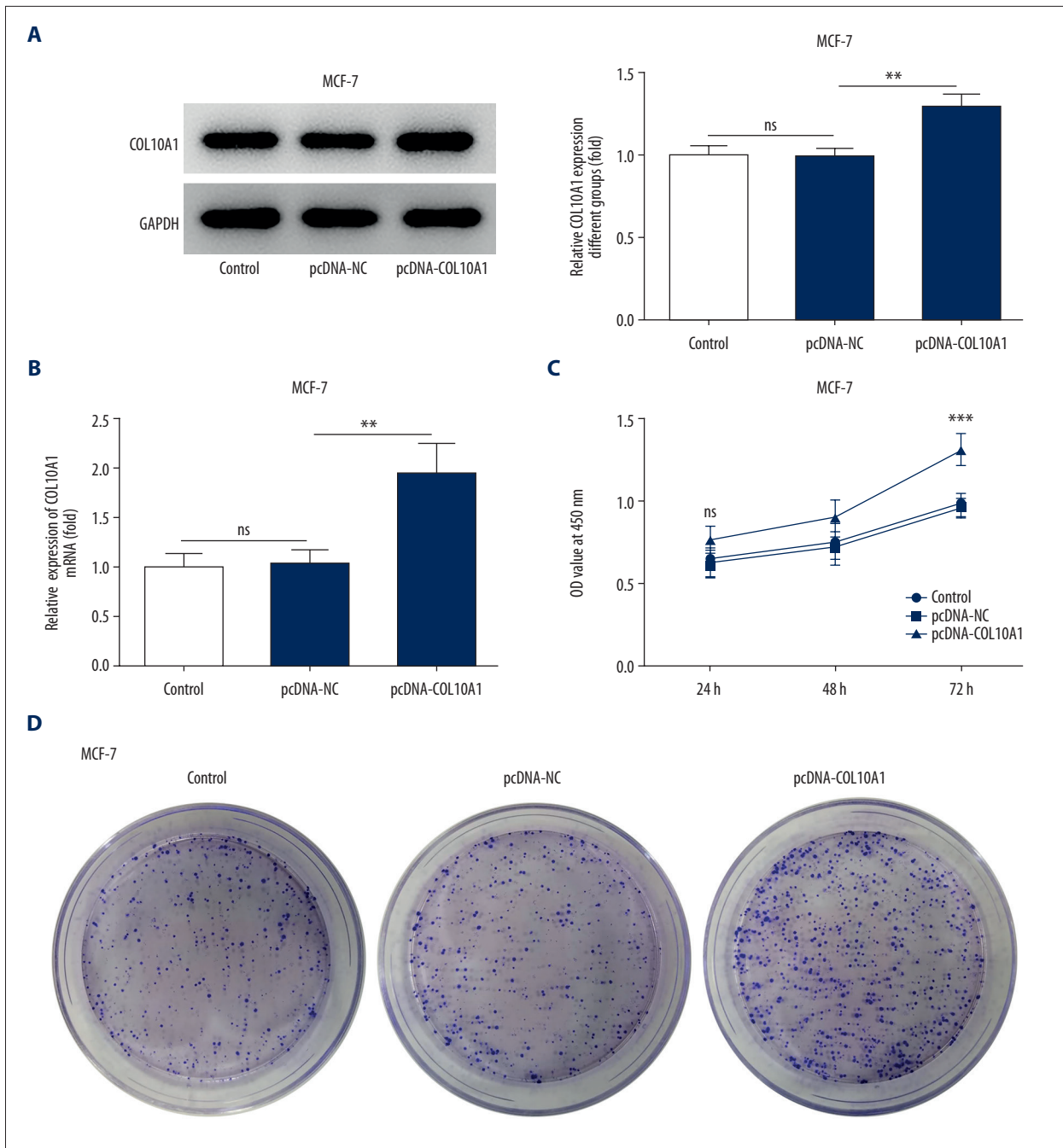
cultivated in DMEM containing 10% FBS. After culturing for 2 weeks at 37°C, the colonies were washed with PBS and fixed with 4% paraformaldehyde for 15 min. Then, the colonies were stained with 0.1% crystal violet (Sigma-Aldrich, MO, USA) for 20 min at room temperature and photographed under an inverted light microscope (Leica, Wetzlar, Germany).

Wound Healing Assay

When cells reached a confluency of 90%, a linear wound was created using a 200- μ l sterile pipette tip. The cells were washed with sterile PBS to remove the floating cells and then cultured in serum-free culture medium for 24 h. Cell migration images (100 \times) were captured under an inverted light microscope (Leica, Wetzlar, Germany).

Transwell Assay

The invasive ability of cells was determined by transwell assays. We coated 100 μ l of Matrigel (Solarbio, Beijing, China) on the upper surface of transwell chambers (Sigma-Aldrich, MO, USA). Then, cells in 200 μ l serum-free medium were seeded into the upper chamber at a density of 1×10^5 cells/well. A total of 500 μ l medium containing 20% serum medium was added into 24-well plate. Following 48-h incubation, the invaded cells were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. Then, the number of migrated cells (100 \times) was counted under an inverted light microscope (Leica, Wetzlar, Germany).



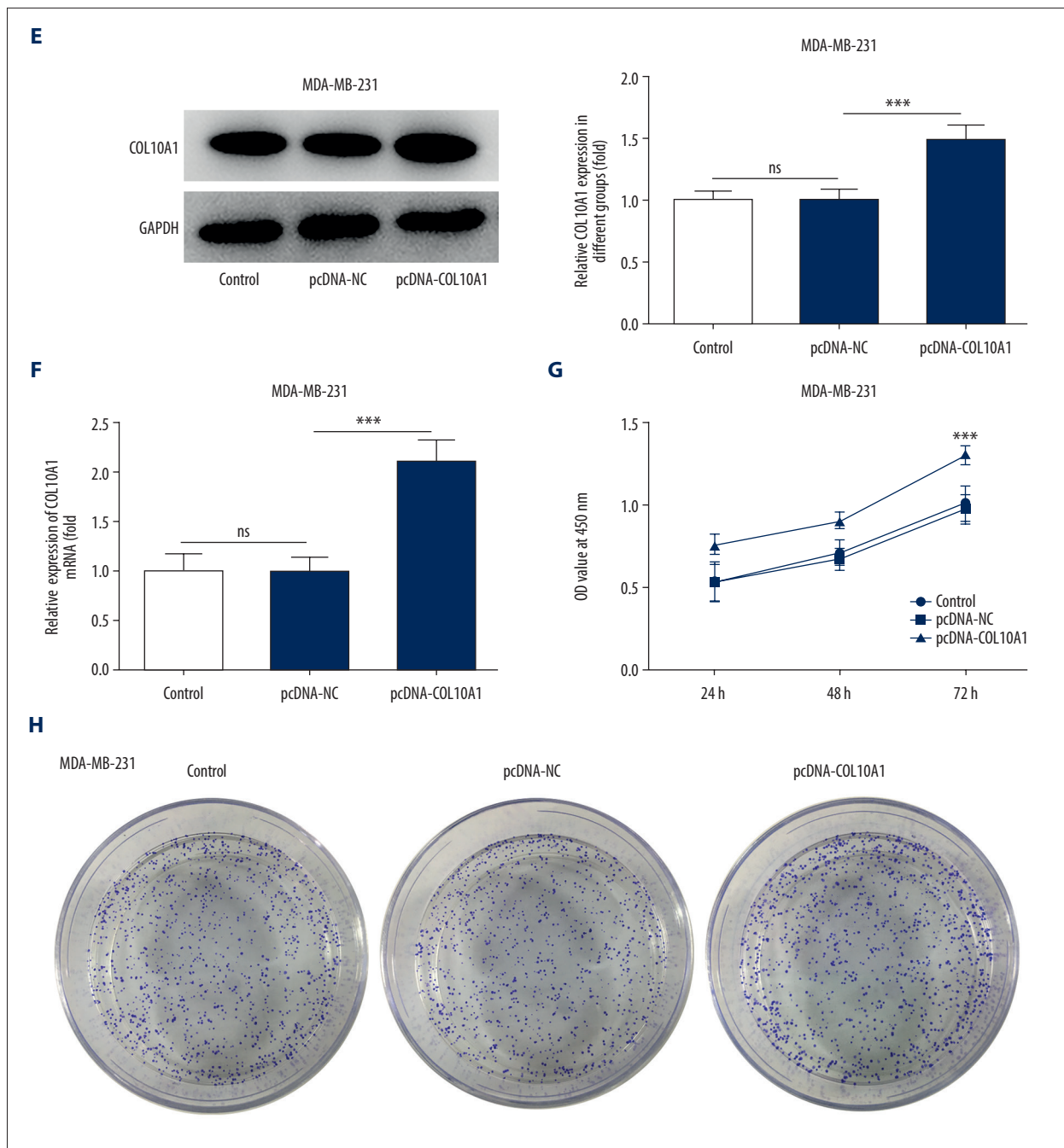
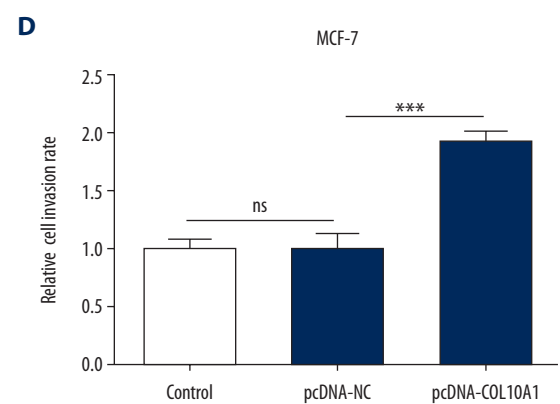
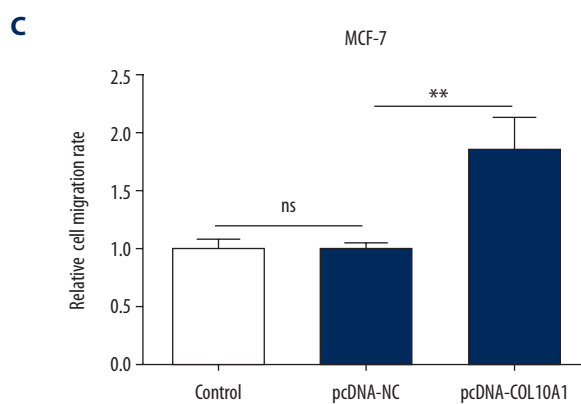
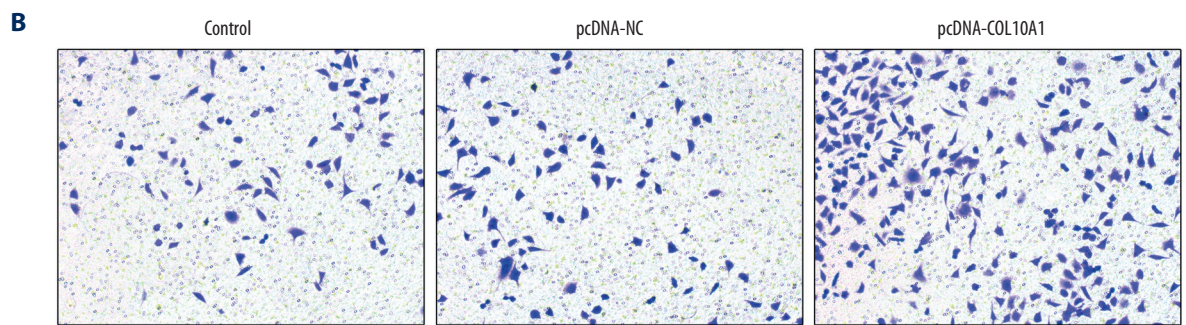
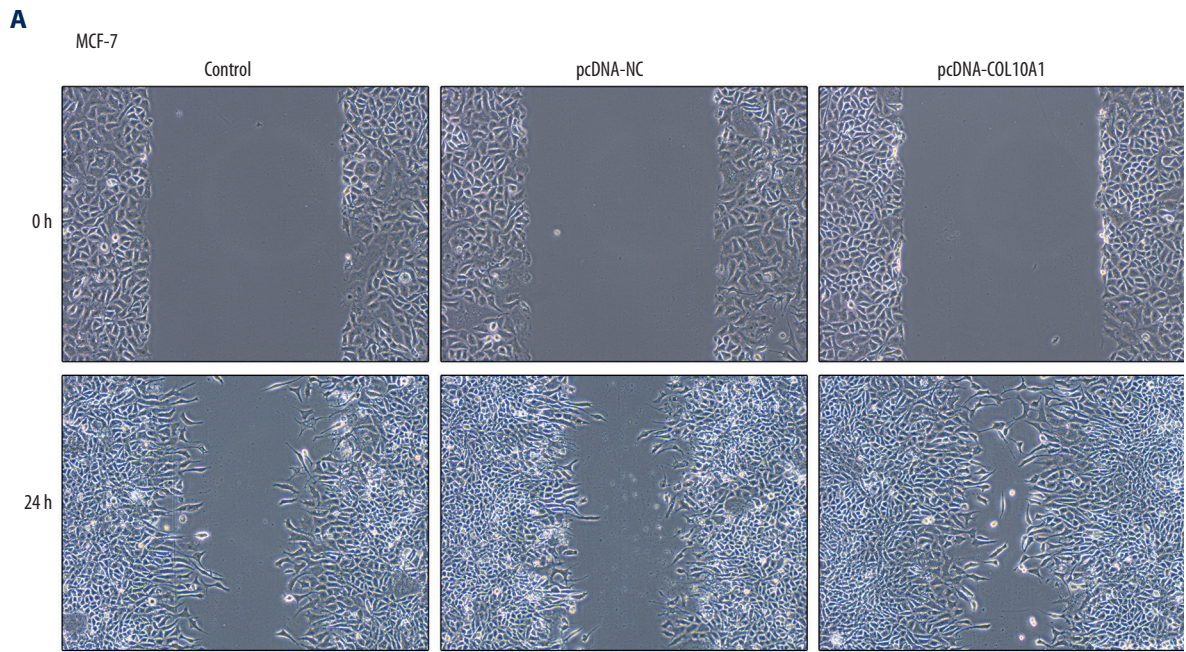


Figure 2. Upregulation of COL10A1 enhanced cell proliferation and clone-forming abilities of breast cancer cells. (A) Western blot analysis was performed to detect the transfection efficiency in MCF-7 cells which were transfected with pcDNA-COL10A1. (B) RT-qPCR analysis was performed to detect the transfection efficiency in MCF-7 cells which were transfected with pcDNA-COL10A1. (C) CCK-8 assay was applied to explore the effect of COL10A1 overexpression on cell viability of MCF-7. (D) Colony formation assay was applied to explore the effect of COL10A1 overexpression on clone-forming ability of MCF-7. (E) Western blot analysis was performed to detect the transfection efficiency in MDA-MB-231 cells which were transfected with pcDNA-COL10A1. (F) RT-qPCR analysis was performed to detect the transfection efficiency in MDA-MB-231 cells which were transfected with pcDNA-COL10A1. (G) CCK-8 assay was applied to explore the effect of COL10A1 overexpression on cell viability of MDA-MB-231. (H) Colony formation assay was applied to explore the effect of COL10A1 overexpression on clone-forming ability of MDA-MB-231. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



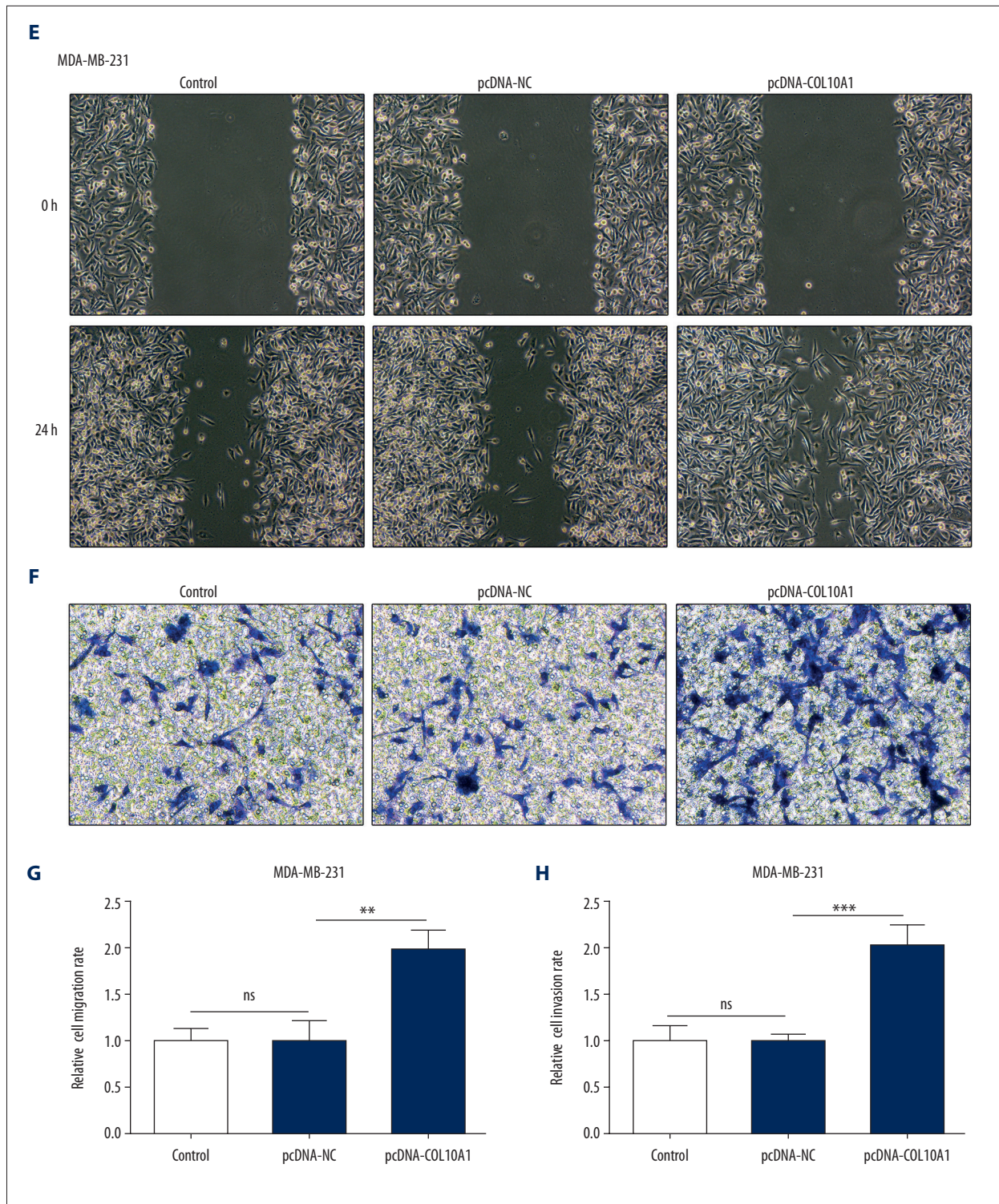
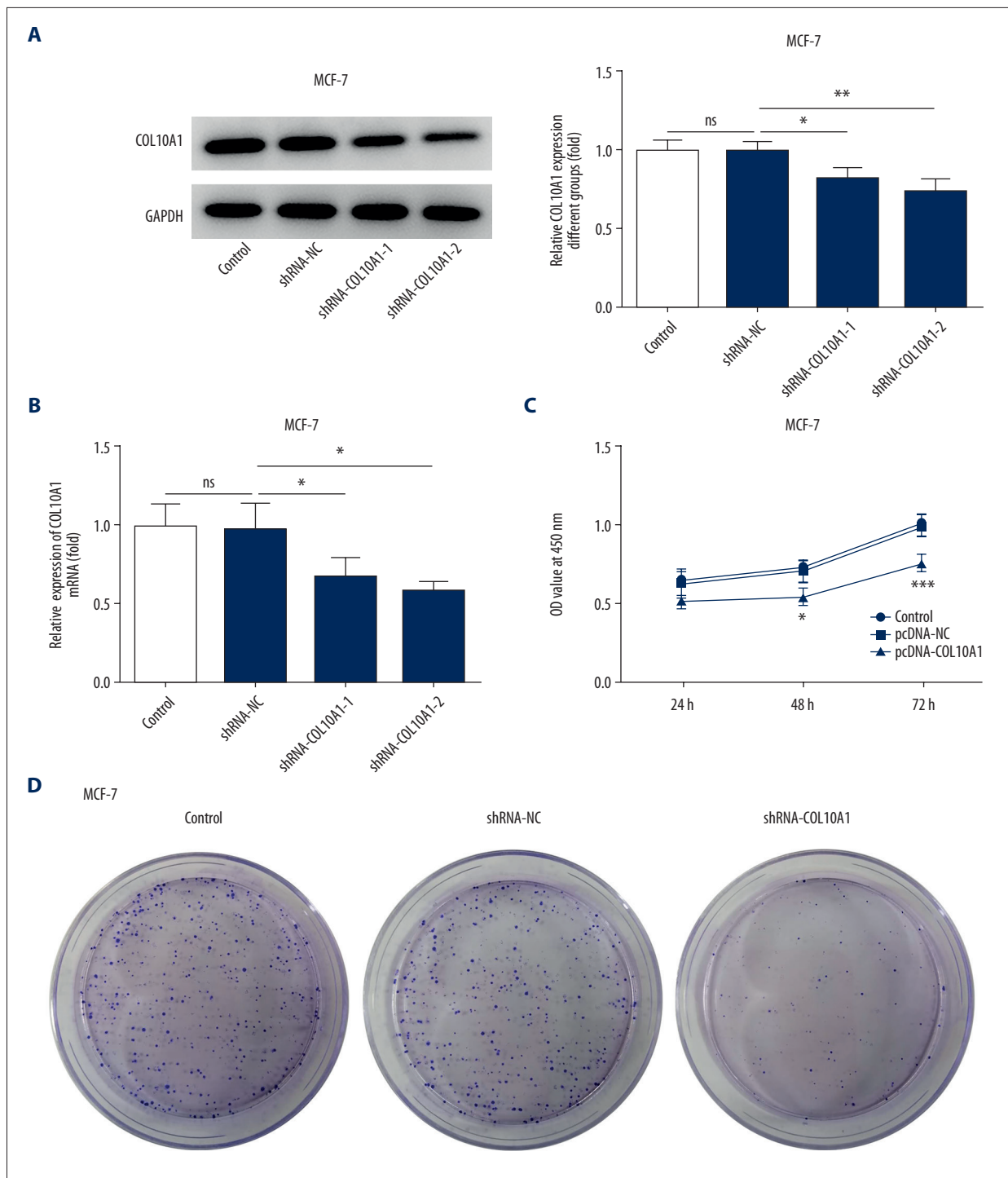


Figure 3. Upregulation of COL10A1 boosted the migration and invasion of breast cancer cells. **(A, C)** Wound healing assay was used to examine the effect of COL10A1 overexpression on MCF-7 cell migration (100×). **(B, D)** Transwell assay was used to examine the effect of COL10A1 overexpression on MCF-7 cell invasion (100×). **(E, G)** Wound healing assay was used to examine the effect of COL10A1 overexpression on MDA-MB-231 cell migration (100×). **(F, H)** Transwell assay was used to examine the effect of COL10A1 overexpression on MDA-MB-231 cell invasion (100×). ** $P < 0.01$, *** $P < 0.001$.



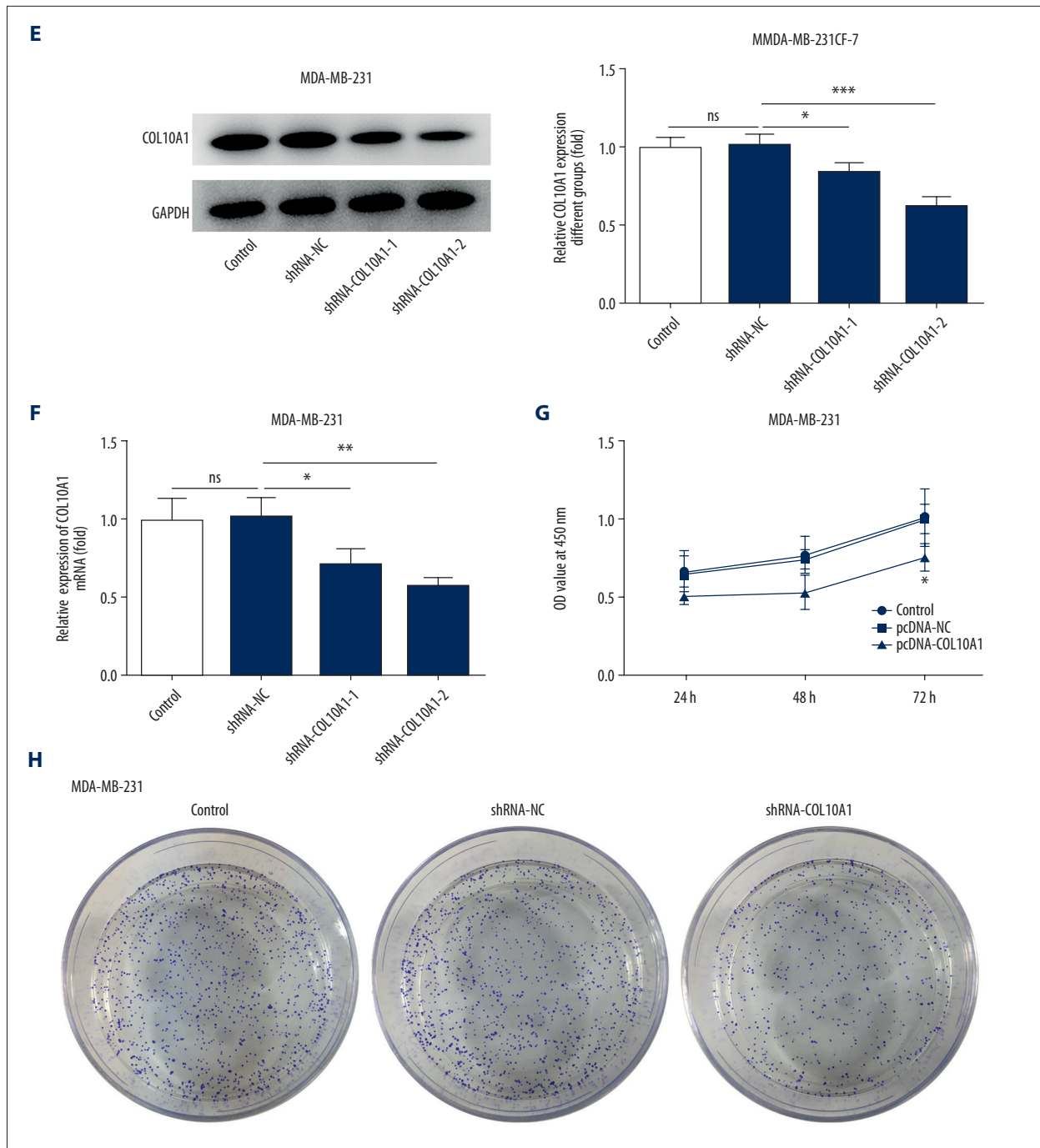
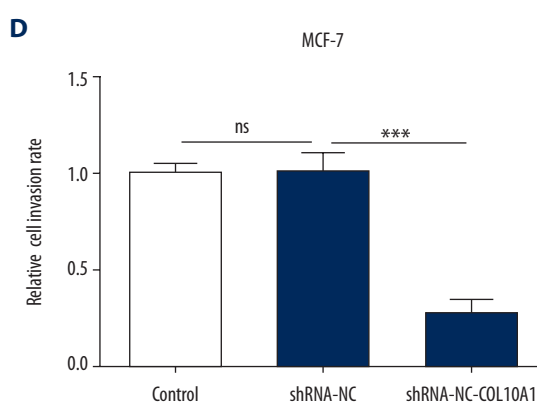
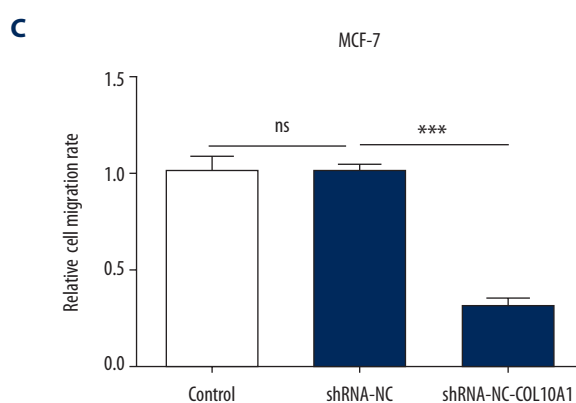
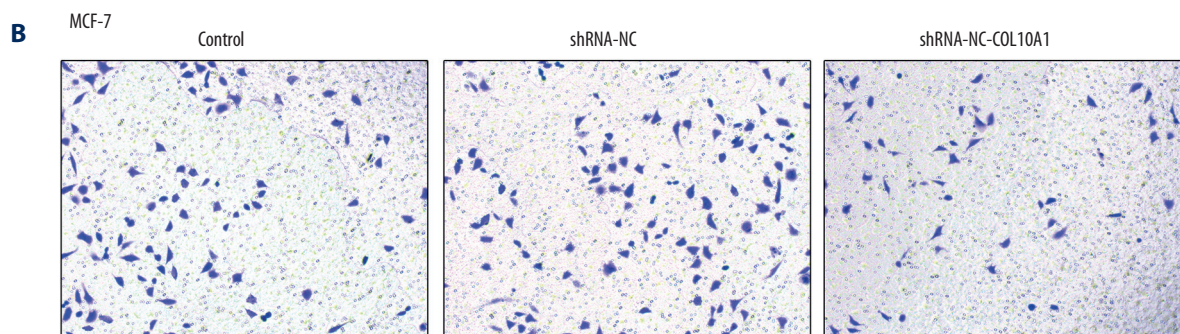
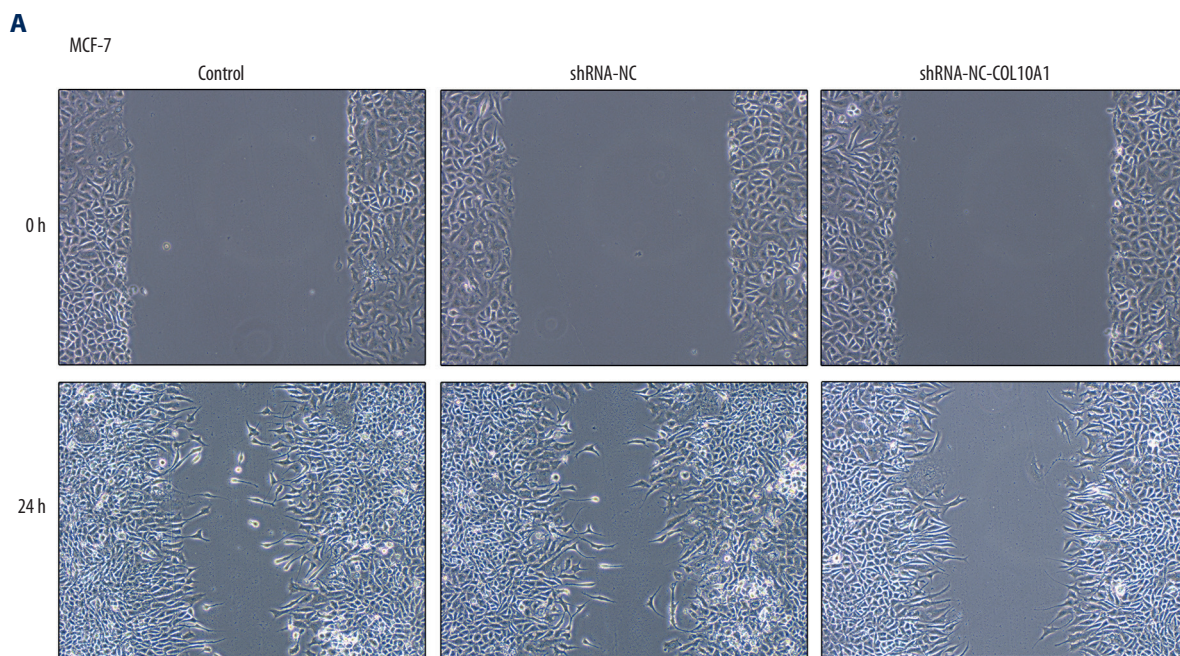


Figure 4. Downregulation of COL10A1 suppressed cell proliferation and clone-forming abilities of breast cancer cells. **(A)** Western blot analysis was performed to detect the transfection efficiency in MCF-7 cells which were transfected with shRNA-COL10A1-1 or shRNA-COL10A1-2. **(B)** RT-qPCR analysis was performed to detect the transfection efficiency in MCF-7 cells which were transfected with shRNA-COL10A1-1 or shRNA-COL10A1-2. **(C)** CCK-8 assay was applied to explore the effect of silenced COL10A1 on cell viability of MCF-7. **(D)** Colony formation assay was applied to explore the effect of silenced COL10A1 on clone-forming ability of MCF-7. **(E)** Western blot analysis was performed to detect the transfection efficiency in MDA-MB-231 cells which were transfected with shRNA-COL10A1-1 or shRNA-COL10A1-2. **(F)** RT-qPCR analysis was performed to detect the transfection efficiency in MDA-MB-231 cells which were transfected with shRNA-COL10A1-1 or shRNA-COL10A1-2. **(G)** CCK-8 assay was applied to explore the effect of silenced COL10A1 on cell viability of MDA-MB-231. **(H)** Colony formation assay was used to explore the effect of silenced COL10A1 on clone-forming ability of MDA-MB-231. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



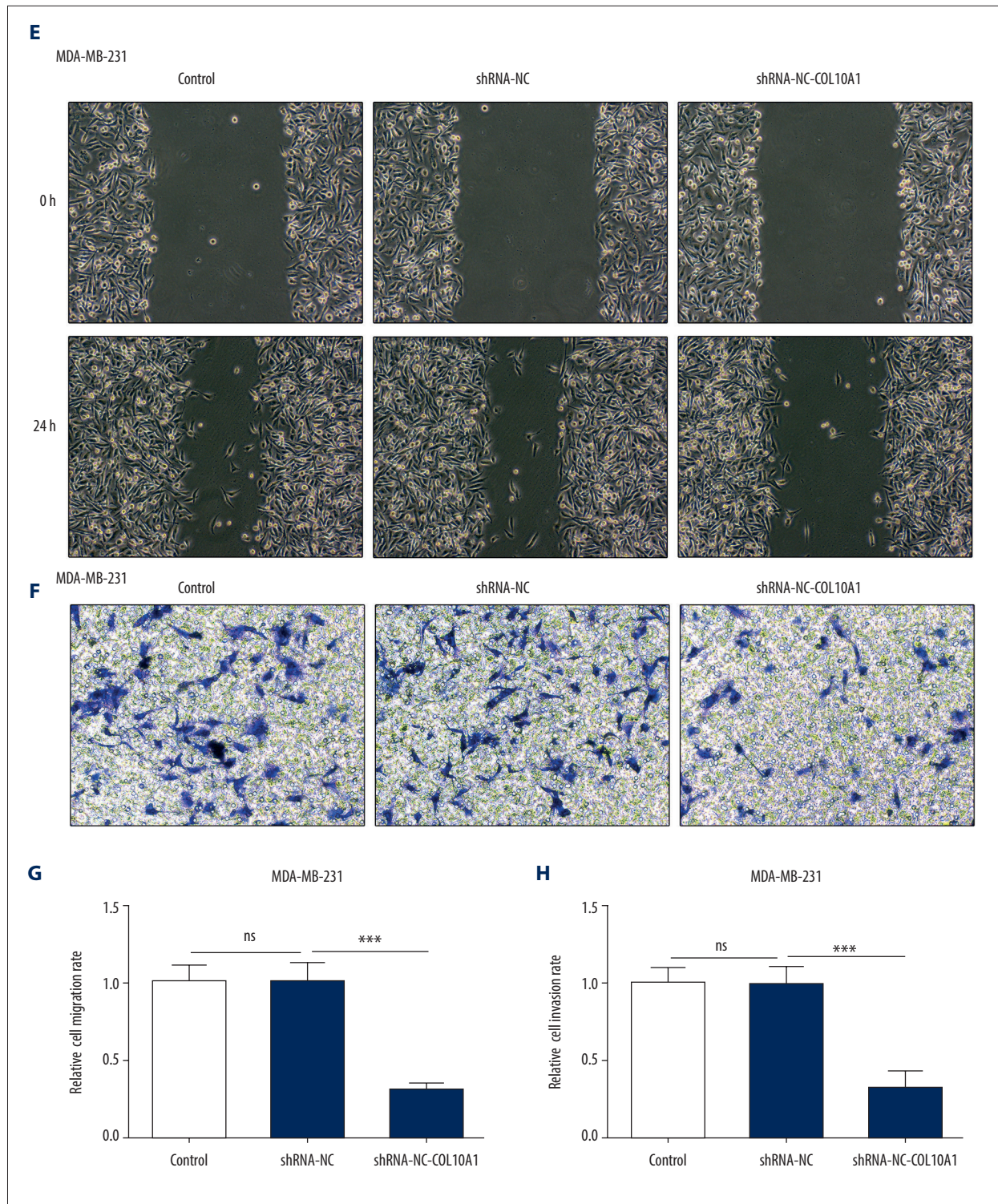


Figure 5. Downregulation of COL10A1 restrained the migration and invasion of breast cancer cells. (A, C) Wound healing assay was used to examine the effect of silenced COL10A1 on MCF-7 cell migration (100×). (B, D) Transwell assay was used to examine the effect of silenced COL10A1 on MCF-7 cell invasion (100×). (E, G) Wound healing assay was used to examine the effect of silenced COL10A1 on MDA-MB-231 cell migration (100×). (F, H) Transwell assay was used to examine the effect of silenced COL10A1 on MDA-MB-231 cell invasion (100×). *** $p < 0.001$.

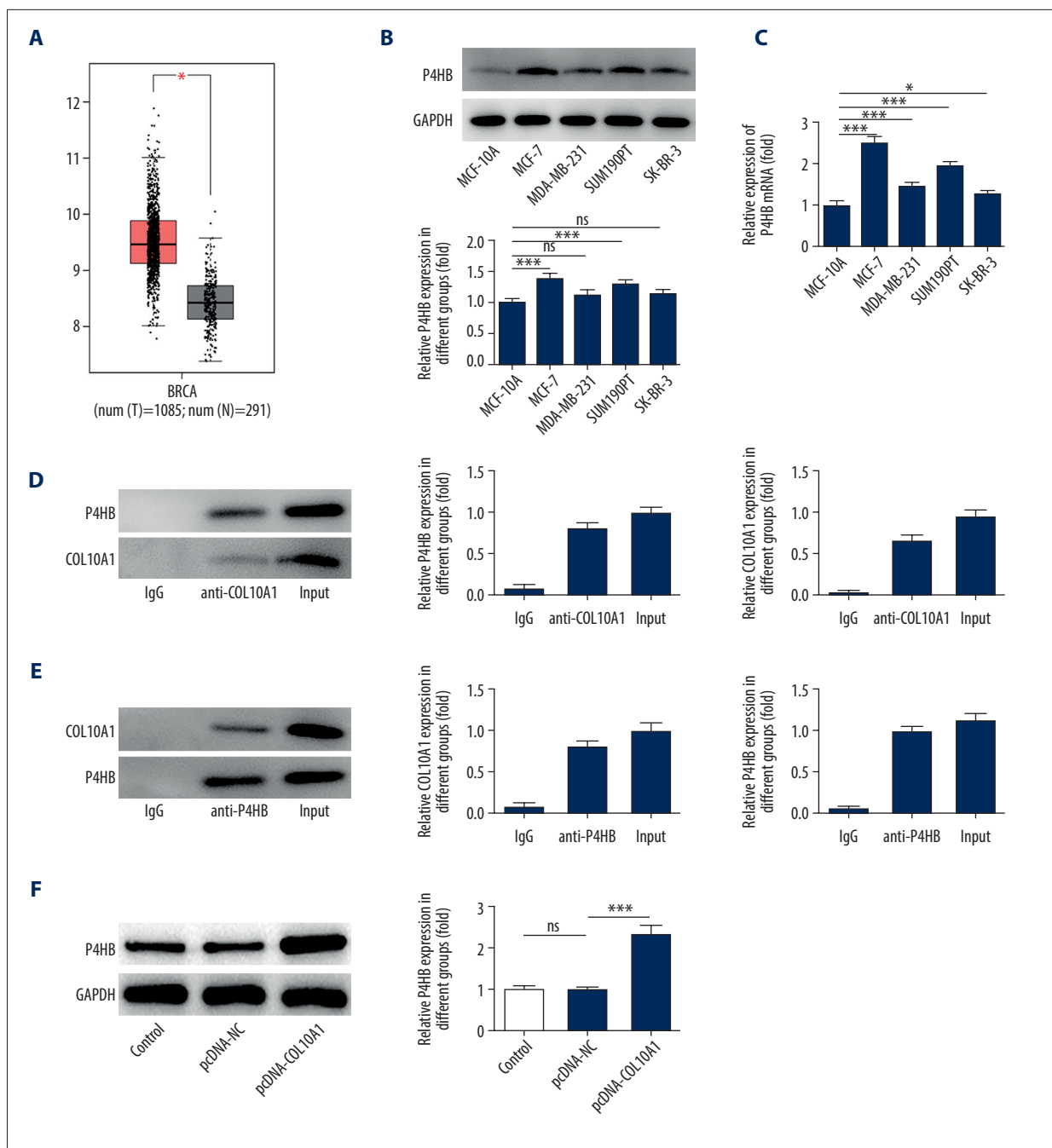
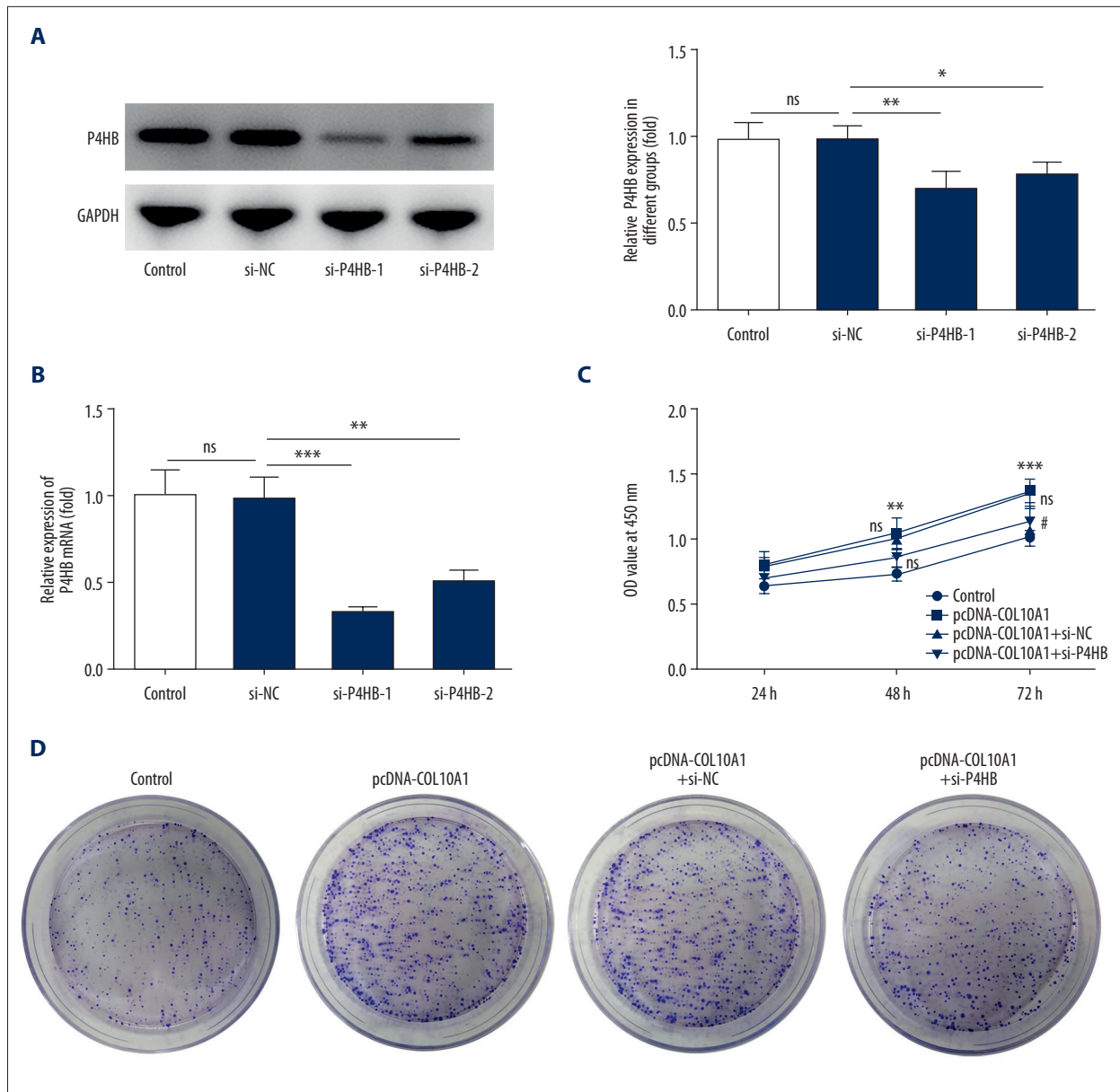


Figure 6. COL10A1 combined with P4HB. **(A)** P4HB expression in tumor tissues or normal tissues of breast cancer patients was analyzed based on GEPIA repository (<http://gepia.cancer-pku.cn/>). **(B)** Western blot analysis of P4HB expression in human breast epithelial cells (MCF-10A) and breast cancer cell lines (MCF-7, MDA-MB-231, SUM190PT, SK-BR-3). **(C)** RT-qPCR analysis of P4HB expression in human breast epithelial cells (MCF-10A) and breast cancer cell lines (MCF-7, MDA-MB-231, SUM190PT, SK-BR-3). **(D, E)** The relation between COL10A1 and P4HB was determined by Co-IP assay. **(F)** Western blot analysis of P4HB expression after transfection with pcDNA-COL10A1. * $P < 0.05$, *** $P < 0.001$.



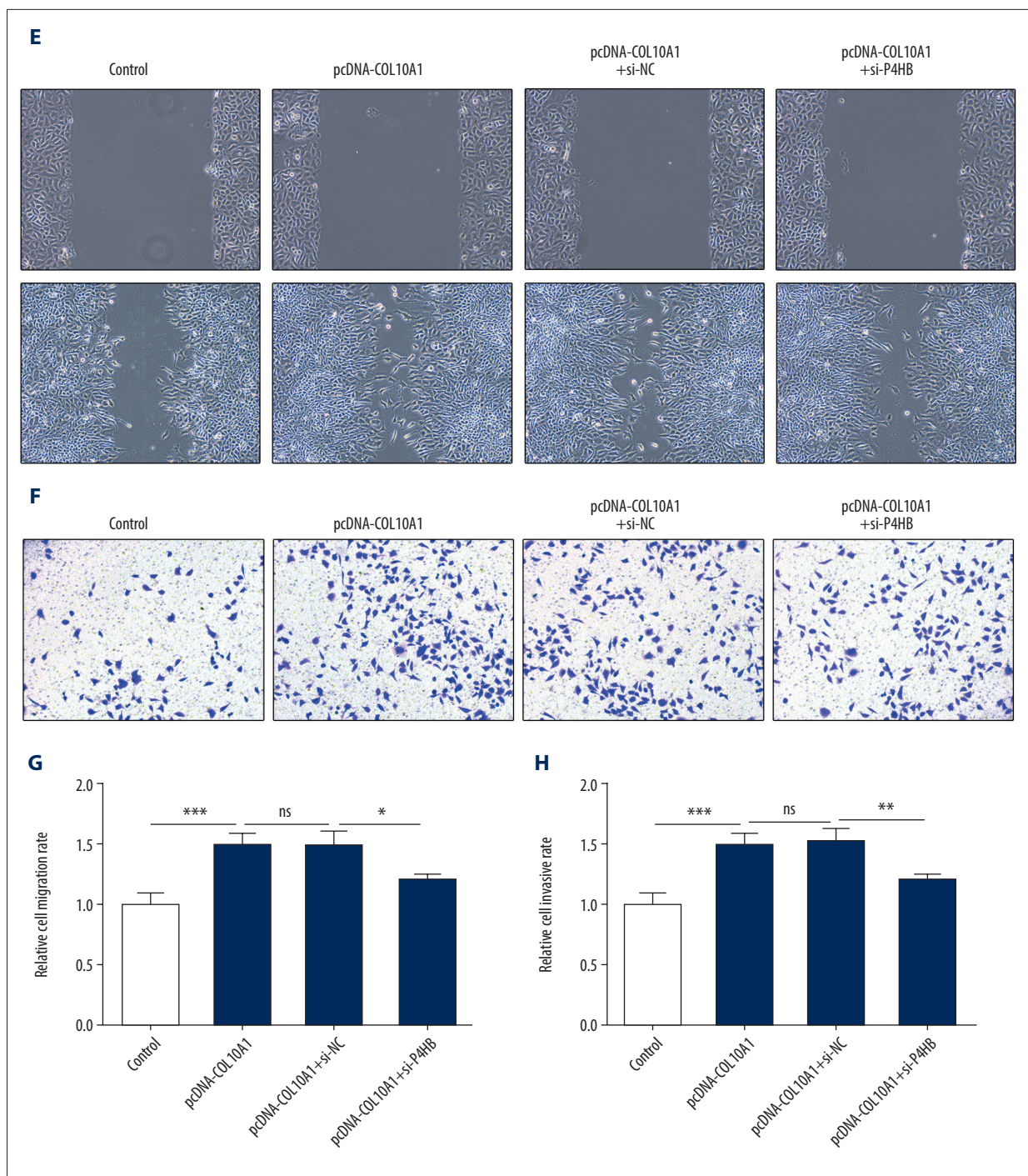


Figure 7. Silencing of P4HB disrupted the effect of COL10A1 overexpression in the progression of breast cancer. (A). Western blot analysis was performed to detect the transfection efficiency in MCF-7 cells transfected with si-P4HB-1 or si-P4HB-2. (B). RT-qPCR analysis was performed to detect the transfection efficiency in MCF-7 cells transfected with si-P4HB-1 or si-P4HB-2. (C). CCK-8 assay was performed to explore the effect of silenced P4HB on the enhanced cell viability induced by overexpressed COL10A1. (D). Colony formation assay was used to explore the effect of silenced P4HB on the elevated clone-forming ability induced by overexpressed COL10A1. (E, G). Wound healing assay was used to examine the effect of silenced P4HB on the enhanced cell migration ability induced by overexpressed COL10A1. (F, H). Transwell assay was used to examine the effect of silenced P4HB on the enhanced cell invasion ability induced by overexpressed COL10A1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Western Blotting Analysis

RIPA lysate (Beyotime, Shanghai, China) containing protease inhibitors was used to extract the total protein from the transfected cells. Then, total protein was quantified by the BCA Protein Assay kit (Beyotime, Shanghai, China) and separated by 8-10% SDS-polyacrylamide gels (PAGE), followed by transfer to PVDF membranes (Beyotime, Shanghai, China). After blocking with 5% non-fat milk for 1 h at room temperature, the membranes were incubated with primary antibodies against COL10A1 (SolarBio, K004843P, 1: 2000), P4HB (Abcam, ab137119, 1: 5000) and GAPDH (Abcam, ab9485, 1: 2500), diluted in blocking buffer at 4°C overnight. Subsequently, the membranes were washed with TBST and incubated with the HRP-conjugated secondary antibodies (Abcam, ab205718, 1: 20 000) at room temperature for 1.5 h. Finally, the protein bands were visualized by ECL chemiluminescence reagent (Beyotime, Shanghai, China). GAPDH was used as a loading control.

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA from breast cancer cells was extracted using the TRIzol reagents (Invitrogen, CA, USA) according to the manufacturer's instructions. Subsequently, the RNA was used to synthesize the first-strand cDNA by using a PrimeScript RT reagent kit (Takara, Tokyo, Japan). RT-qPCR reactions were performed using a SYBR Premix Ex Taq™ II kit (Takara, Dalian, China) on the ABI Prism 7500 sequence detection system (Applied Biosystems, CA, USA). GAPDH was used as a reference gene for mRNA expression. The relative gene levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Co-immunoprecipitation (Co-IP) Assay

To confirm the interaction between COL10A1 and P4HB, cellular proteins were extracted using lysis buffer. Then, cell lysates were incubated with anti-COL10A1, anti-P4HB, or control IgG overnight at 4°C and then cultivated with protein A/G agarose beads (Santa Cruz, CA, USA) for 2 h at 4°C. Immuno-complexes were washed 6 times in extraction buffer and analyzed by western blot.

Statistical Analyses

All statistical analysis was performed using statistical software SPSS 19.0 and experimental data are expressed as mean±standard deviation (SD). Differences between different groups were estimated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $P < 0.05$ was considered as statistically significant.

Results

COL10A1 was Greatly Elevated in Breast Cancer

COL10A1 is highly expressed in tumor tissues compared with normal tissues of breast cancer patients in GEPIA dataset (Figure 1A). Subsequently, RT-qPCR (Figure 1B) and western blot analysis (Figure 1C) were conducted to analyze COL10A1 expression in human breast epithelial cells (MCF-10A) and breast cancer cell lines (MCF-7, MDA-MB-231, SUM190PT, SK-BR-3). COL10A1 expression was dramatically elevated in breast cancer cell lines, especially in MCF-7 and MDA-MB-231 cells. Hence, we selected MCF-7 and MDA-MB-231 for the functional experiments.

Upregulation of COL10A1 Promoted Proliferation, Migration and Invasion of Breast Cancer Cells

To examine the biological functions of COL10A1 in breast cancer, COL10A1 was overexpressed in MCF-7 (Figure 2A, 2B) and MDA-MB-231 (Figure 2E, 2F) cells through transfection with pcDNA-COL10A1. CCK-8 assay and colony formation assay showed enhanced proliferation ability and clone-forming ability in MCF-7 (Figure 2C, 2D) and MDA-MB-231 (Figure 2G, 2H) cells after transfection with pcDNA-COL10A1. Additionally, wound healing assay showed that COL10A1 overexpression could significantly promote MCF-7 (Figure 3A, 3C) and MDA-MB-231 (Figure 3E, 3G) cell migration. Moreover, results from transwell assay supported that upregulation of COL10A1 greatly elevated MCF-7 (Figure 3B, 3D) and MDA-MB-231 (Figure 3F, 3H) cell invasion capability in breast cancer.

Downregulation of COL10A1 suppressed proliferation, migration, and invasion of Breast Cancer Cells

shRNA-COL10A1-1 or shRNA-COL10A1-2 transfection led to the downregulation of COL10A1, and shRNA-COL10A1-2 had stronger transfection efficiency in MCF-7 (Figure 4A, 4B) and MDA-MB-231 (Figure 4E, 4F) cells. Hence, shRNA-COL10A1-2 was chosen for the subsequent experiments. Downregulation of COL10A1 inhibited proliferation and clone-forming ability of MCF-7 (Figure 4C, 4D) and MDA-MB-231 (Figure 4G, 4H) cells. Meanwhile, COL10A1 knockdown arrested the migration of MCF-7 (Figure 5A, 5C) and MDA-MB-231 (Figure 5E, 5G) cells. Silenced COL10A1 decreased the invasion abilities of MCF-7 (Figure 5B, 5D) and MDA-MB-231 (Figure 5F, 5H) cells.

COL10A1 Combined with P4HB

P4HB levels in tumor tissues and normal tissues of breast cancer patients were obtained from the GEPIA repository. In comparison with normal tissues, P4HB expression in tumor tissues of breast cancer was greatly elevated (Figure 6A). Moreover,

P4HB was highly expressed in breast cancer cell lines than that in human breast epithelial cells (Figure 6B, 6C). The interaction between COL10A1 and P4HB was further verified by performing Co-IP assay. P4HB protein existed in the anti-COL10A1 group and COL10A1 protein existed in the anti-P4HB group (Figure 6D, 6E). Therefore, we concluded that COL10A1 could combine with P4HB. In addition, it was observed that COL10A1 overexpression distinctly enhanced P4HB levels and COL10A1 exerted a positive moderating effect on P4HB expression (Figure 6F).

Silenced P4HB Disrupted the Function of COL10A1 Overexpression in the Progression of Breast Cancer

To explore the potential role of P4HB in breast cancer, P4HB was silenced in MCF-7 cells through transfection with si-P4HB-1 and si-P4HB-2 (Figure 7A, 7B). Due to the stronger transfection efficiency, si-P4HB-1 was applied to silence P4HB expression in the following experiments. COL10A1 was confirmed to obviously enhance cell proliferation, migration, and invasion in breast cancer. As we expected, the enhanced proliferation ability and clone-forming ability caused by COL10A1 overexpression was significantly reversed by silencing of P4HB (Figure 7C, 7D). In addition, the promoting effects of overexpressed COL10A1 on cell migration (Figure 7E, 7G) and invasion capabilities (Figure 7F, 7H) in breast cancer were dramatically abolished following P4HB silencing.

Discussion

Breast cancer is a malignant tumor that occurs in the epithelial tissue of the breast gland [1]. The global incidence of breast cancer has increased year by year and the average age at diagnosis has been getting younger [16]. It is likely to recur and metastasize, with poor prognosis [17]. Although therapies for breast cancer have been greatly improved, there are limitations for advanced and triple-negative breast cancers [18].

In recent years, much progress has been achieved in research of a variety of small-molecule compound drugs targeting breast cancer [19]. Novel targeted drugs have strong specificity and low toxic adverse effects, thus playing an important role in the therapeutics of breast cancer [20]. Therefore, the development and clinical application of breast cancer targeted therapy have become an important focus in breast cancer research.

There is sufficient evidence to support the role of COL10A1 in the diagnosis and prognosis of breast cancer, but more basic experiments on its functionary mechanism are still urgently needed for further research and demonstration [10]. High COL10A1 expression is significantly correlated with worse OS, RFS, DFS, and DMFS [10]. COL10A1 is highly expressed in tumor tissues of breast cancer patients, according to the GEPIA repository (<http://gepia.cancer-pku.cn/>). In the present study, we found that COL10A1 expression in breast cancer cell lines was higher than that in human breast epithelial cells. Li et al reported that COL10A1 could promote the metastasis of gastric cancer cells, and COL10A1 was confirmed to be a potential inducer of epithelial-mesenchymal transition (EMT) [21]. Interestingly, we demonstrated that COL10A1 overexpression could facilitate the proliferation, migration, and invasion of breast cancer cells. These results suggest that COL10A1 may be an oncogene for breast cancer.

It is documented that P4HB is an oncogene closely related to the occurrence and progression of several tumor types [13,15]. Xia et al report that P4HB could promote cell migration, invasion, and EMT in hepatocellular carcinoma [22]. Besides, P4HB knockdown reduces the migration and invasion abilities of liver cancer cells by suppressing EMT and β -catenin/Snail pathway [14]. However, the role of P4HB in breast cancer growth and metastasis remains to be elucidated. It was observed that P4HB was highly expressed in breast cancer cell lines. We demonstrated that downregulation of P4HB repressed the promoting effects of overexpressed COL10A1 on the proliferation, migration, and invasion of breast cancer cells. In addition, we clarified the regulatory mechanism of COL10A1 on P4HB. Upregulation of COL10A1 enhanced breast cancer cell proliferation and clone-forming abilities and boosted breast cancer cell migration and invasion by positively modulating P4HB expression.

Conclusions

In summary, we identified a critical role of COL10A1 in the progression of breast cancer for the first time. COL10A1 overexpression facilitated breast cancer growth and metastasis through upregulating P4HB expression. These novel findings may provide a novel target for breast cancer therapy.

Conflict of Interest

None.

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