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Original Article

PNMA5 accelerated cellular proliferation, invasion and migration in colorectal cancer

Jie Lin[1*], Xiaokang Zhang[1,2*], Fan Meng[1*], Fanlin Zeng[1], Weiyou Liu[1,2], Xin He[1,2]

[1]The First Affiliated Hospital of Gannan Medical University, Ganzhou 341000, Jiangxi, PR China; [2]Jiangxi Provincial Branch of China Clinical Medical Research Center for Geriatric Diseases, Gannan Medical University, Ganzhou 341000, Jiangxi, PR China. [*]Equal contributors.

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Abstract: Objectives: Paraneoplastic antigen Ma family (PNMA) is dysregulated in the pathological development of various cancers. However, the actions of PNMA member 5 (PNMA5) in cancers are still unknown. The aim of this study was to explore the biological actions of PNMA5 and its implication in epithelial-mesenchymal transition (EMT) during the progression of colorectal cancer (CRC). Methods: Immunohistochemical staining, western blot and qPCR were used to explore PNMA5 expression in colorectal cancer tissues and cells. In addition, western blot, MTT assays, Colony formation assay, wound-healing, and transwell cell invasion assays were used to investigate the effects of PNMA5 on EMT in colorectal cancer. The lung metastasis models and xenografts in nude mice were established to explore the roles of PNMA5 *in vivo*. Results: It was found that the expression level of PNMA5 in colorectal cancer tissues was significantly up regulated compared to that in the adjacent tissues. The overall survival rates of patients with a higher PNMA5 expression were markedly decreased. In addition, knockdown of PNMA5 expression decreased the proliferation, invasion and migration of both HCT-15 and HCT-116 cells. PNMA5 expression was found to be positively associated with the expression of C-myc, CyclinD1, Ki67, N-cadherin, zinc finger E-box binding homeobox 1 and vimentin, and negatively associated with E-cadherin. It was also found that PNMA5 knockdown attenuated TGF-β-induced EMT in colorectal cancer cells. Finally, it was demonstrated that PNMA5 accelerated colorectal cancer cell proliferation, invasion and migration *in vivo*. Conclusion: The results revealed that PNMA5 increased cellular proliferation, invasion and migration in colorectal cancer. PNMA5 plays a key role in promoting CRC carcinogenesis and progression for patients with CRC.

Keywords: PNMA5, colorectal cancer, EMT, proliferation

Introduction

Colorectal cancer (CRC) is a deadly cancer [1]. By histological classification, CRC can be classified into two groups, adenocarcinoma and adenosquamous carcinoma, of which adenocarcinoma is the main one. The factors contribute to CRC development is unclear. Factors, such as genetics, dietary, and intestinal flora imbalance, might be involved [2]. Aberrant genes expression contributes to the carcinogenesis and progression of CRC. These genes provide potential targets for the therapeutic strategy during CRC treatment. However, a high mortality is still observed in the advanced stage of CRC in patients. A potential prognostic biomarker for developing the therapeutic strategy for treating CRC needs to be explored.

PNMA consists of more than 15 family members. PNMA1-3 is implicated in the development of the Paraneoplastic Disorder [3]. However, PNMAs exhibit functional divergence. Specifically, different PNMAs may be agonists or antagonists in apoptosis signaling in cancer cells [4]. The localization pattern of PNMA5 in the primate brain is different from that of other PNMAs [5]. Reports on the functions and mechanisms of PNMA5 are limited. Decreased PNMA5 expression in glioblastoma has been reported [6]. PNMA5 exhibits a role in meiosis, and it is involved in Src/Erk1/2/Pnma5/Akt/Gsk3β pathway [7]. PNMA5 can be a potential candidate, which is the target for therapeutic management of NSCLC patients with bone metastases at the advanced stage [8]. PNMA5 was found to be highly correlated with the prog-

nosis of colorectal cancer [9]. PNMA5 could promote apoptosis and chemo-sensitivity, both N- and C-terminal domains of PNMA5 appear to be required for pro-apoptotic function [10]. However, the mechanisms of PNMA5 in regulating CRC proliferation, invasion and migration remain unknown. Here, we mainly focused on the activity of PNMA5 in human CRC.

Materials and methods

Clinical specimen collection

Tissue microarray and immunohistochemistry. A piece of CRC tissue microarray was prepared for investigating PNMA5 expression. The tissue microarray (Microarray Number: HcolAde180Sur-04) was obtained from Outdo Biotech (Shanghai, China) and included 90 pairs of CRC and its para-carcinoma tissues. The pathological diagnosis for CRC samples from patients were confirmed at the early stage (Stages IA-IIIA). All patients' follow-up records were collected from January 2006 to July 2015.

Immunohistochemical staining

Immunohistochemistry was conducted according to a previously published protocol [11]. Simply, after being dewaxed and hydrated, the tissue chip was antigenretrieved in a citrate buffer (consisting of 10 mM citric acid at a pH value of 6.0) during the microwaving processes. 5% animal serum was added to block, and then PNMA5 antibody (dilution, 1:200) was coincubated overnight at 4°C. DAB and hematoxylin were prepared for staining. The sections were evaluated using a microscope (Zeis, Oberkochen, Germany).

Two senior pathologists were blindly assigned to evaluate the immunoreactivity independently. The immunostaining examination was evaluated and scored. The grade was set as 0/no reactivity in immunostaining, 1/weak reactivity in immunostaining, 2/moderate reactivity in immunostaining, and 3/strong reactivity in immunostaining. The immunoreactive cells were accounted. Similarly, it was scored and graded as 0 for none, 1 for <20%, 2 for 20-50%, 3 for 51-75%, and 4 for >75%. Finally, the cut-off value was proposed for determination of the low and the high expression using the extent grades × intensity staining grades. The low expression (score value <6) and the high expression (score value ≥6) were defined.

Cell lines and cell culture

NCM460 and human CRC cell lines (HCT-15 and HCT-116) were provided from the Research Center of Clinical Medicine (RCCM) at Zhujiang Hospital (Guangzhou, China). NCM460 cells were cultured in keratinocyte serum-free medium (Gibco-Invitrogen, Carlsbad, CA, USA), and CRC cells were cultured in RPMI-1640 medium (Gibco).

Lentivirus vectors and transfection

CRC cells were performed for transient transfection with the lentivirus vectors carrying two pairs of short hairpin RNA (si-PNMA5-1 and si-PNMA5-2) (Ribobio, Guangzhou, China). After 48 h transfection, RNA and proteins in CRC cells were extracted. A PNMA5-overexpressed Lentivirus vector and an empty vector were prepared from Synbio Technologies (Suzhou, China). The experimental procedures were conducted according to the instructions from the manufacturer.

Quantitative real-time PCR (qRT-PCR)

Trizol (Invitrogen) was available for extracting total RNA. The qRT-PCR protocol was performed in the SsoFast EvaGreen Supermix (BioRad Laboratories, Hercules, CA, USA). GAPDH acted as an internal reference. The sequences for different primers were listed as follows: PNMA5, 5'-AGATGAGGGCCGAAGTATGAC-3' (forward) and 5'-GCTCTAAAGGTGGGGATCT AACT-3' (reverse); GAPDH, 5'-GTCAACGGATTTGGTCGTATTG-3' (forward) and 5'-CTCCTGGAAGATGGTGATGGG-3' (reverse). The main procedures of qRT-PCR included 50 cycles. The data were analyzed by the 2-ΔΔCt method.

Western blotting

RIPA (Biyotime, Shanghai, China) was employed for the extraction of the proteins from the cell lysate of CRC cells, and the BCA Kit (Biyotime) was introduced to measure the protein concentration. Samples were implicated in SDS-PAGE separation, transferred onto PVDF membranes, and probed with the antibodies. The primary antibodies were listed as follows: PNMA5 antibody (1:1000) (Abcam, Cambridge, MA), C-myc antibody (1:1000) (Abcam, Cambridge, MA), CyclinD1 antibody (1:1000) (Ab-

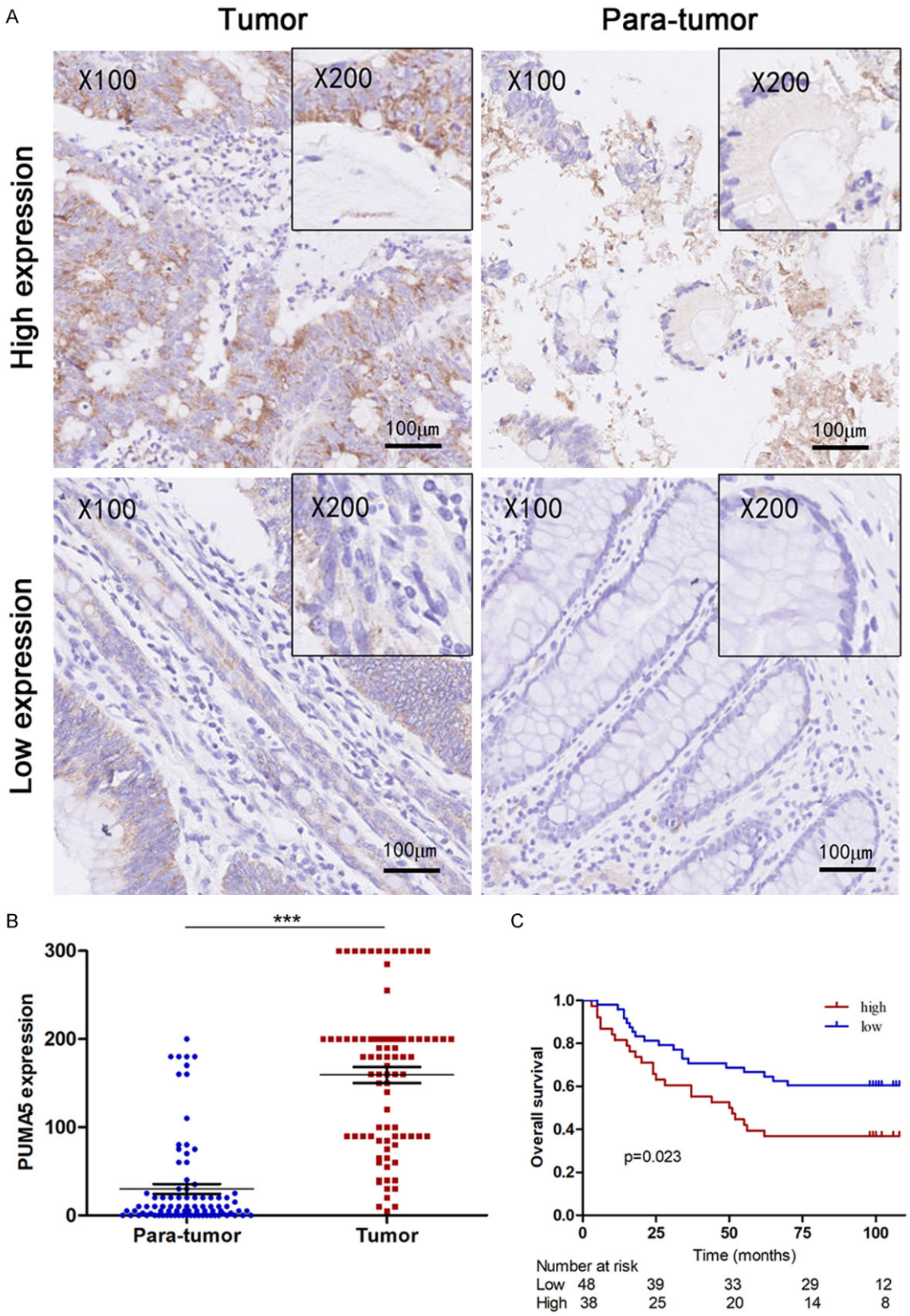

Figure 1. PNMA5 expression in CRC tissues was detected. A, B. PNMA5 protein expression was detected by immunohistochemical examination (× 100, × 200) in tumor and adjacent non-tumor tissues. C. Kaplan-Meier analysis of overall survival was done for the two groups, *P<0.05; **P<0.01; ***P<0.001; compared with the para-tumor.

Table 1. Differential expression of PNMA5 in cancer and adjacent tissues

	n	PNMA5 expression		Chi-square Value	*p* value
		High	Low		
cancer	86	38	48	45.874	<0.0001***
Adjacent tissues	87	1	86		

***P<0.001.

Table 2. Correlation between PNMA5 expression and clinicopathological characteristics

	variables	PNMA5 expression		total	χ^2	*p* value
		low	high			
Age (year)					2.557	0.110
	≤65	22	11	33		
	>65	26	27	53		
Sex					0.148	0.701
	Female	22	19	41		
	male	26	19	45		
Grade					1.786	0.181
	II	32	23	55		
	III	11	15	26		
T stage					1.948	0.163
	T1/T2	7	2	9		
	T3/T4	40	35	75		
N stage					0.699	0.403
	N0	32	22	54		
	N1/N2	16	16	32		
M stage					0.023	0.879
	M0	46	37	83		
	M1	1	1	2		
TNM stage					0.743	0.389
	I/II	31	21	52		
	III/IV	16	16	32		
Tumor size					4.166	0.041*
	≤4.5 cm	23	10	33		
	>4.5 cm	24	27	51		

*P<0.05.

cam), Ki67 antibody (1:1000) (Abcam), ZEB1 antibody (1:1000) (Abcam), E-cadherin antibody (1:1000) (Abcam), N-cadherin antibody (1:1000) (Abcam), vimentin antibody (1:1000) (Cell Signaling, Danvers, MA), and GAPDH antibody (1:1000) (Cell Signaling).

Cell proliferation assays

CRC cells were transiently transfected with lentivirus vectors (si-PNMA5-1, si-PNMA5-2, and NC, respectively), were grown for 24 h. According to the protocol from the manufacturer, 20 μl MTT solution (5 mg/mL) (BOSTER, Wuhan, China) was added and then incubated at 37°C for 4 h. The wavelength of 570 nm was set for detection of the absorbance.

Colony formation assays

CRC cells were transfected with lentivirus vectors (si-PNMA5-1, si-PNMA5-2, and NC, respectively). After 14 days culture, 75% ethanol was used to fix cells and crystal violet staining was employed. The number of clones/well was counted, and images were taken.

Wound-healing assays

CRC cells (about 3 × 10^5 cells/well) transfected with lentivirus vectors (si-PNMA5-1, si-PNMA5-2, and NC), respectively, were cultured. When cell culture reached approximately 90% confluence, a scratch was performed. Finally, cells were cultured in 2% culture medium. At the time point of 0 h and 24 h, the scratch width was analyzed.

Transwell cell invasion assays

5 × 10^4 cells/100 μl suspension in RPMI-1640 supplemented with 5% FBS were cultured in the upper chamber, which was pre-coated with Matrigel. In contrast, medium supplemented with 20% FBS was plated in the lower chamber. Cells across the membrane into the lower chamber within 48 h were analyzed using a microscope.

In vivo experiments

Twenty-five male BALB/c nude mice (20±2 g) were aquired from the Medical Laboratory Animal Center (MLAC) of Guangdong Province. In each group, five mice were randomly selected. Tumor growth was evaluated by detecting the volumes of the xenografts once a week. Specifically, the formula for calculating the volumes was defined: (volume) =1/2 × (long axis) × (short axis) 2. The tumors were surgically dissected at the 28th day after injection. Initally, the mice were injected intravenously with 3.0 × 10^6 PNMA5 overexpression cells. After one month, the lung tissues were collect-

Table 3. Summary of univariate and multivariate Cox regression analyses of overall survival

variables	Univariate analysis				Multivariate analysis			
	p value	HR	95% CI		*p value*	HR	95% CI	
			Lower limit	Upper limit			Lower limit	Upper limit
expression	0.026*	1.982	1.084	3.624	0.251	1.455	0.767	2.76
Sex	0.398	1.299	0.708	2.382				
Age	0.038*	2.07	1.042	4.11	0.140	1.723	0.836	3.554
Tumor size	0.027*	2.085	1.085	4.007	0.046*	2.08	1.012	4.274
Grade stage	0.008**	2.325	1.252	4.319	0.118	1.677	0.877	3.206
TNM stage	0.001***	2.773	1.515	5.078	0.007**	2.48	1.278	4.815
T stage	0.358	1.735	0.536	5.61	0.821	1.183	0.275	5.084
N stage	0.001***	2.902	1.585	5.314	NA	NA	NA	NA

*P<0.05; **P<0.01; ***P<0.001.

ed for metastatic nodules counting and further fixed in 10% formalin for histopathological analysis. All animal experiments were performed in accordance with the principles and procedures outlined in the Gannan Medical University Guide for the Care. Approval was obtained from the First Affiliated Hospital of Gannan Medical University Animal Ethics Committee (approval number: LLSC-2021122201).

Statistical analysis

SPSS (version 22.0) was used for statistical analysis. Student's t test and/or chi-squared test were statistically employed for data analysis. Results were indicated as mean ± SD. P< 0.05 was considered statistically significant.

Results

PNMA5 expression was increased in CRC tissues

Firstly, immunohistochemistry analysis showed that PNMA5 expression was greatly enhanced in CRC tissues compared with that in the matched adjacent non-tumor tissues (**Figure 1A**, **1B**, P<0.001). We compared the expression of PNMA5 in cancer tissues and adjacent tissues (**Table 1**). The relation between clinicopathological traits and the expression of PNMA5 in patients with CRC was analyzed in **Table 2**. Specifically, Cox multivariate analysis showed that PNMA5 expression did not reach significant difference. PNMA5 expression could be not considered as a negative prognostic factor for patients with CRC; tumor size and TNM stage can be used for independent prognosis for patients with CRC (**Table 3**). To investigate the roles of PNMA5 expression in the prognosis of CRC, the relation between PNMA5 expression and patients' survival rate was evaluated using Kaplan-Meier analysis and the log-rank test (**Figure 1C**). Enhanced PNMA5 expression decreased the survival time of patients with CRC. These findings indicated that PNMA5 served as a potential prognosis predictor in patients with CRC.

PNMA5 expression was increased in CRC cells

Next, we observed that the expression of PNMA5 in CRC cells were dramatically increased compared to that in NCM460 cells (**Figure 2A**, **2B**, both P<0.05). To investigate the actions of PNMA5 on the capacity of migration and invasion, the expression of PNMA5 in CRC cells was decreased via transfection with lentivirus vectors (si-PNMA5-1, si-PNMA5-2, and NC). The efficiency of transfection was verified by qPCR and Western blotting assays (**Figure 2C-F**, both P<0.05).

Knockdown of PNMA5 expression inhibited the proliferation, migration and invasion of CRC cells

MTT assays showed that knockdown of PNMA5 expression in CRC cells inhibited cell proliferation (**Figure 3A**, **3B**, both P<0.05). Similarly, attenuated expression of PNMA5 reduced the number of the cell colonies in CRC cells (**Figure 3C**, P<0.001). The protein expression of PNMA5 was positively related to that of C-myc, CyclinD1, and Ki67 (**Figure 3D**). These indicated that knockdown of PNMA5 expression inhib-

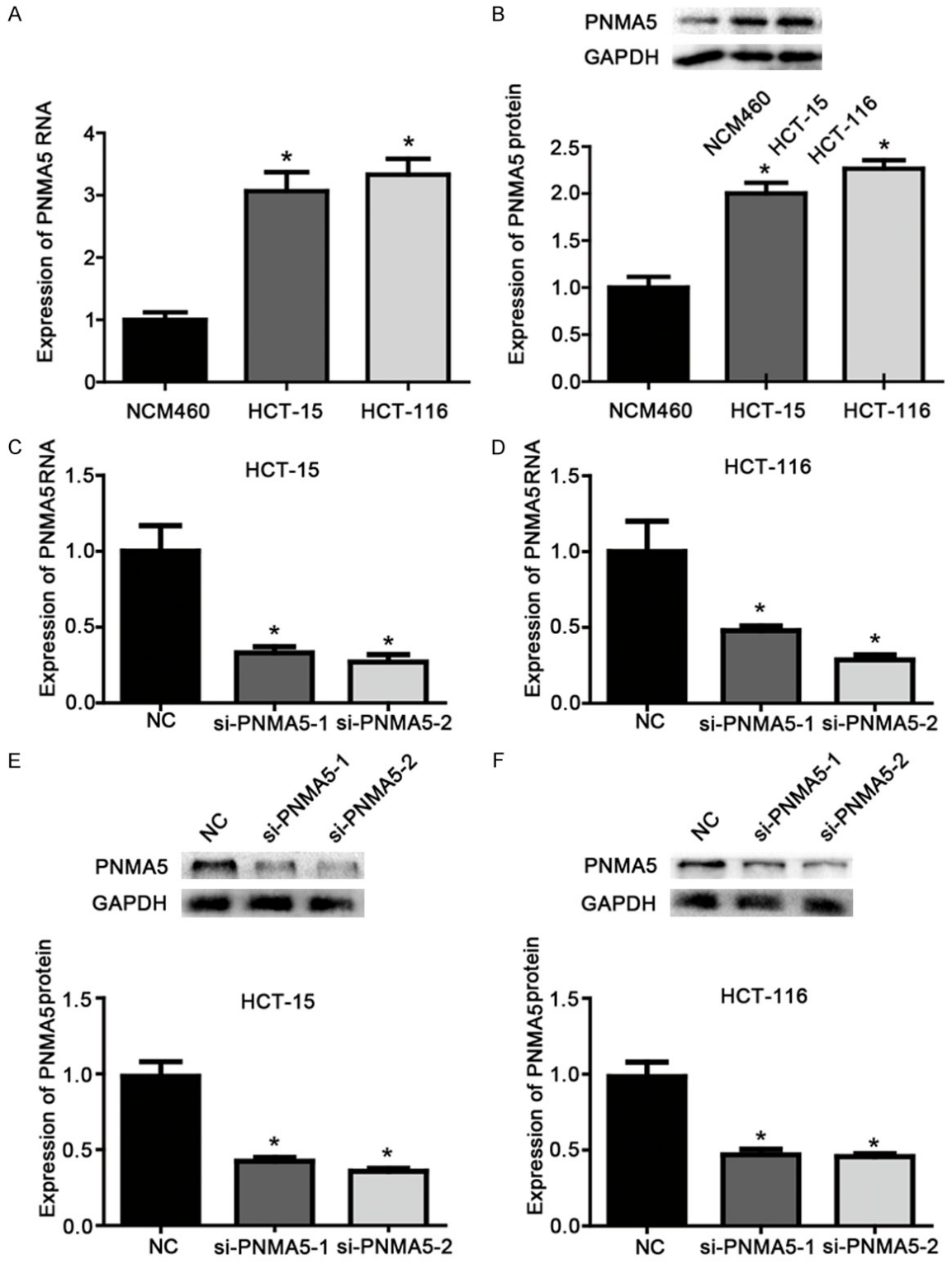

Figure 2. PNMA5 expression in CRC cells was detected. A, B. qRT-PCR and western blotting were used to detect the expression of PNMA5 in CRC and NCM-460 cells. C-F. qRT-PCR and western blotting were used to verify the knock-down efficiency of PNMA5 expression in HCT-15 and HCT-116 cells, *P<0.05, compared with compared with the NC group; NC: negative control.

ited CRC cells growth *in vitro*. In addition, PNMA5-siRNA-transfected CRC cells showed lower invasive potentials (**Figure 4A**, **4B**, both P<0.05). In addition, the healing ability of CRC

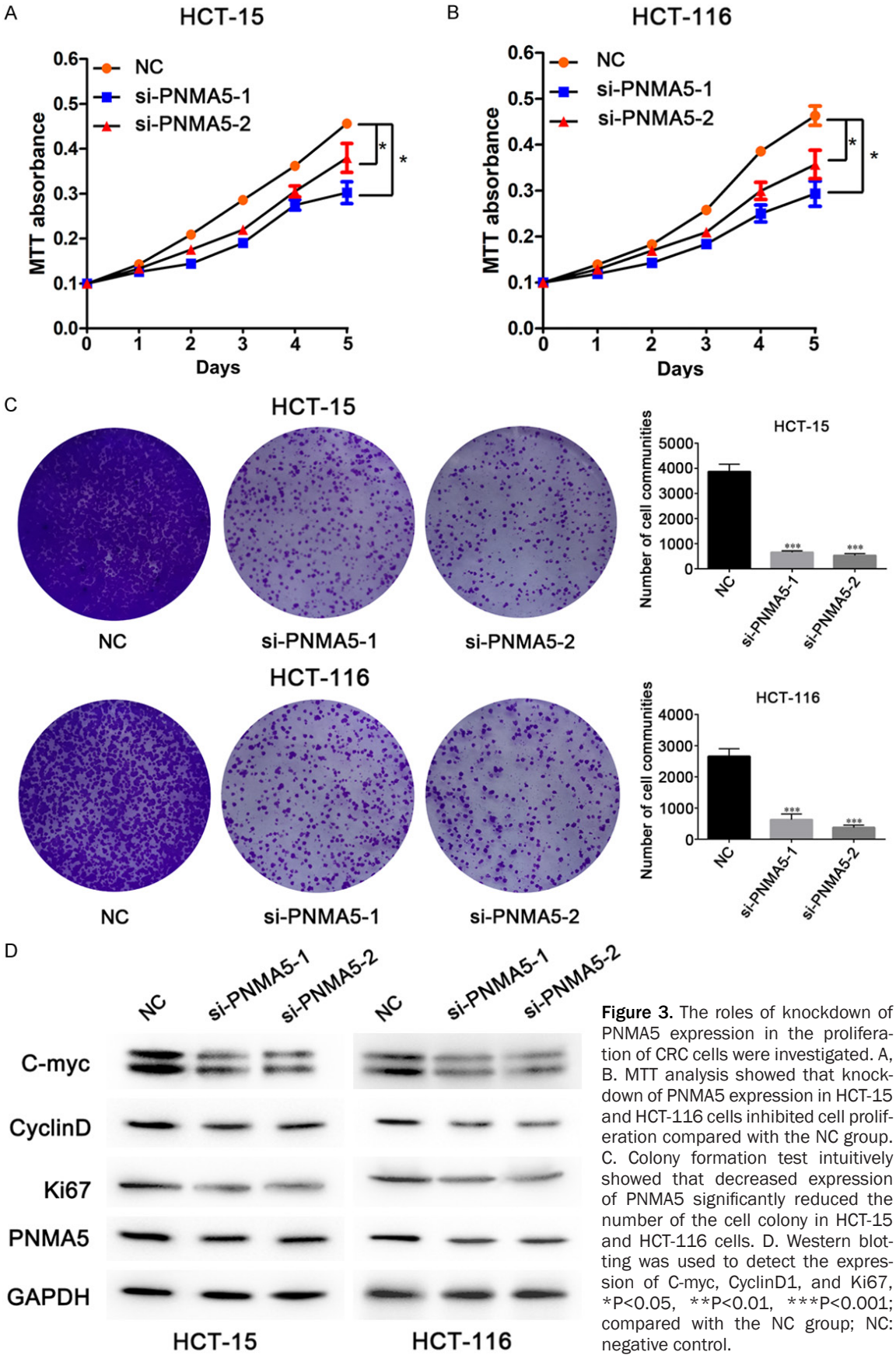

Figure 3. The roles of knockdown of PNMA5 expression in the proliferation of CRC cells were investigated. A, B. MTT analysis showed that knockdown of PNMA5 expression in HCT-15 and HCT-116 cells inhibited cell proliferation compared with the NC group. C. Colony formation test intuitively showed that decreased expression of PNMA5 significantly reduced the number of the cell colony in HCT-15 and HCT-116 cells. D. Western blotting was used to detect the expression of C-myc, CyclinD1, and Ki67, *P<0.05, **P<0.01, ***P<0.001; compared with the NC group; NC: negative control.

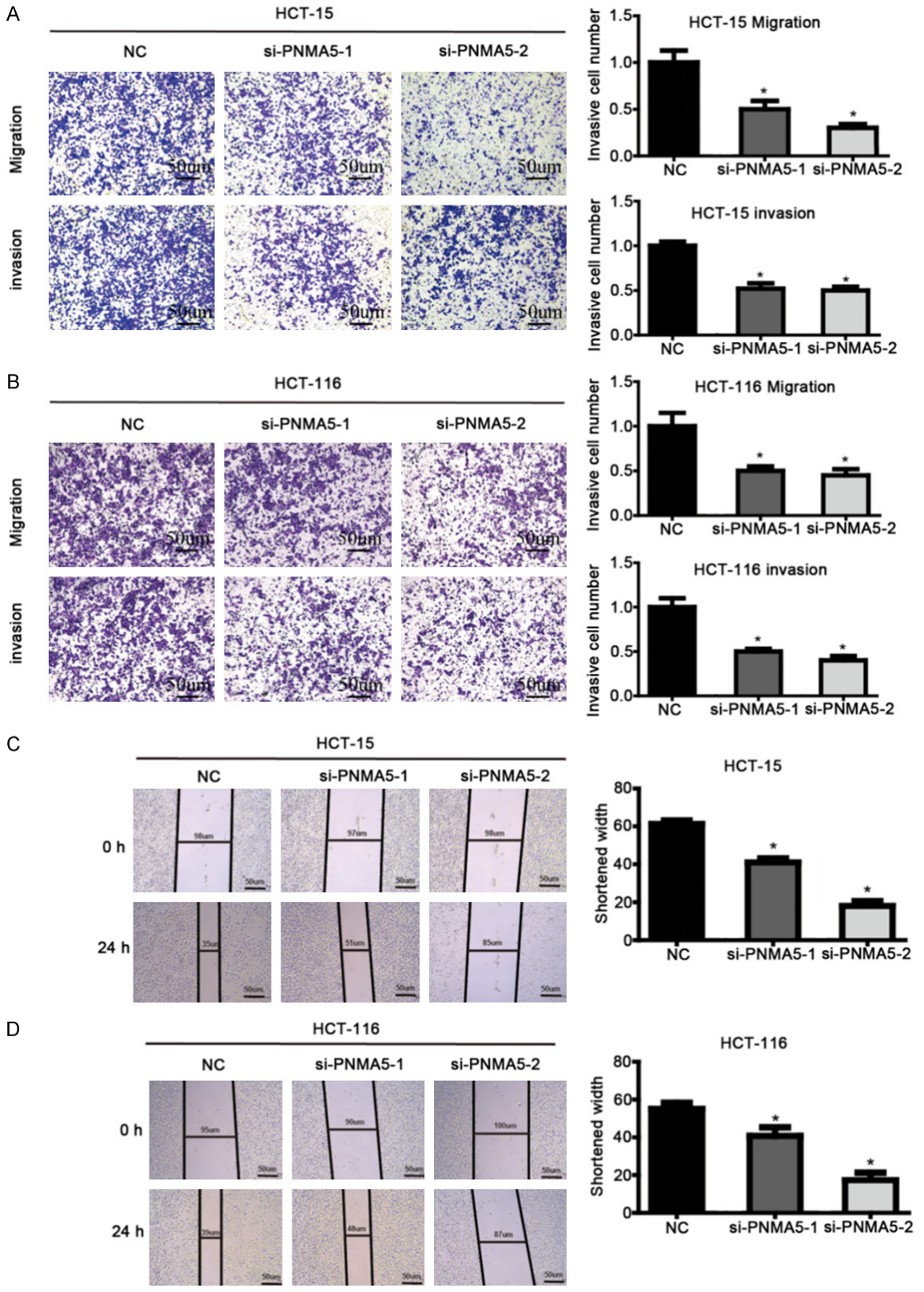

Figure 4. The effects of knockdown of PNMA5 expression on the migration and the invasion of CRC cells were investigated. A, B. Transwell cell invasion assays showed that knockdown of PNMA5 expression inhibited HCT-15 and HCT-116 cells invasion. C, D. Knockdown of PNMA5 expression attenuated the migration of HCT-15 and HCT-116 cells in wound-healing assays, *P<0.05, compared with compared with the NC group; NC: negative control.

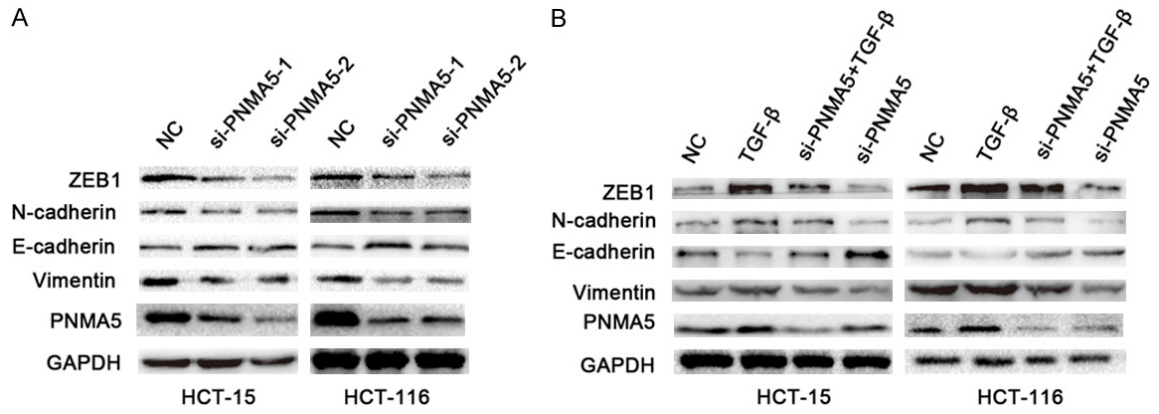

Figure 5. The effects of knockdown of PNMA5 expression on TGF-β-mediated EMT were investigated. A. The expression of ZEB1, N-cadherin, Vinmentin, and E-cadherin in PNMA5 siRNAs-transfected HCT-15 and HCT-116 cells was detected by western blotting. CRC cells were transfected with PNMA5 siRNAs lentivirus vectors or the NC in the presence or absence of TGF-β (2 ng/mL). B. The expression of ZEB1, N-cadherin, Vinmentin, and E-cadherin was detected using western blotting in HCT-15 and HCT-116 cells, respectively.

cells transfected with PNMA5 siRNA was significantly inhibited (**Figure 4C**, **4D**, both P<0.05).

Knockdown of PNMA5 expression inhibited CRC cells migration and invasion via suppressing TGF-β-mediated EMT

EMT exhibits significant biological functions in tumor invasion and metastasis [12]. To investigate the actions of PNMA5 on EMT in CRC cells, the expression of EMT markers in CRC cells was determined. PNMA5 was positively related to the expression of N-cadherin, Zeb1, and Vinmentin, and was negatively associated with E-cadherin (**Figure 5A**). TGF-β enhances the growth and development of cancers by increasing tumor-related angiogenesis and fibroblasts activity and promoting the tumor to escape from inhibitory immunoreactivity [13, 14]. Knockdown of PNMA5 expression inhibited TGF-β-mediated EMT in CRC cells (**Figure 5B**). To further determine whether knockdown of PNMA5 inhibited CRC cell migration and invasion by suppressing TGF-β-regulated EMT, four group cells were involved in detecting the capacity of migration and invasion. The results showed that knockdown of PNMA5 expression inhibited the migration and invasion of CRC cells via suppressing TGF-β-mediated EMT (**Figure 6A-D**, both P<0.05).

PNMA5 accelerated proliferation and metastasis in vivo

Next, the potential roles of PNMA5 *in vivo* were validated. Three group cells (HCT-15) transfected with PNMA5 lentivirus vectors (si-PNMA5-1, si-PNMA5-2, and NC) were implanted into the mice by subcutaneous injection. The development of tumor cells in si-PNMA5-treated mice was slower than those in NC-treated mice (**Figure 7A**, **7B**, P<0.05). We also conducted a tail vein metastasis assay. The CRC cells transfected with PNMA5 siRNA or control vector were administered by injection through the tail vein. Thirty days after administration, the lung metastasis in the mice was observed. The tumor volumes in the model groups injected with PNMA5-overexpressed cells were larger, compared with those in the NC group (**Figure 7C-E**, P<0.01). The tumor-metastatic lungs were collected for immunohistochemical analysis, which showed that PNMA5 expression was positively associated with that of N-cadherin and Vinmentin and negatively with that of E-cadherin (**Figure 7F**). Collectively, PNMA5 accelerated proliferation and metastasis in CRC *in vivo*.

Discussion

With the alternation of diets and lifestyles, CRC morbidity is prone to increase [15]. Although the precise causes of CRC are unknown, the interaction between genetics and lifestyle factors may contribute to its development. Many risk factors, including unhealthy diets, obesity, and sedentary lifestyles, have become popular. Whether these factors, particularly for young people, can trigger the initiation of diseases by inducing genotoxicity is still unclear [16]. Many patients with CRC are diagnosed in

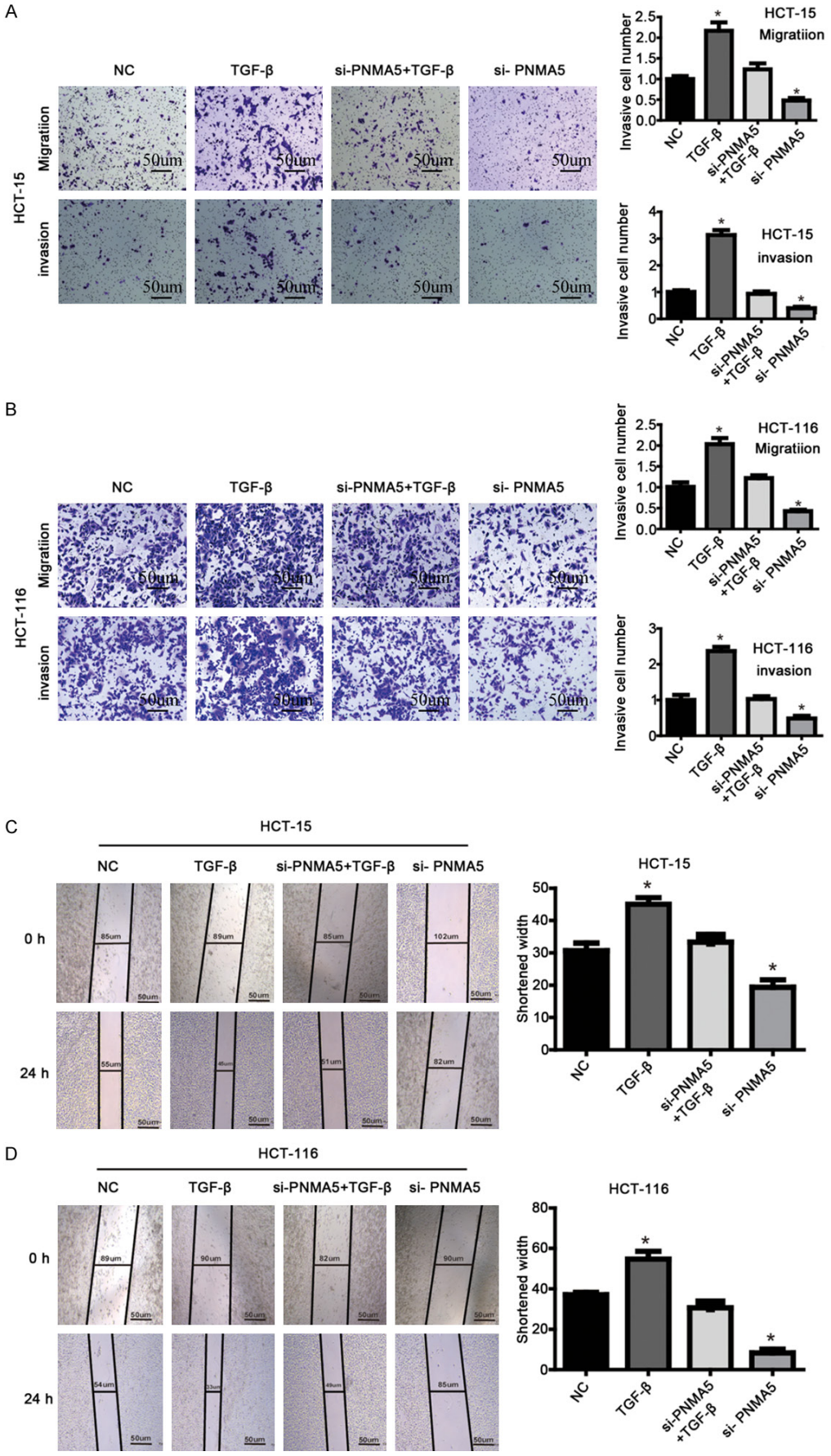
A
NC
TGF-β
si-PNMA5+TGF-β
si- PNMA5
HCT-15
Migratiion
invasion
50um
Invasive cell number
HCT-15
Migratiion
HCT-15
invasion
B
HCT-116
Migratiion
invasion
HCT-116
Migratiion
HCT-116
invasion
C
HCT-15
0 h
24 h
Shortened width
HCT-15
D
HCT-116
HCT-116

Figure 6. The effects of knockdown of PNMA5 expression on TGF-β-mediated migration and invasion of CRC cells were investigated. A, B. Transwell invasion assays showed that knockdown of PNMA5 expression inhibited TGF-β-mediated EMT in HCT-15 and HCT-11 cells. C, D. Wound-healing assays showed that knockdown of PNMA5 expression inhibited TGF-β-mediated EMT in HCT-15 and HCT-116 cells, *P<0.05, compared with compared with the NC group; NC: negative control.

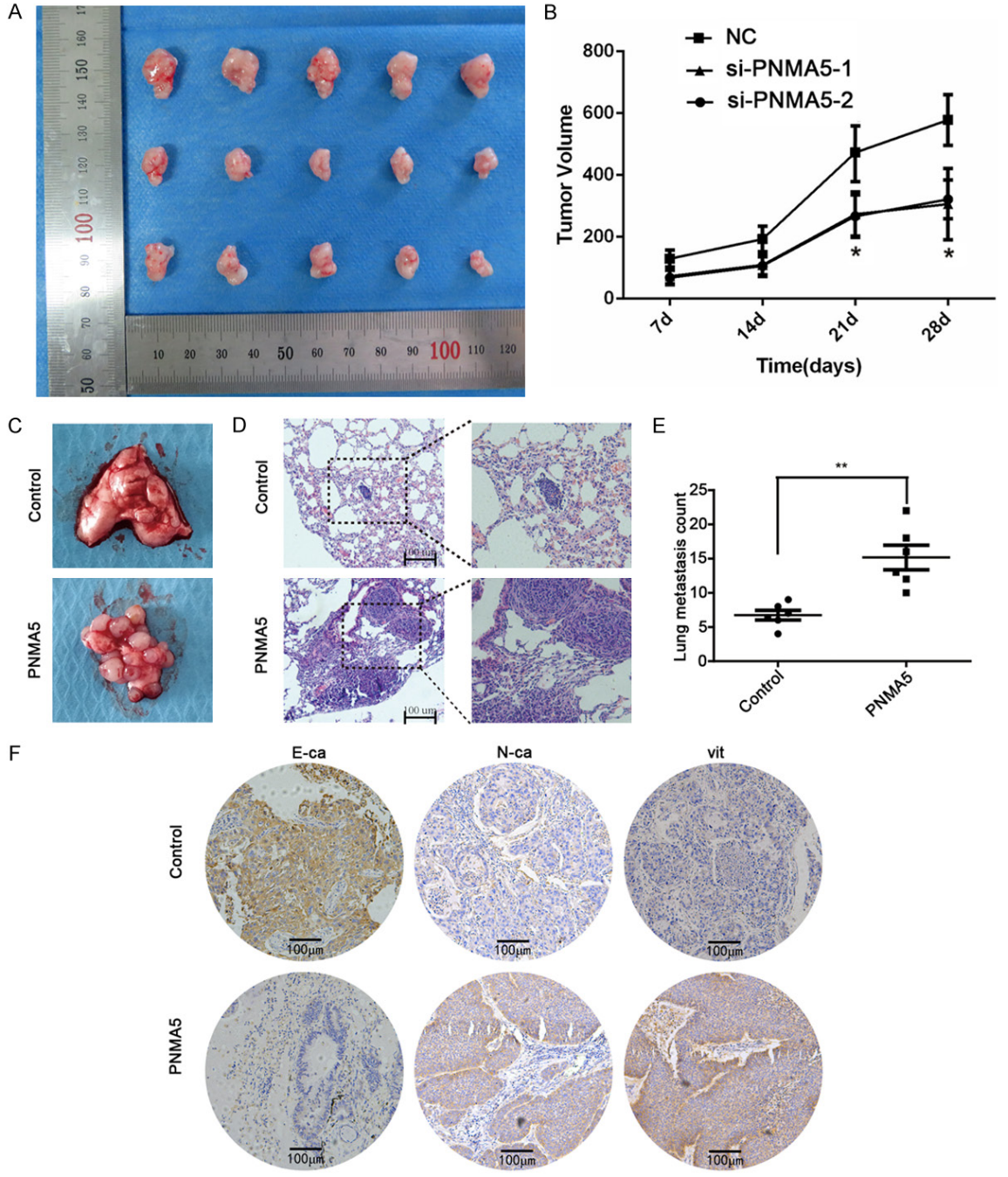

Figure 7. The roles of PNMA5 in the proliferation and metastasis in vivo. Three group cells (HCT-15) transfected with PNMA5 lentivirus vectors (si-PNMA5-1, si-PNMA5-2 and NC) were implanted subcutaneously into the flanks of nude mice. A, B. The volume and size of tumor xenograft in si-PNMA5-treated mice were detected. PNMA5-overexpressed HCT-15 cells were injected into mice through the caudal vein, and the two groups of mice's lung were taken out after 1 month. C, E. The volumes in PNMA5-overexpressed transfection group were significantly larger, compared with those in the control group. D, F. H&E staining and immunohistochemical examination showed the expression of N-cadherin, Vinmentin, and E-cadherin, *P<0.05; **P<0.01; ***P<0.001; compared with the NC group; NC: negative control.

an advanced stage at their first visit to the clinic. This poses great challenge to the treatment of CRC. The dissatisfaction with pharmacological treatment for advanced CRC contributes to the challenge [17].

Abnormal gene expression is essential in the pathological development of CRC. We found, in this study, that PNMA5 was dysregulated in CRC patients through employing the bioinformatics technology. However, the specific mechanisms of PNMA5 in mediating CRC development are still under investigation. In this article, we found that PNMA5 expression contributes to the metastasis of CRC. To explore the mechanisms of PNMA5 in orchestrating the invasion and the metastasis of CRC, we tested the expression of EMT-related markers. The results showed that PNMA5 could promote EMT in CRC [18]. Metastases contribute to more cancer-associated mortality than the primary tumors. Increased EMT activity facilitates cells to migration and invasion [19, 20]. Consistently, mediation of EMT in CRC cell lines was involved in this study.

TGF-β exhibits significant biological functions for cell proliferation, differentiation, and EMT [21-23]. *Lu et al* reported that YY1 affected the EMT of bladder cancer through TGF-β signaling pathway [24]. *Zhang et al* suggested that down regulation of SEPT9 expression could block the development of glioma by suppression of TGF-β1-induced EMT [25]. In our study, when the expression of PNMA5 was decreased, TGF-β-mediated EMT was blocked. Subsequently, cellular invasion and migration were suppressed.

Collectively, our results validated that PNMA5 could accelerate cellular proliferation, invasion, and migration in CRC. Knockdown of PNMA5 expression exhibited the opposite effects via suppressing TGF-β-mediated EMT. Thus, PNMA5 could be considered as a useful candidate for the prognosis in patients with CRC.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Xin He, The First Affiliated Hospital of Gannan Medical University, Ganzhou 341000, Jiangxi, PR China. Tel: +86-797-8283913; E-mail: hexin@gmu.edu.cn

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