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Neural precursor cell expressed, developmentally downregulated 8 promotes tumor progression and predicts poor prognosis of patients with bladder cancer

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Neddylation has been researched in many different human carcinomas. However, the roles of neural precursor cell expressed, developmentally downregulated 8 (NEDD8) in bladder cancer are still unknown. Our study was the first study which systematically investigated the possible functions of NEDD8 in bladder cancer (BC) progression. We carried out immunohistochemistry to explore associations between the expression of NEDD8 in tumor tissues and clinical outcomes of patients. RT-qPCR and western blot were used to detect the expressional levels of genes. The biological abilities of cell proliferation, migration and invasion were researched by in vitro and in vivo experiments. Results were as follows: Data from The Cancer Genome Atlas (TCGA) database showed that NEDD8 was overexpressed in BC tissues and was associated with poor patient survival. Results of immunohistochemistry found that NEDD8 was significantly associated with poor clinical outcomes of BC patients. Suppression of NEDD8 could inhibit the proliferation, migration and invasion of tumor cells. Knocking down NEDD8 could induce apoptosis and G2 phase arrest of cell cycle progression. In vivo, suppression of NEDD8 restricted growth and metastasis of tumors in mice. In conclusion, NEDD8 has important roles in regulating the progression of BC cells and was associated with poor prognosis of patients; hence, it may become a potential therapeutic target of BC.

KEYWORDS
bladder cancer, NEDD8, prognosis, tumor progression, tumor tissue

1 | INTRODUCTION

Bladder cancer (BC) is a common urinary system malignancy with 81 190 new cases and 17 240 deaths estimated in the USA in 2018.[1] Approximately 70%-75% of newly diagnosed patients present with non-muscle invasive bladder cancer (NMIBC), and 25%-30% are invasive (MIBC).[2] Although many treatments such as surgery, chemotherapy, radiotherapy and drug-targeting therapy have been applied in the interventional treatment of bladder cancer, survival of patients remains unsatisfactory.[3] Even the most effective neoadjuvant chemotherapy produces favorable responses in only about 25% of BC patients.[4] Few advances in the treatment of BC have been found in recent decades.[5,6] Therefore, a novel effective treatment strategy is urgently required in the management of BC.

The ubiquitin-proteasome system is a well-characterized post-translational protein modification profile, which regulates many

critical cellular processes by transferring ubiquitin to substrate proteins.[7] Neural precursor cell expressed, developmentally downregulated 8 (NEDD8) is a ubiquitin-like small molecule that can be activated by a NEDD8-activating enzyme (NAE) in a similar way to the ubiquitin system.[8,9] Neddylation is the process of combining NEDD8 to substrate proteins. Mature NEDD8 is activated by NAE and transferred to E2-enzyme and conjugated to E3-ligase.[10] It has been reported that neddylation plays essential roles in promoting the progression of many different human carcinomas. Hence, inhibition of protein neddylation has become an attractive anticancer strategy.[10,11] Neddylation inhibitor such as MLN4924 was found to play important roles in inhibiting tumor proliferation and metastasis.[12-14] In bladder cancer, MLN4924 suppressed proliferation and migration of tumors in in vitro and in vivo experiments.[15] However, the role of NEDD8 in bladder cancer remains unknown.

Data from a public database found that NEDD8 were overexpressed in BC tissues and was associated with the prognosis of patients. Therefore, in the present study, we investigated the roles of NEDD8 in BC progression by in vitro and in vivo experiments. Based on the results, we hope to better understand the pathogenesis and develop a new treatment strategy for BC.

2 | MATERIALS AND METHODS

2.1 | The Cancer Genome Atlas database

The Cancer Genome Atlas (TCGA; https://cancergenome.nih.gov/) is an online bioinformatics database that provides biological information on different cancers. We downloaded clinical data and mRNA expressional data of patients with BC from TCGA database. Expression of NEDD8 in bladder tumors and controls was then compared. Prognostic value of NEDD8 in overall survival (OS) and disease-free survival (DFS) of BC patients was analyzed.

2.2 | Patients

A total of 115 patients pathologically and clinically diagnosed with BC were included in our study. Tumor tissues were obtained at the Second Hospital of Tianjin Medical University when the patients were undergoing their first surgery (transurethral resection of bladder tumor [TURBT] or cystectomy). Patients who underwent neoadjuvant treatment were excluded. Clinical and pathological data of patients including age, gender, tumor TNM stage, grade, lymph node metastasis, and distant metastasis were recorded. Tumors were classified according to the 2004 WHO criteria as well as to 2009 UICC TNM staging.[16] Follow up was recommended every 3 months during the first year and every 6 months during subsequent years. The present study was approved by the Ethics Committee of the Second Hospital of Tianjin Medical University and written informed consent was obtained from each patient.

2.3 | Immunohistochemistry and scoring

Tumor tissues and adjacent normal tissues were collected at the Second Hospital of Tianjin Medical University. Sections (4 μm) were cut from paraffin blocks and incubated with NEDD8 antibody (1:100 dilution, SAB4501973, rabbit; Sigma, St Louis, MO, USA) overnight at 4°C. Then, the second antibody was added for 30 minutes at room temperature. Staining intensity was scored as follows: no staining, 0; weak staining, 1; moderate staining, 2; strong staining, 3. Percentages of positive cells were categorized as follows: no staining, 0; 1%-25% of stained cells, 1; 25%-50%, 2; 51%-85%, 3; 85%-100%, 4. Immunohistochemical (IHC) staining was evaluated by two pathologists. Final scores were calculated by multiplying the proportion and intensity. According to the distributions of final scores, we divided the expression of NEDD8 into high (scores 3-6) and low (scores 0-2) expressional groups.

2.4 | Cell culture

Human bladder cancer T24 and 5637 cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Both cell lines were cultured in RPMI-1640 medium supplied with 10% FBS and 1% antibiotic (penicillin/streptomycin) according to the instructions. All cells were maintained at 37°C with 5% CO_2 atmosphere.

2.5 | Stable knocking-down cell lines

The effective shRNA sequence was selected to establish stable NEDD8 knocking-down cell in both cell lines. The sequence of shRNA was 5′-GGCATCATATATCCTCTCACT-3′. Lentivirus carried with shRNA plasmid was used to infect tumor cells. RT-qPCR and western blot were used to detect the efficiency of knocking down in both cell lines. Stable knocking-down cells were selected and used in later experiments.

2.6 | Reverse transcription-qPCR

Total RNA of cells was extracted by Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed to cDNA by the cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Then, quantitative real-time PCR was carried out and the primers of NEDD8 were as follows: NEDD8-forward primer 5′-CGCTGACCGGAAAGGAGATT-3′ and NEDD8-reverse primer 5′-CAGAGCCAACACCAGGTGAA-3′. Expression fold changes were calculated using $2^{-\Delta\Delta Ct}$ methods.[17]

2.7 | Western blot

Cells from different groups were harvested, and proteins were extracted and concentrated using the bicinchoninic acid (BCA) method. The lysates were resolved by 12% SDS-PAGE and transferred to

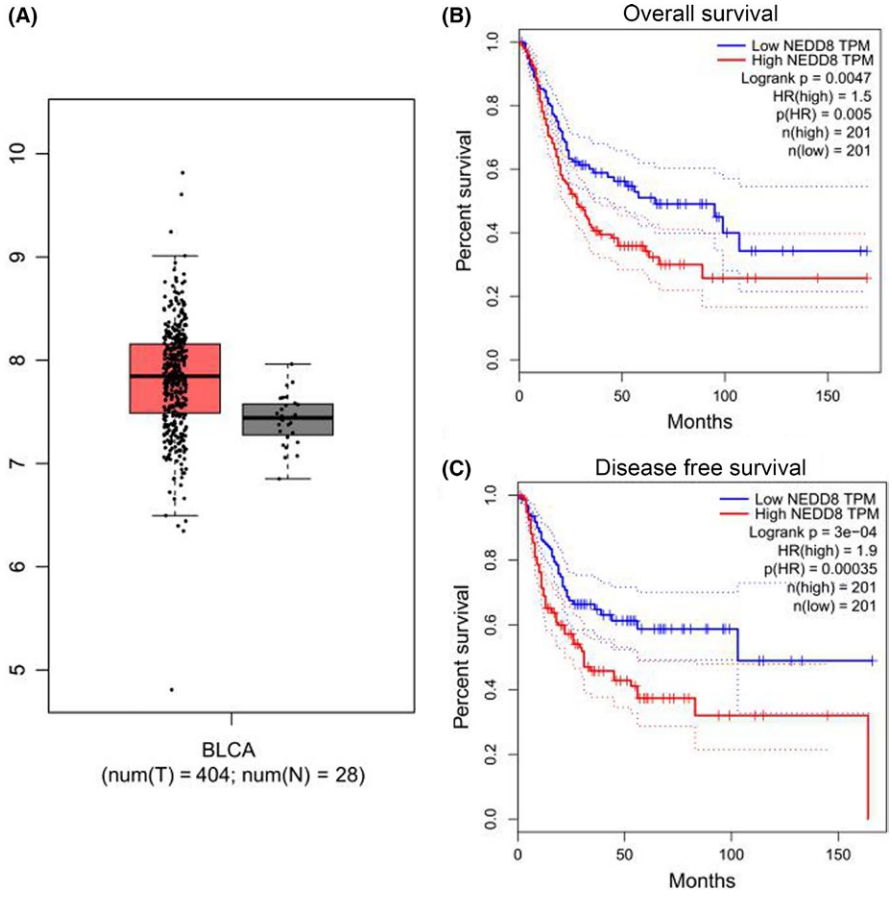

FIGURE 1 Bioinformatics analysis of neural precursor cell expressed, developmentally downregulated 8 (NEDD8) from The Cancer Genome Atlas (TCGA) database. A, Expression of NEDD8 in bladder cancer tissues and normal controls. B,C, Kaplan-Meier analysis of the associations between NEDD8 mRNA expression and overall survival and disease-free survival of bladder cancer patients. TPM, transcripts per million

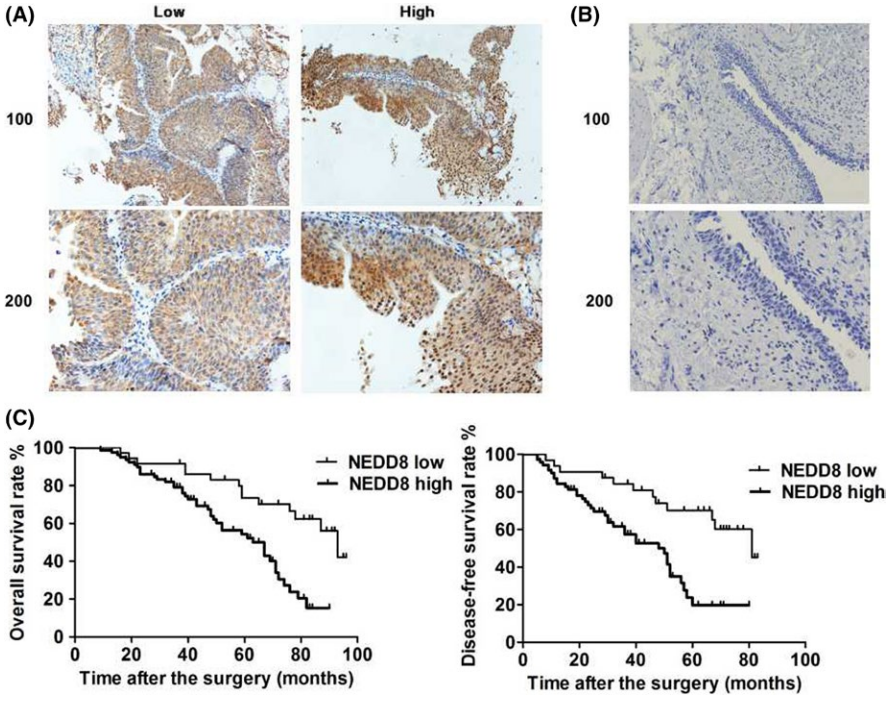

FIGURE 2 Immunohistochemical analysis of correlation between neural precursor cell expressed, developmentally downregulated 8 (NEDD8) and clinical outcomes of bladder cancer (BC) patients. A, Typical staining of high and low expression of NEDD8 in BC tissues. B, Negative staining of NEDD8 in normal bladder tissues. C, Kaplan-Meier analysis of the association between NEDD8 protein expression and overall survival and disease-free survival of patients

a PVDF membrane. Then, the membranes were blocked and incubated with primary antibodies: NEDD8 antibody (1:1000 dilution; Cell Signaling Technology [CST], Danvers, MA, USA), anti-β-actin (1:1000 dilution; Abcam, Cambridge, UK), rabbit anti-Ki67 (1:1000 dilution; Abcam), mouse antiproliferating cell nuclear antigen (PCNA) (1:500 dilution; Abcam), anti-caspase-3 (1:500 dilution;

TABLE 1 Relationship between neural precursor cell expressed, developmentally downregulated 8 and clinicopathological characteristics in 115 patients with bladder cancer

		NEDD8 expression			
		Low	High		
Variable	All n = 115	n = 36	n = 79	χ^2	*P*
Age (years)					
<65	58	16	42	0.752	.386
≥65	57	20	37		
Gender					
Male	62	20	42	0.057	.811
Female	53	16	37		
Tumor stage					
T2	40	20	20	9.969	.002*
T3/T4	75	16	59		
Tumor grade					
Low	33	13	20	1.408	.235
High	82	23	59		
Vascular invasion					
Yes	59	12	47	6.774	.009*
No	56	24	32		
Lymph node metastasis					
Yes	16	6	10	0.332	.565
No	99	30	69		
Recurrence					
Yes	33	13	20	1.408	.235
No	82	23	59		
Distant metastasis					
Yes	49	10	39	4.714	.030*
No	66	26	40		

NEDD8, neural precursor cell expressed, developmentally downregulated 8.
*$P < 0.05$.

CST), anti-caspase-7 (1:500 dilution; CST), anti-MMP-2 (1:500 dilution; CST), and anti-MMP-9 (1:500 dilution; CST) overnight at 4°C. Then, the second antibody was added for another 1 hour.

2.8 | Colony formation array

Cells (500 cells/well) were seeded into 60-mm dishes and cultured for 10 days at 37°C in 5% CO_2. After fixation in methanol for 15 minutes, the cells were stained with Giemsa for another 10-30 minutes. Numbers of colonies were counted under a microscope. The arrays were carried out in triplicate.

2.9 | MTT array

Proliferation of both T24 and 5637 cells was detected by MTT array. Cells with densities of 5×10^3 cells/well were seeded into 24-well plates, and 50 μL of 5 mg/mL MTT solution was added and incubated for 4 hours at 37°C. Then, the medium was removed and DMSO was added. Absorbance of the cells was measured by microplate reader at 595 nm. The experiment was carried out in triplicate.

2.10 | Apoptosis and cell cycle analysis

Cells in shNEDD8 and control groups were harvested for apoptosis and cell cycle analysis. For apoptosis array, cells were stained by the Annexin V-FITC apoptosis kit (Sigma). Apoptotic cells were identified and measured by flow cytometry according to the manufacturer instructions. Cells calculated with cell cycle staining kit (MultiSciences, Tianjin, China) were used for cell cycle analysis. The arrays were carried out in triplicate.

2.11 | Wound-healing array

T24 and 5637 cells (2×10^5/well) were seeded into 6-well plates and cultured for 24 hours. A lesion was created by a 10-μL pipette tip and washed with PBS. After culturing for 24 hours, migration of cells was visualized by a microscope. The migration rate of cells was evaluated by the width between the edges of both sides. The arrays were carried out three times.

2.12 | Transwell array

Cells (1×10^5/well) were seeded into the upper chamber, and 2 mL medium with 10% FBS was added to the lower chamber. Membrane with Matrigel substrate (BD Biosciences, Franklin Lakes, NJ, USA) was used for filtering. Then after culturing for 24 hours, cells filtered through the membrane were stained with 0.5% crystal violet solution and calculated under the microscope. Three repeat arrays were carried out.

2.13 | Animal study

All animal experiments were approved by the animal care and use committee of the Second Hospital of Tianjin Medical University. Sixteen 4-week-old BALB/c nude mice were purchased from Slac Laboratory Animal Co. Ltd (Shanghai, China). T24 cells (1×10^7) effectively transfected by shNEDD8 and controls were suspended in PBS and injected s.c. into the right armpits of nude mice (8 in each group). After 2 weeks of tumor formation, volumes of tumors were measured daily. On the 29th day, mice were killed and final tumor tissues were obtained. To investigate the correlation between NEDD8 and tumor metastasis, T24 cells were injected into the tail vein of mice. After 4 weeks, metastasis in lung was detected and the tumors were separated.

2.14 | Statistical analysis

All data were analyzed by SPSS 20.0 statistics software. Quantitative data were evaluated by mean ± SD. According to whether the

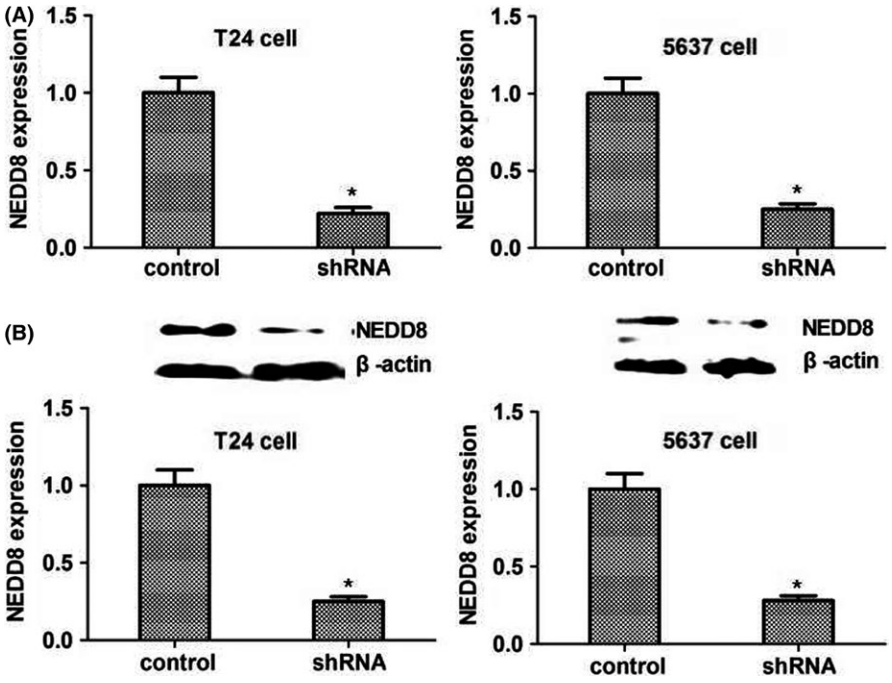

FIGURE 3 Stable knocking down of T24 and 5637 cell lines. A,B, RT-qPCR and western blot were used to confirm the efficiency of knocking down. Expression of neural precursor cell expressed, developmentally downregulated 8 (NEDD8) was dramatically inhibited in the shRNA group in both mRNA and protein levels. *$P < 0.05$

variance was homogeneity, parameter test and non-parametric test were used, respectively. Associations between the expression of NEDD8 and clinical characters were analyzed by chi-squared tests. Kaplan-Meier method and log-rank tests were carried out to estimate the prognostic value of NEDD8 in patient survival. $P < .05$ for the difference was considered significant.

3 | RESULTS

3.1 | Bioinformatics analysis of NEDD8 from TCGA database

Data downloaded from TCGA database were used to predict the roles of NEDD8 in BC. We found NEDD8 was overexpressed in bladder tumor tissues compared to the adjacent normal controls (Figure 1A). Then, to investigate the associations between NEDD8 and the prognosis of BC patients, we divided the patients into high and low NEDD8 expressional groups according to the median value. Results showed that high expression of NEDD8 was associated with worse OS and DFS of patients (Figure 1B,C). Results of TCGA suggested that NEDD8 was related to the prognosis of BC patients and may play an important role in tumor progression.

3.2 | High expression of NEDD8 predicted poor clinical outcomes of patients with BC

One hundred and fifteen patients with bladder cancer were collected to investigate the associations between the expression of NEDD8 and clinicopathological characteristics. Immunohistochemistry was carried out to evaluate the expression of NEDD8 protein in tumor tissues. Typical staining of strong and weak expression of NEDD8 was seen (Figure 2A). Results showed that NEDD8 was significantly associated with tumor stage ($P = .002$), distant metastasis ($P = .030$) and vascular invasion ($P = .009$) of BC patients. However, no associations were found between NEDD8 and other common clinical features such as patient age, gender, tumor pathological stage, grade and lymph node metastasis (Table 1). Then, Kaplan-Meier analysis was carried out to analyze the prognostic value of NEDD8 in OS and progression-free survival (PFS) of patients. According to distribution of NEDD8 expression, we divided patients into two groups (high and low). Results showed that high expression of NEDD8 was significantly related to short OS ($P < .05$) and PFS ($P < .05$) of patients with BC, which was consistent with the conclusions from TCGA database (Figure 2B,C). In all, we concluded that NEDD8 was more likely to be an unfavorable factor, which was associated with the poor clinical outcomes of BC patients.

3.3 | Slicing of NEDD8 inhibited cell proliferation of BC cell lines

Expression of NEDD8 in the shRNA group and controls was measured by RT-qPCR and western blot. NEDD8 in the shRNA group was dramatically suppressed in both mRNA and protein levels (Figure 3). Therefore, an effective NEDD8 knocking-down cell model was successfully constructed in both T24 and 5637 cell lines. In order to explore the role of NEDD8 in BC cell proliferation, colony formation and MTT arrays were carried out. Knocking down of NEDD8 induced significant inhibition of cell proliferation ($P < .05$) (Figure 4A,B). To explore the mechanism of NEDD8 in tumor cell proliferation, expression of proliferation-related proteins was detected. Suppression of NEDD8, expression of Ki67 and PCNA proteins were significantly decreased ($P < .05$) (Figure 4C,D). According to these results, we concluded that the suppression of NEDD8 could dramatically inhibit the proliferation of BC cells by regulating relevant proteins such as Ki67 and PCNA.

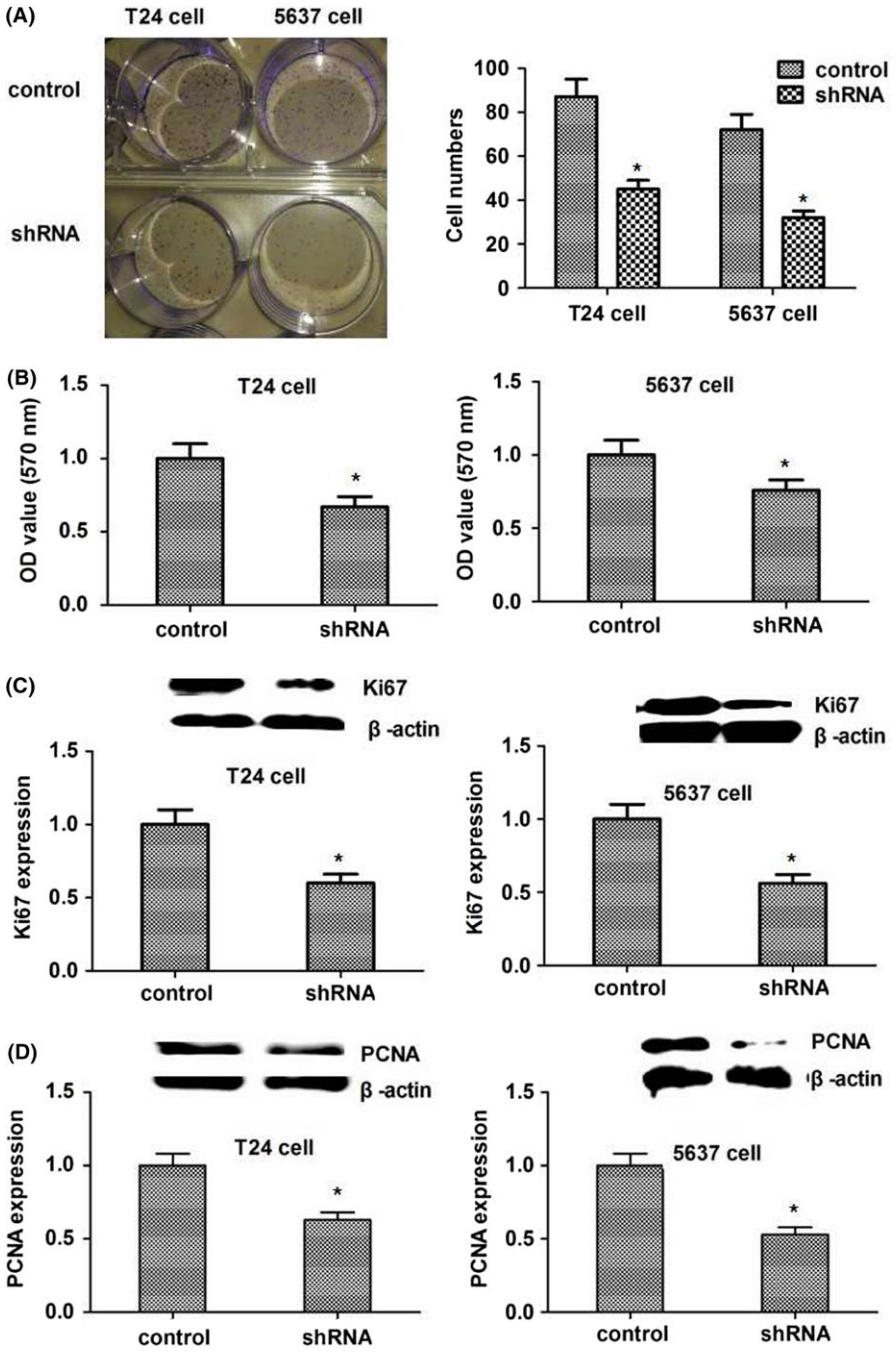

FIGURE 4 Slicing of neural precursor cell expressed, developmentally downregulated 8 (NEDD8) inhibited the cell proliferation of both cell lines by regulating Ki67 and proliferating cell nuclear antigen (PCNA) proteins. A,B, Colony formation and MTT arrays were carried out to investigate the proliferation of tumor cells. Numbers of colonies and cells in the shNEDD8 group were significantly decreased compared to controls. C,D, Knocking down the expression of NEDD8, proliferation-relevant proteins Ki67 and PCNA were obviously downregulated. *$P < 0.05$

3.4 | Knocking down NEDD8 induced cell cycle arrest at G2 phase and promoted apoptosis of BC cells

Flow cytometry was applied to analyze the effect of NEDD8 on cell cycle progression. As shown in Figure 5, cells in G2 phase were increased when we knocked down the expression of NEDD8 ($P < .05$). Cells in S phase were decreased in the shNEDD8 group compared to controls ($P < .05$). Changes in G0/G1 phase were non-significant. Then, cell apoptosis array was carried out. Results showed that when we suppressed NEDD8, the ratios of apoptotic cells were obviously increased ($P < .05$) (Figure 6A). Regarding the mechanism, we found that knocking down NEDD8 could significantly decrease the expression of apoptosis-relevant proteins caspase-3 and caspase-7 ($P < .05$) (Figure 6B,C). Taken together, suppression of NEDD8 could induce cell cycle arrest at G2 phase and promote apoptosis of BC cells by regulating apoptosis-relevant protein caspase-3 and caspase-7.

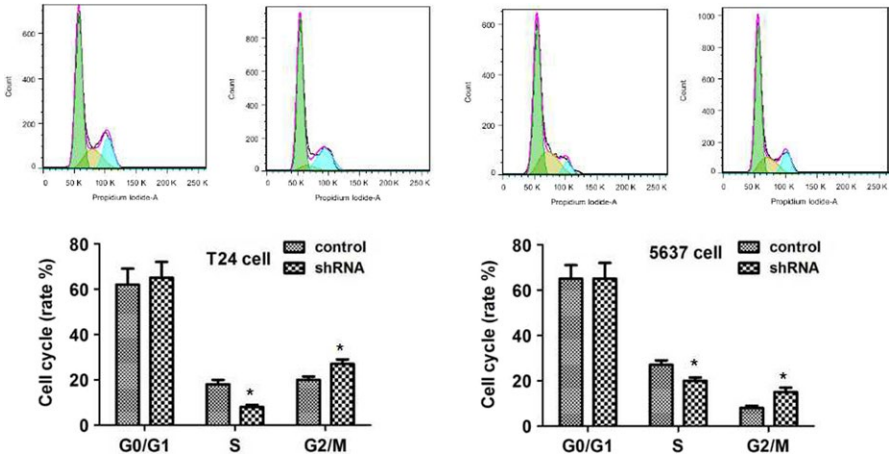

FIGURE 5 Knocking down neural precursor cell expressed, developmentally downregulated 8 (NEDD8) induces cell cycle arrest at G2 phase. Flow cytometry was used to analyze the cell cycle of tumor cells. When knocking down NEDD8, cells in S phase were decreased and cells in G2 phase were dramatically increased. *$P < 0.05$

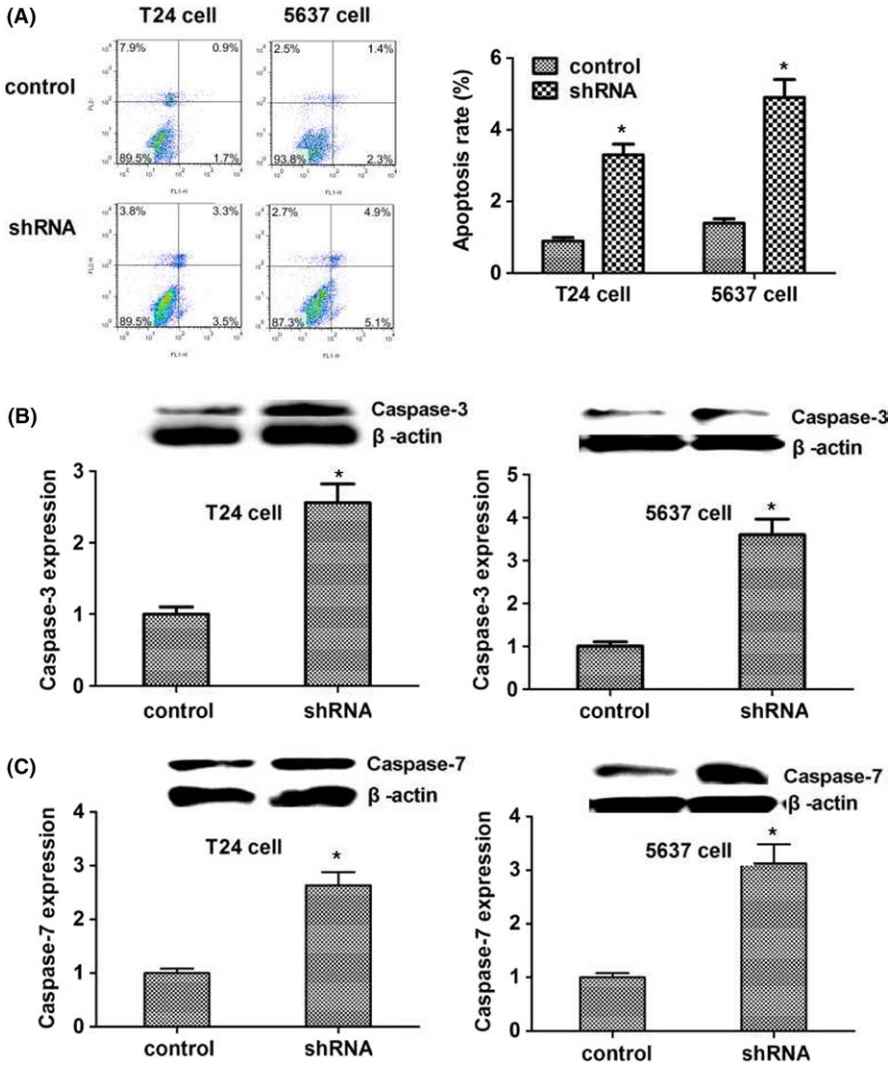

FIGURE 6 Knocking down neural precursor cell expressed, developmentally downregulated 8 (NEDD8) induces cell apoptosis by regulating caspase-3 and caspase-7 proteins. A, Apoptotic cells in shNEDD8 group were increased significantly. B,C, When we suppressed the expression of NEDD8, the expression of caspase-3 and caspase-7 apoptotic proteins was obviously inhibited. *$P < 0.05$

3.5 | Knocking down NEDD8 suppressed migration and invasion of BC cell lines

Wound-healing and Transwell arrays were carried out to evaluate the influence of NEDD8 on the migration and invasion activities of BC cells. Results of wound-healing array showed that knocking down NEDD8 inhibited the migration of cells compared to controls ($P < .05$) (Figure 7A). Invasion of cells in the shNEDD8 group was significantly decreased ($P < .05$) by Transwell (Figure 7B). Then, to further investigate the mechanism of the effect of NEDD8 on the migration and invasion of cells, we detected the expression of many migration- and invasion-relevant proteins in shNEDD8 cells. Results

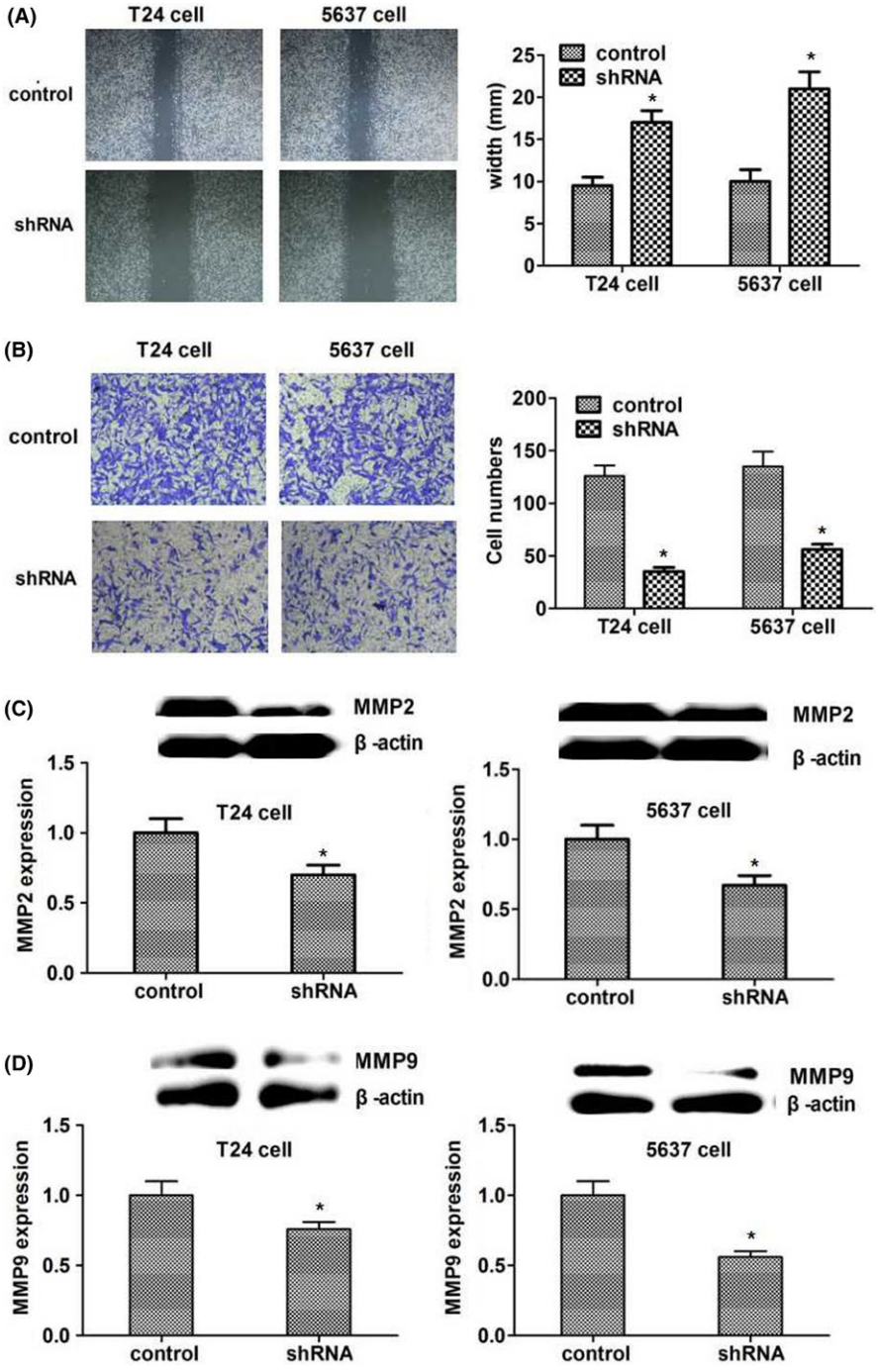

FIGURE 7 Knocking down neural precursor cell expressed, developmentally downregulated 8 (NEDD8) suppresses the migration and invasion of cells by regulating MMP-2 and MMP-9 proteins. A, Wound-healing array shows that the distance of migration in the shNEDD8 group was decreased. B, Transwell array was used to measure the invasion of tumor cells. Compared to controls, cells of the lower chamber in the shNEDD8 group were reduced significantly. C,D, Expression of MMP-3 and MMP-9 proteins was downregulated when we knocked down NEDD8. *$P < 0.05$

showed that expression of MMP-2 and MMP-9 in the shNEDD8 group was significantly downregulated ($P < .05$) (Figure 7C,D). Therefore, we summarized that knocking down NEDD8 could suppress the migration and invasion of BC cells by regulating the expression of MMP-2 and MMP-9 proteins.

3.6 | Suppression of NEDD8 inhibited tumor growth and metastasis in vivo

To further investigate the influence of NEDD8 on the tumor growth of BC, T24 cells transfected with shNEDD8 and controls were injected into mice. Growth of tumors was monitored by measuring tumor volume. After 29 days, mice were killed and tumor tissues were harvested. Results showed that tumors in the shNEDD8 group grew more slowly than those of controls. Volume of tumors in the shNEDD8 group was dramatically smaller than that of controls (Figure 8A). Then, metastatic tumors from lungs of mice were detected and harvested. Results found that metastatic tumors in shNEDD8 groups were smaller than those of controls ($P < .05$), which confirmed an effective inhibition of metastasis when we knocked down the expression of NEDD8 (Figure 8B). To confirm the effective knocking down of NEDD8 in tumor tissues of mice, expression of NEDD8 in different groups was detected by western blot

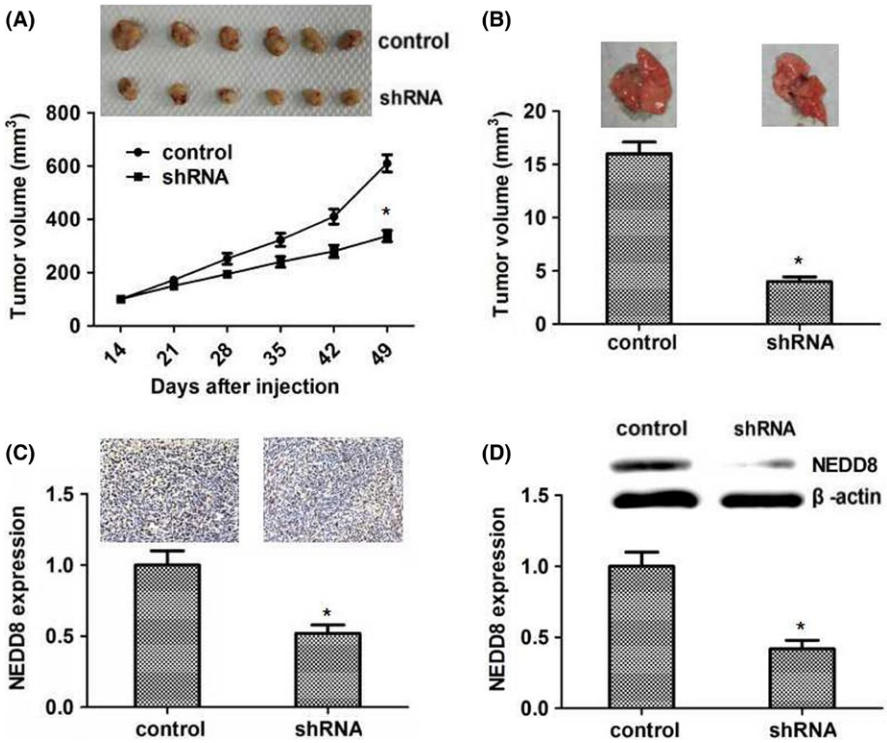

FIGURE 8 In vivo animal experiment. A, Growth curves of tumors from day 14 to day 49 was recorded after injection of transfected T24 cells. Tumors in the shNEDD8 group grew slowly, and tumor sizes were smaller than that of controls. B, Lung metastatic tumors in different groups were harvested after 4 weeks of tumor formation. Tumors in the shNEDD8 group were obviously smaller than those of controls. C,D, Western blot and immunohistochemical staining were done to confirm effective knocking down of NEDD8 in tumor tissues of mice. *P < 0.05

and immunohistochemistry. Results showed that NEDD8 was significantly decreased in the shNEDD8 group (P < .05) (Figure 8C,D). From the above, we concluded that suppression of NEDD8 could effectively inhibit tumor growth and metastasis in mice.

4 | DISCUSSION

Neddylation has been researched in many different human carcinomas. However, the role of NEDD8 in BC is still unknown. The present study was the first study that systematically investigated the possible functions of NEDD8 in BC progression. Data from TCGA database showed that NEDD8 was overexpressed in BC and was associated with patient survival. In our study, we found that NEDD8 was significantly related to poor clinical outcomes of patients diagnosed with BC in our hospital. Suppression of NEDD8 expression could inhibit proliferation, migration, and invasion of tumor cells. Knocking down NEDD8 induced apoptosis and G2 phase arrest of cells. In vivo, knocking down NEDD8 could restrict tumor growth and lung metastasis in mice. Together, these results showed that NEDD8 plays an essential role in regulating the progression of BC cells.

The ubiquitin-proteasome pathway has been reported to play essential roles in human carcinomas, and inhibitors of this pathway have become new drug targets in the treatment strategy.[18-20] Neddylation is a process that combines the ubiquitin-like molecule NEDD8 with substrate proteins.[10,11] Targeting neddylation in cancer therapy has been researched for decades.[21-23] For instance, MLN4924, a small molecule inhibitor of NAE, has been advanced into phase I clinical trials for certain solid tumors.[24] MLN4924 shows antitumor activities in numerous types of cancer.[12,14,25-27] In bladder cancer, MLN4924 could suppress the proliferation of tumor cells in vitro and in vivo.[15] In our study, silencing NEDD8 could dramatically inhibit the proliferation, migration and invasion of BC cells, which was consistent with the functions of previously inhibiting NAE. Therefore, by combining the results of our study and those of Kuo et al,[15] we suspect that neddylation plays a vital role in bladder tumor progression and that NEDD8 may become an effective target in BC treatment.

Neural precursor cell expressed, developmentally downregulated 8 has been identified to attach to the protein of the cullin family and combine with cullin-RING ubiquitin E3 ligases (CRL).[28,29] It was reported that NEDD8 plays a critical role in various cellular functions in cancers.[30,31] Xie's study found that elevated NEDD8 expression was an independent unfavorable factor in predicting OS and DFS of patients with nasopharyngeal carcinoma (NPC). Knocking down the expression of NEDD8 by shRNA and NAE inhibitor MLN4924 could dramatically suppress proliferation and induce cell apoptosis and cell cycle arrest in NPC cells.[31] Similarly, in our study, high expression of NEDD8 was associated with poor prognosis of patients with BC. Suppression of NEDD8 could inhibit proliferation and induce cell apoptosis and G2 phase cell-cycle arrest. Together, we summarized that suppression of NEDD8 could inhibit tumor proliferation by inducing cell cycle arrest and apoptosis in BC cells.

In summary, the present study has confirmed that NEDD8 has important roles in the tumorigenesis of BC and is associated with poor prognosis of patients, which may become a useful biomarker and serve as a potential therapeutic target of BC in the future.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Da-Wei Tian, Zhou-Liang Wu, Li-Ming Jiang, Jie Gao, Chang-Li Wu, and Hai-Long Hu. Carried out the experiments: Da-Wei Tian, Zhou-Liang Wu, Li-Ming Jiang, and Jie Gao. Contributed reagents/materials/analysis tools: Da-Wei Tian, Zhou-Liang Wu, Li-Ming Jiang, Jie Gao, Chang-Li Wu, and Hai-Long Hu. Wrote the paper: Da-Wei Tian.

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