Prostate cancer invasion is promoted by the miR-96-5p-induced NDRG1 deficiency through NF-κB regulation

Invaze karcinomu prostaty je podporována nedostatkem NDRG1 vyvolaným miR-96-5p prostřednictvím regulace NF-κB

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Summary

Background: The N-myc downstream-regulated gene 1 (*NDRG1*) has been discovered as a significant gene in the progression of cancers. However, the regulatory mechanism of *NDRG1* remained obscure in prostate cancer (PCa). ***Methods:*** The *miR-96-5p* and *NDRG1* expression levels were evaluated in PCa cell lines, and prostate tissues, and validated in public databases by real-time polymerase chain reaction, western blot analysis, and immunohistochemistry. The function of *miR-96-5p* and *NDRG1* were investigated by scratch assay and transwell assays *in vitro*, and mouse xenograft assay *in vivo*. The candidate pathway regulated by *NDRG1* was conducted by the next-generation gene sequencing technique. Immunofluorescence and luciferase assays were used to detect the relation between *miR-96-5p*, *NDRG1*, and NF-κB pathway. ***Results:*** Overexpressing *NDRG1* suppresses the migration, invasion, and epithelial-mesenchymal transition (EMT) *in vitro*, and inhibits metastasis *in vivo*. Moreover, *miR-96-5p* contributes to *NDRG1* deficiency and promotes PCa cell migration and invasion. Furthermore, *NDRG1* loss activates the NF-κB pathway, which stimulates p65 and *IKBa* phosphorylation and induces EMT in PCa. ***Conclusions:*** *MiR-96-5p* promotes the migration and invasion of PCa by targeting *NDRG1* and regulating the NF-κB pathway.

Key words

prostate cancer – NDRG1 – miR-96-5p – NF- κB – EMT

Souhrn

Východiska: N-myc downstream-regulovaný gen 1 (*NDRG1*) má významnou funkci při progresi nádorů. U karcinomu prostaty (prostate cancer – PCa) však regulační mechanizmus *NDRG1* zůstává nejasný. ***Materiál a metody:*** Hladiny exprese *miR-96-5p* a *NDRG1* byly hodnoceny v buněčných liniích PCa a v tkáních prostaty a validovány ve veřejných databázích pomocí polymerázové řetězové reakce v reálném čase, analýzy western blot a imunohistochemie. Funkce *miR-96-5p* a *NDRG1* byla zkoumána pomocí testů hojení ran a transwell testů *in vitro* a testu myšího xenoimplantátu *in vivo*. Dráha regulovaná pomocí *NDRG1* byla testována technikou sekvenování nové generace. K detekci vztahu mezi *miR-96-5p*, *NDRG1* a NF-κB dráhou byl použit imunofluorescenční test a test s luciferázou. ***Výsledky:*** Nadměrná exprese *NDRG1* potlačuje migraci, invazi a epiteliálně-mezenchymální přechod (EMT) *in vitro* a inhibuje metastázy *in vivo*. Navíc *miR-96-5p* přispívá k deficitu *NDRG1* a podporuje migraci a invazi buněk PCa. Kromě toho ztráta *NDRG1* aktivuje dráhu NF-κB, která stimuluje fosforylaci p65 a IKBa a indukuje EMT v PCa. ***Závěr:*** *MiR-96-5p* podporuje migraci a invazi PCa tím, že cílí na *NDRG1* a reguluje dráhu NF-κB.

Klíčová slova

karcinom prostaty – NDRG1 – miR-96-5p – NF-κB – EMT

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Introduction

Prostate cancer (PCa) was the second most common malignant tumor in males. Based on the Globocan 2020 estimates of cancer, there were 1,414,259 new cases of PCa worldwide. Moreover, there were also 375,304 patients who died of PCa [1]. Most of the patients were in an advanced stage at the time of the first diagnosis, with lymph nodes or distant metastasis, especially in eastern Asian countries [2]. Although several treatments have been applied recently, the prognosis of PCa remains not well. Therefore, it is necessary to look for reliable biomarkers that play a crucial role in tumor growth and metastasis to distinguish early-stage tumors.

N-myc downstream-regulated gene-1 (*NDRG1*) is located on chromosome 8q24.3, which contains 16 exons and 15 introns. It is an important member of the *NDRG* family [3,4]. Epithelial-mesenchymal transition (EMT) is known as a crucial phenotypic conversion. In the process of EMT, cells can change from an epithelial state to a mesenchymal state in a highly plastic and dynamic way, modify the adhesion molecules expressed by cells and make them have migration and invasive behavior [5,6]. Some previous studies have demonstrated that *NDRG1* potentially inhibited cancer cell migration, invasion, and EMT by affecting the indispensable molecules involved in metastasis [7,8]. Similar studies suggested that *NDRG1* could bind to β-catenin and E-cadherin directly, indicating that these proteins formed a complex. Under the stimulation of WNT3a, ectopic overexpression of *NDRG1* maintained β-catenin on the plasma membrane of colon and PCa cells, which blocked the activation of the Wnt pathway [9,10]. In addition, Xi et al. have recently found an effective way to inhibit TNF-α and LYRIC-induced EMT through *NDRG1* [11]. *NDRG1* also modulated EMT by regulating the level of related protein expression. Elevating E-cadherin and inhibiting vimentin, N-cadherin, slug, and snail-1 have been established by Lee et al. in oral squamous cancer cells [12]. It has also been suggested that *NDRG1* potentially attenuates EMT by inhibiting the expression of *Smad2* and suppressing the latter-related protein in nasopharyngeal cancer [13]. Though several molecular pathways illustrating the function of *NDRG1* have been partially elucidated, the underlying mechanism responsible for *NDRG1* and EMT in PCa is still needed further exploration.

MicroRNAs (miRNA) are small endogenous non-coding RNA that binds to a specific sequence of target gene 3 'untranslated region (3'UTR) and affects the post-transcriptional regulation of the target gene, which might result in mRNA degradation or translation inhibition [14]. The dysregulation of miRNA in cancer is widely confirmed, and several types of research have shown that the level of miRNA expression was related to tumor metastasis [15,16]. *MiR--96* is a member of the *miR-183* cluster and has been demonstrated to act as an oncogene or tumor suppressor in tumors [17–21]. Furthermore, recent literature has identified that *miR-96* also modulated EMT by regulating the level of related protein expression. Notably, He et al. showed that *miR-96* regulated the protein of the EMT-related gene *FOXQ1*, which could be modulated by TGF-β1 in bladder cancer [22]. A study by Wang et al. implicated that *miR-96-5p* promoted proliferation and EMT by directly regulating *FOXF2* expression in oral squamous cell carcinoma [23]. Moreover, the circPTPRA and RASSF8 suppressed EMT and metastasis of non-small cell lung carcinomas also by competitive sponging *miR-96-5p* [24]. *MiR-96-5p* was markedly upregulated in PCa compared to normal tissue and it acted as an oncogene in some previous studies [25–28]. However, the mechanisms of *miR-96-5p* as a tumor promoter in PCa have not been fully elucidated. The NF-κB pathway has been studied for many years and its significant role in immunity, inflammation and other physiological processes has been fully confirmed. Previous studies showed that EMT was potently administrated by several signaling pathways, such as TGF-β, Wnt, and ErbB [29,30]. While accumulating studies have indicated that the NF-κB signaling pathway also played a crucial role in inducing and maintaining EMT in a variety of tumors [31–34]. Furthermore, some evidence has illustrated that activation of the NF-κB pathway was also related to the metastasis of PCa [16,35].

Materials and Methods

Study design

This research aimed at evaluating the prostate cancer migration and invasion by miR-96-5p-induced *NDRG1* deficiency through regulation of the NF-κB signaling pathway, with a randomized clinical trial (registered at Iranian Registry of Clinical Trials, N22021073034422), which was conducted in the Molecular Genetic and Reproductive Biology Department, Kowsar poly-clinic, Tehran, Iran. The study was approved by the Ethics Committee of Kowsar polyclinic Research Institute and all participants signed informed written consent prior to the experiment whose prostate tissues were taken.

Clinical samples and cell lines

Twenty paired PCa and adjacent normal prostate tissue were collected during surgery at the Molecular Genetic and Reproductive Biology Department, Kowsar polyclinic, Tehran, Iran. RWPE-1, LNCaP, C4-2, PC3, and DU145 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). RWPE-1 cells were grown in keratinocyte-SFM (Invitrogen, USA). LNCaP, C4-2, PC3, and DU145 were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/mL) (Solarbio, Beijing, China). All cell lines were incubated under a humidified atmosphere at 37 °C with 5% CO_2. This study has complied with the ARRIVE guidelines and was carried out in accordance with the U.K. Animals Act, 1986, EU Directive 2010/63/EU for animal experiments, as well as respecting the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Twelve replicates per each condition of the experiment were evaluated.

Western blot

After cleaning with cold PBS twice, the tissues or cells were collected, and then extracted the whole cell lysate with RIPA buffer (Solarbio, Beijing, China) concentration was measured by a de-

tection kit (Solarbio, Beijing, China) according to the manufacturer's guidance. The same amount of protein in each sample was placed in 10% gel by SDS-PAGE and then transferred to the nitrocellulose membrane. To block the membrane, 5% non-fat milk or 5% BSA (Solarbio, Beijing, China) was utilized, then incubated with primary antibodies anti-NDRG1, diluted 1 : 10 000, Ki-67, diluted 1 : 1 000, Abcam; p65, p-p65, IkBa, p-IkBa, diluted 1 : 1 000, Cell Signaling Technology; E-cadherin, N-cadherin diluted 1 : 1 000, GAPDH, diluted 1 : 2 000, vimentin, diluted 1 : 10 000 Proteintech). After incubation with the corresponding secondary peroxidase-conjugated antibody, the protein bands were displayed with chemiluminescence dissolvent (Solarbio, Beijing, China).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted by utilizing TRIzol reagent (Invitrogen, USA) following the manufacturer's guidance. Then 5 µg total RNA was transcribed to cDNA according to the protocol supplied with the HiFiScript cDNA Synthesis Kit (Cwbio, Taizhou, China). The mRNA expression was measured by SYBR Green qPCR master mix. The expression levels of all mRNA were normalized to GAPDH or U6. GAPDH is constitutively expressed in almost all tissues in high amounts. For this reason, GAPDH has been chosen as a control for protein normalization. All assays were conducted three times independently. The primers for qRT-PCR were listed as followed:

- *NDRG1* forward: 5′-GTCCTTATCAACGTGAACCCTT-3′
- *NDRG1* reverse: 5′-GCATTGGTCGCTCAATCTCCA-3′
- *GAPDH* forward: 5′-GCTCTCTGCTCCTCCTGTTC-3′
- *GAPDH* reverse: 5′-ACGACCAAATCCGTTGACTC-3′
- *miR-96-5p* forward: 5′-CCTCGATTTGGCACTAGCAC-3′
- *miR-96-5p* reverse: 5′-TATGGTTGTTCTGCTCTCTGTCTC-3′
- *U6* forward: 5′-CGCTTCGGCAGCATATAC-3′
- *U6* reverse: 5′-TTCACGAATTTGCGTGTCATC-3′

Immunofluorescence

Each group of cells was seeded on 24--well plates using glass coverslips (Beijing Solarbio, China), fixed with 4% paraformaldehyde, and infiltrated with 0.5% Triton X-100. Then, 5% BSA was used to block cells for 1 hour and incubated with the primary antibody. *NDRG1*, (diluted 1 : 200, Abcam), p-P65 (diluted 1 : 1 600, CST) at 4°C overnight, followed by incubation with FITC-labeled or TRITC-labeled secondary antibody (diluted 1 : 200, Proteintech, China) for 2 hours. Finally, the cells were stained with DAPI, and the images were detected under a confocal microscope (FV500; Olympus).

Immunohistochemical analysis

Tissue microarray was purchased from Alenabio, Xi'an (PR803d). The six tissues with lymph node metastasis were also collected from the Molecular Genetic and Reproductive Biology Department, Kowsar poly-clinic, Tehran, Iran. Standard immunohistochemistry (IHC) protocols were applied with specific antibodies following previously reported [36].

Luciferase reporter assay

The luciferase reporter assay was conducted with the Dual-Luciferase Reporter Assay kit (Promega, USA) pmirGLO; pmirGLO-WT and pmirGLO-MUT for NDRG1-3′UTR were cotransfected into 293T cell lines, together with *miR-96-5p* or *miR-183-5p* mimics, by using Lipoferctamine™ 2000 (Invitrogen, USA). After transfection in 48 hours, Renilla luciferase activity acts as a standard of the relative luciferase activity. Three independent assays were performed.

Cell transfection

The cells were transfected with the control, siRNA, miRNA mimics/inhibitors were purchased from GenePharma (Shanghai, China) using LipofectamineTM 2000 (Thermo Fisher, USA) following the manufacturer's guidance. siRNAs for *NDRG1* were defined as siNDRG1#1 and siNDRG1#2, siNDRG1#3, respectively. All siRNA are listed as followed: si1: NDRG1-homo-688, si2:NDRG1-homo-835, and si3: NDRG1-homo-956. To overexpress *NDRG1*, full-length *NDRG1* was cloned into a modified LV18 lentiviral vector (GenePharma, Shanghai, China), while an empty vector served as a control and was screened with puromycin. Luciferase express LV11 lentiviral vector (GenePharma, Shanghai, China) was transfected and screened with neomycin scratch assay. LNCaP, C4-2, PC3, and DU145 cells were added into 6-well plates with a density of 6×10^5 cells per well. When the cells are fully attached, the 2- mm scratch will divide the well into three equal parts. Next, the cells were cultured in a serum-free medium for 48 hours. The cells were detected and photographed at 0, 24 and 48 hours, respectively. The scratch healing rate was calculated by ImageJ software.

Migration and invasion assays

Cell migration and invasion abilities were identified by transwell chambers which consist of an 8-mm membrane filter covered with or without Matrigel (BD Biosciences). The cells were cultured with serum-free medium for 24 hours before use. The cells ($1–2\times10^5$) in serum-free medium were seeded to the upper chambers. Lower chambers were supplied with a medium containing 10% FBS. After incubation for 24–48 hours, the cells at the bottom of the chamber were fixed with 4% paraformaldehyde, infiltrated with 0.5% Triton X-100, stained with DAPI, and counted by a fluorescence microscope.

Animal experiment

Animal experiments have been approved by the Ethics Committee of the medical supervisory team, Molecular Genetic and Reproductive Biology Department, Kowsar poly-clinic, Tehran, Iran. This study has complied with the ARRIVE guidelines and was carried out in accordance with the U.K. Animals Act, 1986, EU Directive 2010/63/EU for animal experiments, as well as respecting the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). BALB/c nude male mice (5–6 weeks old) were raised in a room under sterile conditions. The DU145 cells with high expression of *NDRG1* were di-

rectly subcutaneously injected to establish the model of subcutaneous xenograft (N = 5 mice/group, 3.0×10^6 cells / 100 µL). The cells were added to Matrigel (BD Biosciences) with a culture medium (1:1 diluted). D-luciferin/D-PBS at a concentration of 15 mg/mL was given through intraperitoneal injections with 10 µL/g for imaging after 6 weeks of feeding and then all primary tumors were carried out. The two mice with suspected metastatic foci were kept feeding for 8 weeks. Primary tumors and suspected metastatic foci were excised, embedded, and fixed. Hematoxylin & eosin staining and immunohistochemistry analyses were conducted.

Statistical analysis

SPSS v.21.0 software (USA) and GraphPad Prism Software 7.0 (USA) were used for data assessment. Mean ± SD was used to show experimental results. Student's t-test or one-way ANOVA was used to reveal the differences between the groups. P-values < 0.05 were considered statistically significant.

Results

NDRG1 expression is decreased in PCa and affected the prognosis of patients. To investigate the clinical significance of *NDRG1* in PCa, the mRNA sequencing dataset of PCa from the Oncomine database [37] was evaluated. The dataset showed that *NDRG1* expression was decreased in PCa compared with the normal tissues and showed a declined trend with Gleason score increase (Fig. 1A, B). The expression of *NDRG1* was also downregulated in mRNA and protein levels in PCa cells (DU145, LNCaP, C4-2, PC3) compared with normal prostate epithelial cells (RWPE-1) (Fig. 1C, D). Most of the proteins are located in the cytoplasm (Fig. 1E). Our study further measured the expression levels of *NDRG1* in 20 paired PCa patients and an 80-point tissue chip combined with six metastatic specimens. The *NDRG1* expression level in PCa was also decreased. There was a tendency for a gradual decrease of *NDRG1* level in normal tissue, benign prostatic hyperplasia tissue, localized PCa, and metastatic PCa tissue judged by immunohistochemistry (Fig. 1F–H). Furthermore, the patients with a high level of *NDRG1* had higher overall survival than the low according to the dates from GSE16560 (N = 281) [38].

NDRG1 suppresses EMT, migration, and invasion in PCa cells

To determine the function of *NDRG1* in human PCa cells, we transfected lentivirus to overexpress *NDRG1* in two cell lines (LNCaP, DU145) with relatively low expression of *NDRG1* (Fig. 2A) and siRNA to silenced *NDRG1* in relatively high expression of NDRG1 cell lines (C4-2, PC3). To achieve a better-silenced effect, we selected three siRNA to identify in two cell lines (Fig. 3A). The C42 and PC3 were knocked out with siRNA1 and siRNA2, respectively. Relative to control cells, *NDRG1*-overexpressing cells significantly inhibited migration and *NDRG1* knockdown cells significantly promoted migration in the scratch assay (Fig 2. C, D, 3C, D). Transwell assays were also performed and the result indicated a consistent trend in migration. The overexpression group showed a strong ability to inhibit invasion and the knocking-down group showed a high ability to promote invasion, especially in the PC3 cell line (Fig. 2E, F, 3E, F). Furthermore, western blot analysis implicated that upregulating *NDRG1* increased the expression of E-cadherin and decreased vimentin and N-cadherin in LNCaP and DU145 cells (Fig. 2B). Conversely, silencing *NDRG1* had an opposite effect on these EMT markers (Fig. 3B). These results demonstrate that *NDRG1* suppresses migration, invasion, and EMT in PCa cells *in vitro*. *MiR-96-5p* expression is increased in PCa and promotes EMT in PCa cells. The *miR-96-5p* expression was analyzed by miRNA sequencing dataset of PCa from The Cancer Genome Atlas (TCGA) [39]. The data suggested that *miR-96-5p* was significantly upregulated in PCa compared with adjacent normal tissues (Fig. 4A). We further investigated the expression levels of *miR-96-5p* in different cell lines. Compared with the RWPE1 cell line, the expression of *miR-96-5p* was significantly increased (Fig. 4B). In the tumor tissues, we found the same trend (Fig. 4C). Then, transfection of PC3 cells was performed with mimic and inhibitor of *miR-96-5p*. The wound-healing assays revealed that overexpression or knocking down of *miR-96-5p* can promote or inhibit migration, respectively (Fig. 4D). Transwell assays further confirmed that *miR-96-5p* could promote migration and invasion of PCa (Fig. 4E). *NDRG1* is directly targeted by *miR-96-5p*, and *NDRG1* is important for miR-96-5p-mediated EMT. Our previous study found that *miR--182-5p* could target the expression of *NDRG1* [40]. Further exploration of the possible mechanism that contributed to NDRG1 downregulation in PCa, we used bioinformatics databases TargetScanHuman 7.2 to predict potential miRNA targets in *NDRG1* mRNA [41]. Interestingly, we found *miR-96-5p* and *miR-183-5p* may both have the opportunity to combine with *NDRG1* mRNA 3′-UTR binding sites. Moreover, *miR-183~96~182* as a cluster had been considered to play a significant role in tumor cell survival, proliferation, and migration. Further sequence analysis indicated the presence of only one putative binding site for *miR-96-5p* and *miR-183-5p* in the *NDRG1* 3′-UTR. Luciferase assay was carried out to detect the changes of *miR-96-5p* (Fig. 5B) and *miR-183-5p* transiently transfected with *WT NDRG1* 3′-UTR and mutated *NDRG1* 3′-UTR in 293T cells. As shown in Fig. 2, luciferase activity was remarkably repressed only in *WT NDRG1* 3′-UTR. While there was no significant change in fluorescence value when mutated *NDRG1* 3′-UTR co-transfected with *miR-96-5p* in 293T cells. Further verification of the regulatory relationship, western blot was conducted in different treatment groups in PC3 cells. Fig. 5C showed that *miR-96-5p* inhibitor could upregulate *NDRG1* expression while *miR-96-5p* mimics downregulate *NDRG1* expression. Moreover, Spearman's correlation analysis of the TCGA database demonstrated that *miR-96-5p* mRNA level was negatively correlated with *NDRG1* mRNA level in PCa tissues ($r = -0.192$, $P < 0.001$; Fig. 5D) by using the star base database. Clinical samples also demonstrated that the expression of mRNA levels was negatively correlated with each other ($r^2 = 0.397$, $P < 0.01$; Fig. 5E). There were significant differences in the ability of migration and invasion between the

control group and the group transfected with *miR-96-5p* inhibitor or si*NDRG1* in PC3 cells (Fig. 5F, G). Western blot indicating protein changes also illustrated that *NDRG1* was crucial to *miR-96-5p* mediated EMT (Fig. 5F, G).

Downregulating NDRG1 activates the NF-κB signaling pathway promoting EMT in PCa cells

To further assess the mechanism of *NDRG1* promoting the metastasis of PCa, the next generation sequence was used to compare overexpressed *NDRG1* cells in DU145 with normal cells (Fig. 6A). By comparing the go enrichment analysis of downstream pathway changes, we found that the PI3K-AKT pathway has been significantly modified (Fig. 6B). It has been confirmed that NF- κB could be activated as a downstream pathway of pAKT in many pieces of research. So, we further explored the relationship between *NDRG1* and NF-κB pathway. We analyzed the subcellular localization of phosphorylated p65 (p-p65) in PC3 and C4-2 cells using immunofluorescence. Knocking down the expression of *NDRG1* could increase the number of p-p65 and promote its nuclear translocation (Fig. 6C). Western blot also demonstrated that downregulating *NDRG1* increased the expression of p-p65 and phosphorylated IkBa (p-IkBa) in C4-2 and PC3 cells (Fig.6D). To further verify whether *NDRG1* can mediate EMT through the NF-κB pathway, we added pyrrolidinedithiocarbamate ammonium (PDTC) with 1.0 ng/mL, an inhibitor of NF-κB pathway. When PDTC was added in PC3 cells, the content of p-p65 and p-IkBa was induced as well as the expression of vimentin and N-cadherin. However, there was an increased level of p-p65 and p-IkBa when downregulating *NDRG1*. Moreover, inhibition of NF-κB signaling by PTDC impaired the stimulatory effect of *NDRG1* down-expression on EMT in PCa cells (Fig. 6E, F). The above data indicate that downregulating *NDRG1* activates the NF-κB signaling pathway promoting EMT in PCa cells.

Elevating *NDRG1* inhibits PCa metastasis *in vivo*

To determine the function of *NDRG1* on the metastasis of PCa *in vivo*, we used lentivirus to overexpress *NDRG1* in luciferase-labeled vector DU145 cells and established the xenograft model by directly subcutaneous injection of tumor cells into nude mice. As shown in Fig. 7A, compared with the control group, the metastatic ability of the overexpression group was lower. After feeding for 6 weeks, there were two mice in the control group had distant metastases, while no suspicious lesions were found in the treatment group under the imaging system. We also discovered that the tumor volume and weight were significant differences between the two groups. Upregulating *NDRG1* inhibited tumor proliferation at the same time (Fig. 7B–D). Hematoxylin & eosin staining showed the tumor *in situ* and one typical distant metastasis lesion near the spine. The red arrow indicated the metastatic tumor area (Fig. 7E). Furthermore, the immunohistochemistry of tumor tissue and western blot demonstrated that upregulating *NDRG1* dramatically reduced the level of Ki-67 and vimentin, while increasing E-cadherin (Fig. 7F, G). Consequently, these findings demonstrate that elevating *NDRG1* inhibits PCa proliferation and metastasis *in vivo*.

Discussion

The main findings of our study display novel insights that *miR-96-5p* induced *NDRG1* deficiency and activated NF-κB signal pathway, which further promoted the EMT of PCa. Here, we observed that *NDRG1* expression was decreased in PCa tissues, and high expression of *miR-96-5p* correlated with PCa cell migration and invasion. Our results further indicated that *miR-96-5p* via directly targeting NDRG1 promoted EMT in PCa cells, leading to the development of PCa metastasis. Therefore, our study uncovered novel insights that *miR-96-5p* promotes EMT through *NDRG1* and NF-κB signal pathways, elucidating the tumor suppressor gene of *NDRG1* and the oncogenic function of *miR-96-5p* in PCa. The role of *NDRG1* in inhibiting the progression of metastasis has been described in different cancer research [42–44]. Other studies also partly elucidated the mechanism of decreased levels of NDRG1 in PCa and its possible ways of regulating expression [36,40]. In fact, available evidence has indicated that *NDRG1* might act as a tumor suppressor in several PCa cell lines through different molecular mechanisms [9,11]. However, the mechanism of *NDRG1* regulating migration and invasion is still partly unknown. In our study, we illustrated that inhibiting the activation of the NF-κB pathway could change the EMT markers induced by *NDRG1* depletion, and *miR-96-5p* inhibitors overexpression also reversed the effect of *NDRG1* downregulation to the EMT markers. We also demonstrated that overexpression of *NDRG1* inhibited the proliferation and metastasis of PCa *in vivo*. Higher *miR-96-5p* expression was also accompanied by lower *NDRG1* expression in PCa tissues, which further illustrates the negative relation between the two molecules in PCa progression. More importantly, these demonstrate that *miR-96-5p* regulates *NDRG1* expression and *NDRG1* regulates EMT by modulating NF-κB activation, in part, state that *NDRG1* suppressive function in cell migration, invasion, and EMT mediate by *miR-96-5p* and NF-κB. *MiR-183* cluster is a significant gene located on the short arm of chromosome 7 (7q32.2). Three mature miRNAs (*miR-96, -182*, and *-183*) were generated from a single polycistronic transcript. The expression of the *miR-183* cluster has been documented in several cancers. Most studies assumed that the *miR-183* cluster has shown an oncogenic function in cancers, while some have suggested inhibition of these effects [43]. Other studies have illustrated that *miR-182-5p* induced *NDRG1* deficiency and promoted proliferation and metastasis in PCa cells [40]. In this study, we further explored the effect of *miR-96-5p* and *miR-183-5p* on *NDRG1*. Luciferase activity was remarkably repressed only in miR-96-5p. Several previous studies have suggested that *miR-96-5p* was upregulated in different kinds of tumors and a high level of *miR-96-5p* promoted cancer cell migration and invasion via different mechanisms and also predicted poor survival. Siu et al. illustrated that *miR-96-5p* could target the tumor suppressor ETV6, downregulated EMT markers' expression in PCa [27]. Moreover, TGF-β could regulate

the expression of *miR-96* through Smad-dependent transcription. *MiR-96* also promoted bone metastasis in PCa [26]. Notably, a study by Long et al. showed that *miR-96* controlled the AR signaling pathway and promoted PCa progression by adjusting the RARγ network [42,43]. In our study, these findings reveal that *miR-96-5p* is elevated in PCa and promoted EMT by regulating *NDRG1* expression to control NF-κB pathway activity. A large number of studies have illustrated that the NF-κB signal was significantly activated in several kinds of human tumors, which are closely associated with tumor progression and metastasis [31,42]. In glioma cancer, the activation of the NF-κB signal played a significant role in promoting cell migration and invasion [42,44]. In colorectal cancer, *DCLK1* promoted EMT via the PI3K/Akt/NF-κB pathway [32]. In addition, a study by Mei et al. has demonstrated that the level of *miR-145-5p* was induced by inhibiting the Sp1/NF-κB pathway, which limited the migration and invasion of esophageal squamous cancer cells [33]. Accumulating evidence has illustrated that NF-κB signaling played a crucial role in promoting the invasion and metastasis of PCa [16]. Shang et al. showed that lncRNA-PCAT1 induced CRPC progression by positively regulating AKT/NF-κB signaling [32]. Notably, Zhang et al. discovered that migration and invasion enhancer 1 was an NF-κB induced gene, which enhanced the proliferation and invasion of human PCa cells [23]. In the present study, we revealed that *miR-96-5p* activates the NF-κB pathway by directly targeting *NDRG1*, which promotes EMT in PCa cells. Moreover, we also demonstrated that NF-κB signaling activity plays a significant role in the invasion and migration of PCa cells.

Conclusions

In summary, we demonstrate that *NDRG1* loss is correlated with cell metastasis and poor prognosis of PCa patients. During the development of PCa, the abnormally high expression of *miR-96-5p* can promote this biological process, inhibit the expression of *NDRG1*, activate the NF-κB pathway, and promote cell EMT. Based on our findings, we propose a new molecular mechanism in which *miR-96-5p* promotes EMT by regulating *NDRG1* expression to control NF-κB pathway activation in PCa. These findings provide a new understanding of the progression of PCa and further guide clinical practices.

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Authors' contributions

Aida Jamshidian and Hengameh Ghaffari Monfared, the corresponding authors of this article declare that all the mentioned individuals in this article are members of this research team and had substantial contributions to the conception and design, acquisition of data, analysis, and interpretation of data, drafting of the article, revising it, and final approval of the version to be published.

Availability of data and materials

The data used in this study are available from the corresponding author upon request.

Consent for publication

By submitting this document, the authors declare their consent for the final accepted version of the manuscript to be considered for publication.

Declaration of interest

The authors report no conflicts of interest in this work.

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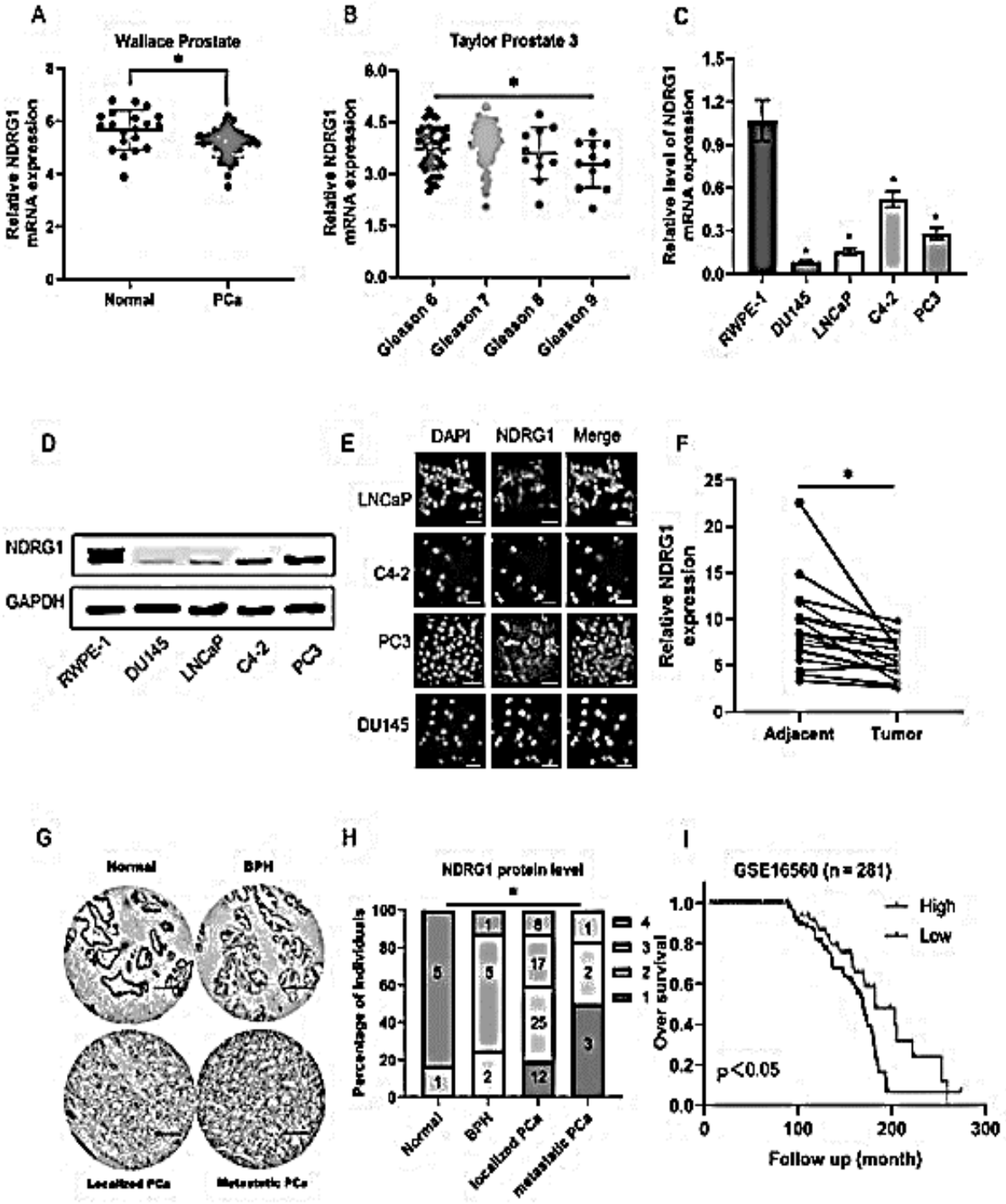

Fig. 1. NDRG1 expression is decreased in prostate cancer (PCa) and affected the prognosis of patients. A, B) *NDRG1* expression was decreased in PCa tissues compared with the normal tissues and showed a declined trend with Gleason score increased by analyzing the Oncomine PCa mRNA sequencing dataset, *P < 0.05; C) real-time PCR analysis of *NDRG1* expression

levels in the normal prostate epithelial cell (RWPE-1), PCa cell lines (LNCaP,C4-2,PC3,DU145), *P < 0.05; D) western blot analysis of each cell line; E) *NDRG1* location and relative level in each PCa cell line by immunofluorescence, scale bar 25 μm; F) 20 paired PCa tissues compared with the matched adjacent normal tissues as assessed by real-time PCR, *P < 0.05; G, H) immunohistochemistry analysis of NDRG1 in an 80-point tissue chip combined with six metastatic specimens including normal prostate tissue, benign prostatic hypertrophy, localized PCa, and metastatic PCa. The *NDRG1* staining intensity was categorized into four groups: 1 (no or poor staining), 2 (weak staining), 3 (intermediate staining), and 4 (strong staining), scale bar 250 μm; I) Kaplan-Meier survival analyses of PCa patients with high or low NDRG1 expression based on GSE16560 dataset (N = 281). The error bars in the bar plots indicate standard deviations.

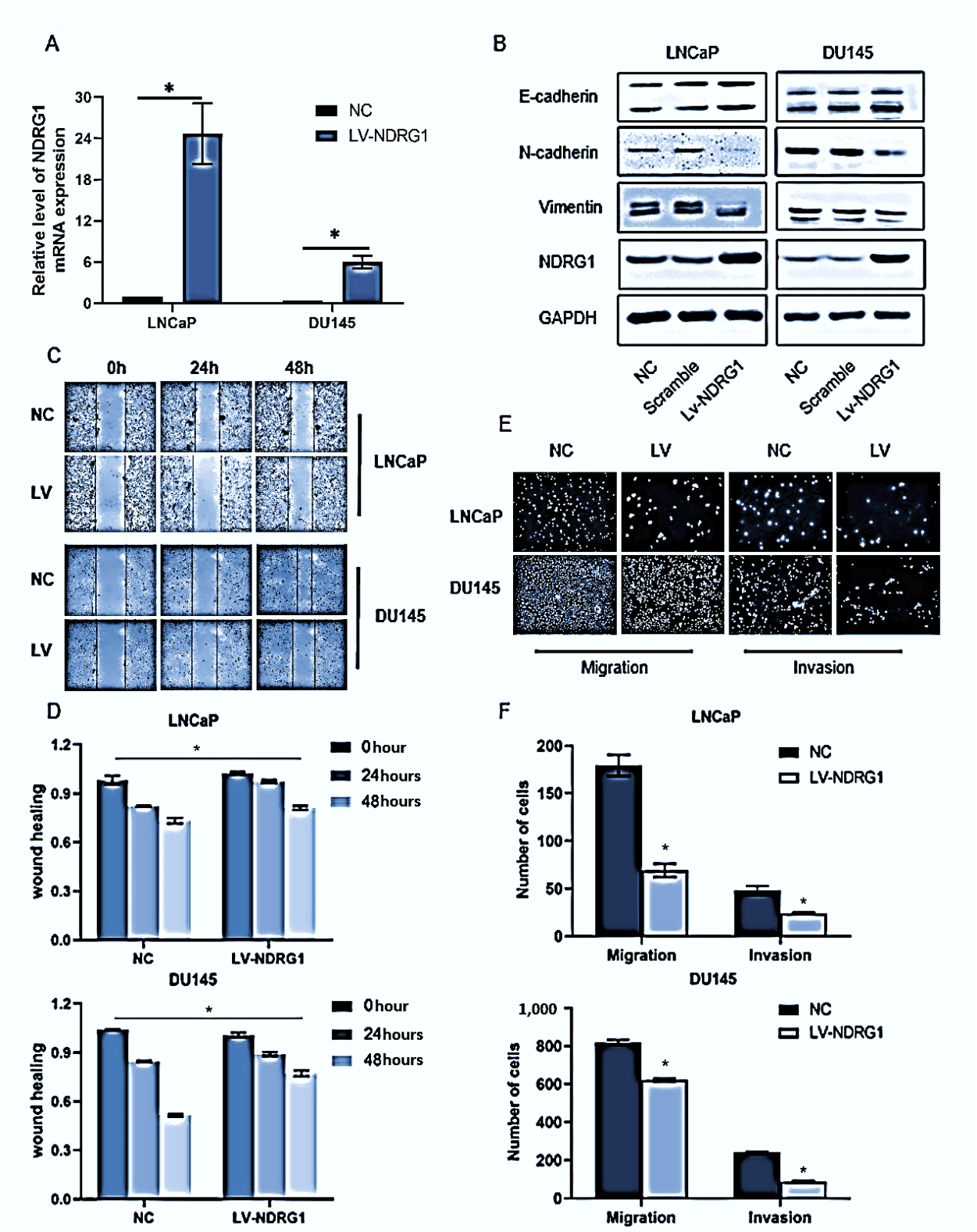

Fig. 2. Overexpression *NDRG1* suppresses epithelial-mesenchymal transition, migration, and invasion in prostate cancer cells. A) Real-time PCR validates the effect of overexpression *NDRG1* in two cell lines (LNCaP, DU145), *P < 0.05; B) overexpression of *NDRG1* increased

E-cadherin expression and decreased vimentin and N-cadherin expression in LNCaP and DU145 cells; C–F) overexpression of *NDRG1* enhanced migration and invasion, *P < 0.05. The error bars in the bar plots indicate standard deviations.

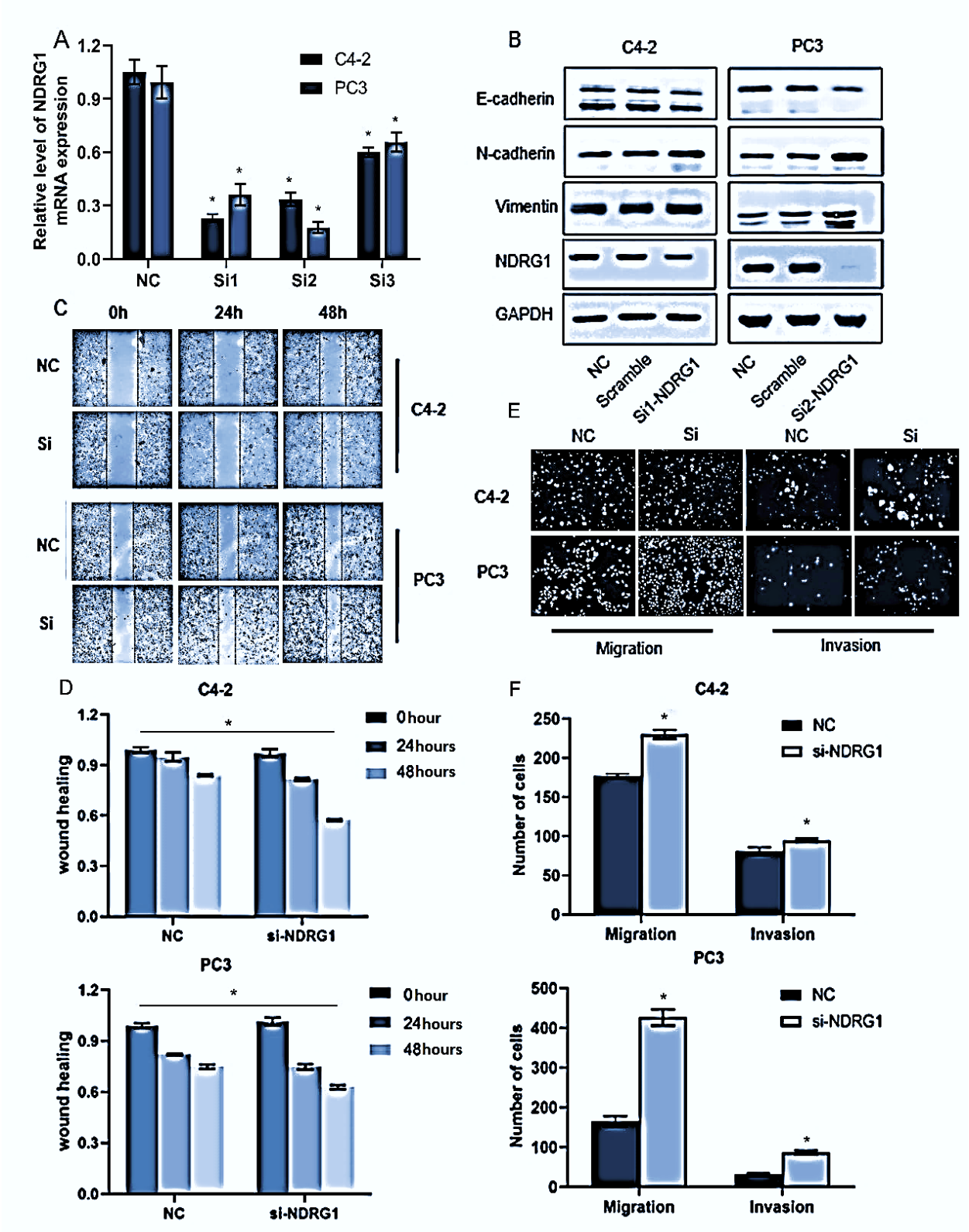

Fig. 3. Silencing *NDRG1* promotes epithelial-mesenchymal transition, migration, and invasion in prostate cancer cells. A) Real-time PCR validates the effect of downregulating *NDRG1* in two cell lines (C4-2, PC3) by transfecting with three siRNA, *P < 0.05; B) silencing

NDRG1 decreased E-cadherin expression and increased vimentin and N-cadherin expression in C4-2 and PC3 cells; C–F) downregulation of *NDRG1* enhanced migration and invasion, *$P < 0.05$. The error bars in the bar plots indicate standard deviations.

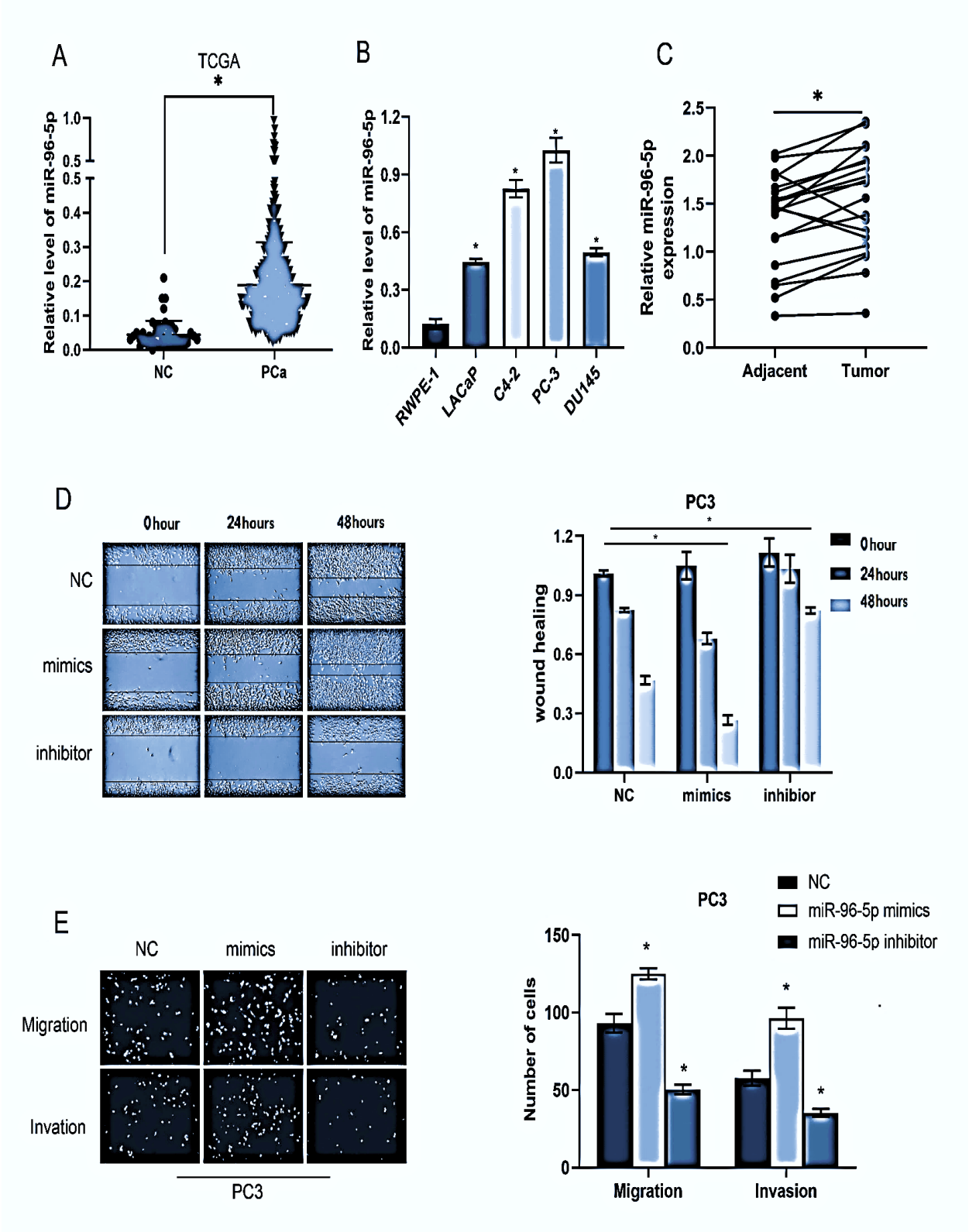

Fig. 4. miR-96-5p expression is increased in prostate cancer (PCa) cells and promotes epithelial-mesenchymal transition in PCa cells. A) MiR-96-5p expression in PCa based on TCGA, *P < 0.05; B) real-time PCR analysis of *miR-96-5p* expression levels in the normal

prostate epithelial cell (RWPE-1), PCa cell lines (LNCaP,C4-2,PC3, DU145), *P < 0.05; C) real-time PCR analysis of *miR-96-5p* expression levels in adjacent tumor tissues and tumor tissues, *P < 0.05; D–E) overexpression of *miR-96-5p* enhanced while silencing *miR-96-5p* suppressed invasion and migration abilities in the PC3 cell line, *P < 0.05. The error bars in the bar plots indicate standard deviations.

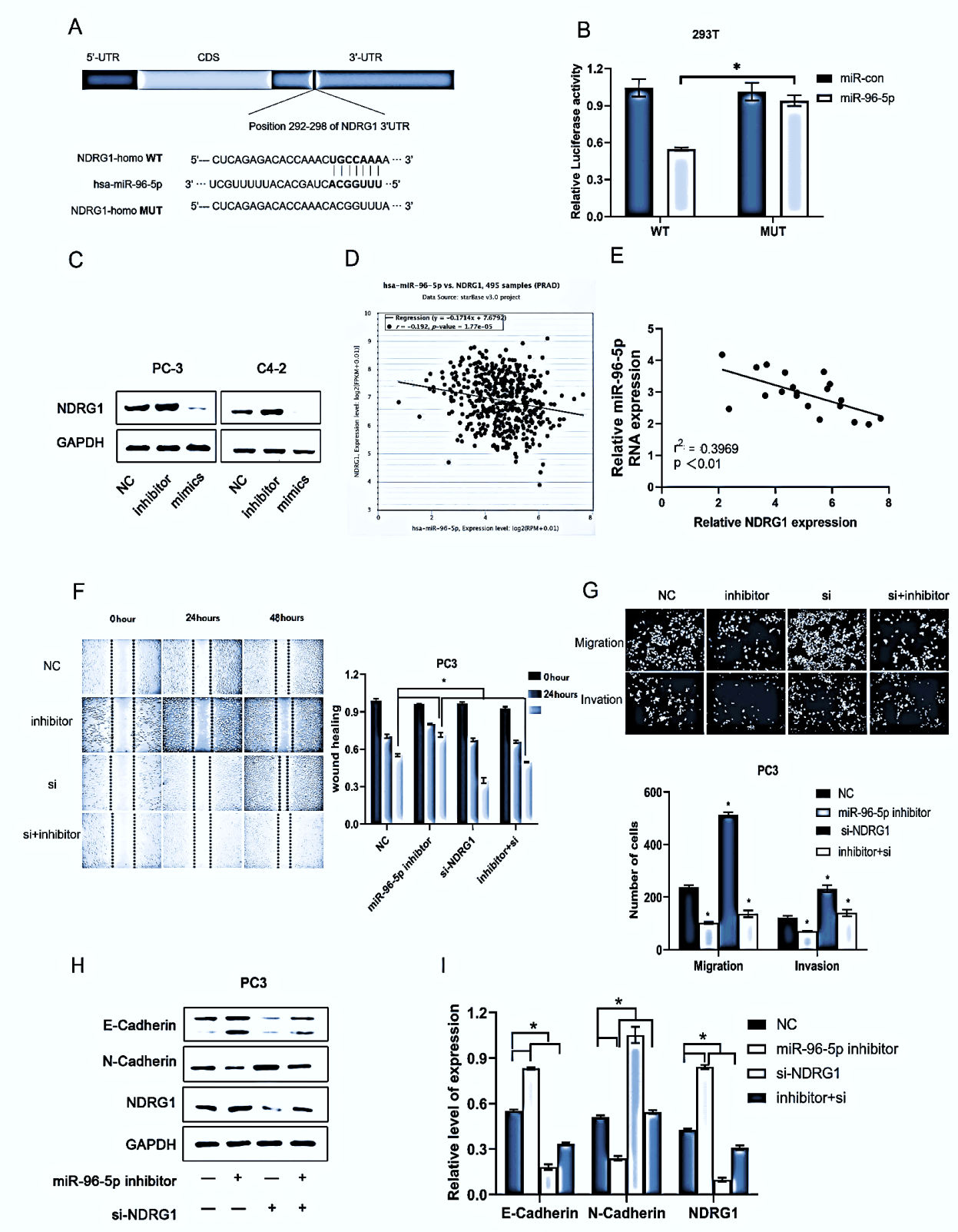

Fig. 5. NDRG1 is directly targeted by miR-96-5p, NDRG1 is important for miR-96-5p-mediated epithelial-mesenchymal transition. A) miR-96-5p-binding sequence in *NDRG1*

3'UTR. A) Mutation was generated in *NDRG1* 3'UTR in the complementary site for *miR-96-5p* binding; B) the luciferase reporter assay was used to validate the relationship between *miR-96-5p* and *NDRG1* in 293T cells, *P < 0.05; C) downregulation of *miR-96-5p* increased NDRG1 expression, while overexpression of *miR-96-5p* decreased *NDRG1* expression; D,E) Spearman's correlation analysis of the TCGA database and clinical samples showed that *miR-96-5p* expression was negatively correlated with *NDRG1* mRNA level in prostate cancer tissues; F,G) the ability of migration and invasion in PC3 cells transfected with *miR-96-5p* inhibitor, siNDRG1, or co-transfection compared with the control group; H,I) western blot indicating proteins changed in PC3 cells, *P < 0.05. The error bars in the bar plots indicate standard deviations.

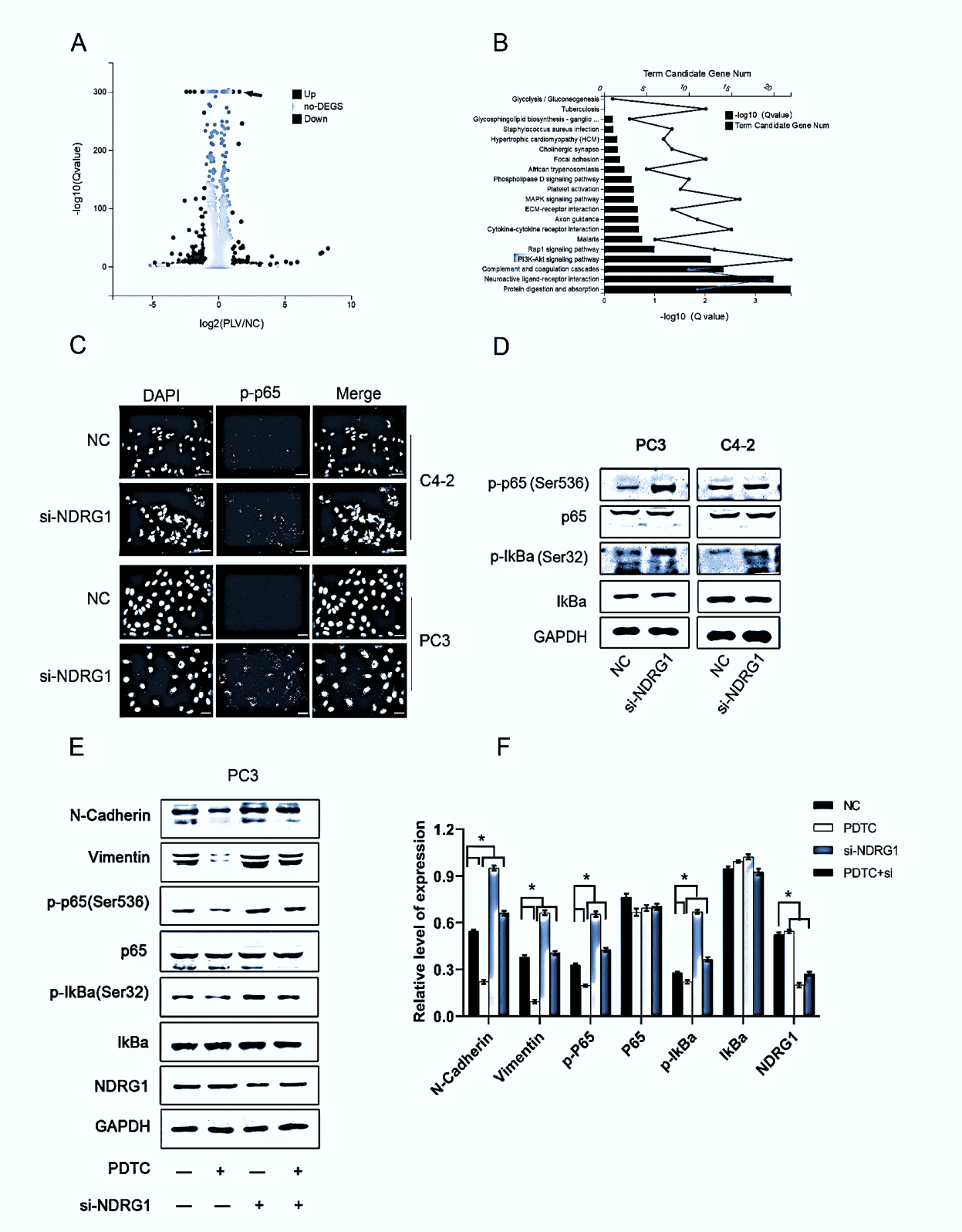

Fig. 6. Downregulating *NDRG1* activates NF-kB signaling pathway promoting epithelial-mesenchymal transition in prostate cancer cells. A) Volcano plot of genes changed between the treatment group and control group in DU145 cells (the red arrow refers to overexpressed

NDRG1); B) go enrichment analysis of the downstream pathway changes gene number; C) immunofluorescence shows that knockdown of the expression of *NDRG1* could increase the number of phosphorylated p65 (p-p65) and increase its nuclear translocation in C4-2 and PC3 cell scale bar 25 μm; D) western blot analysis of NF-kB pathway proteins in C42 and PC3 cells when suppressed *NDRG1* level. E,F) western blot used to assess the effects of *PDTC* or *NDRG1* on the protein levels of NF-kB pathway proteins in PC3 cells, *$P < 0.05$. The error bars in the bar plots indicate standard deviations.

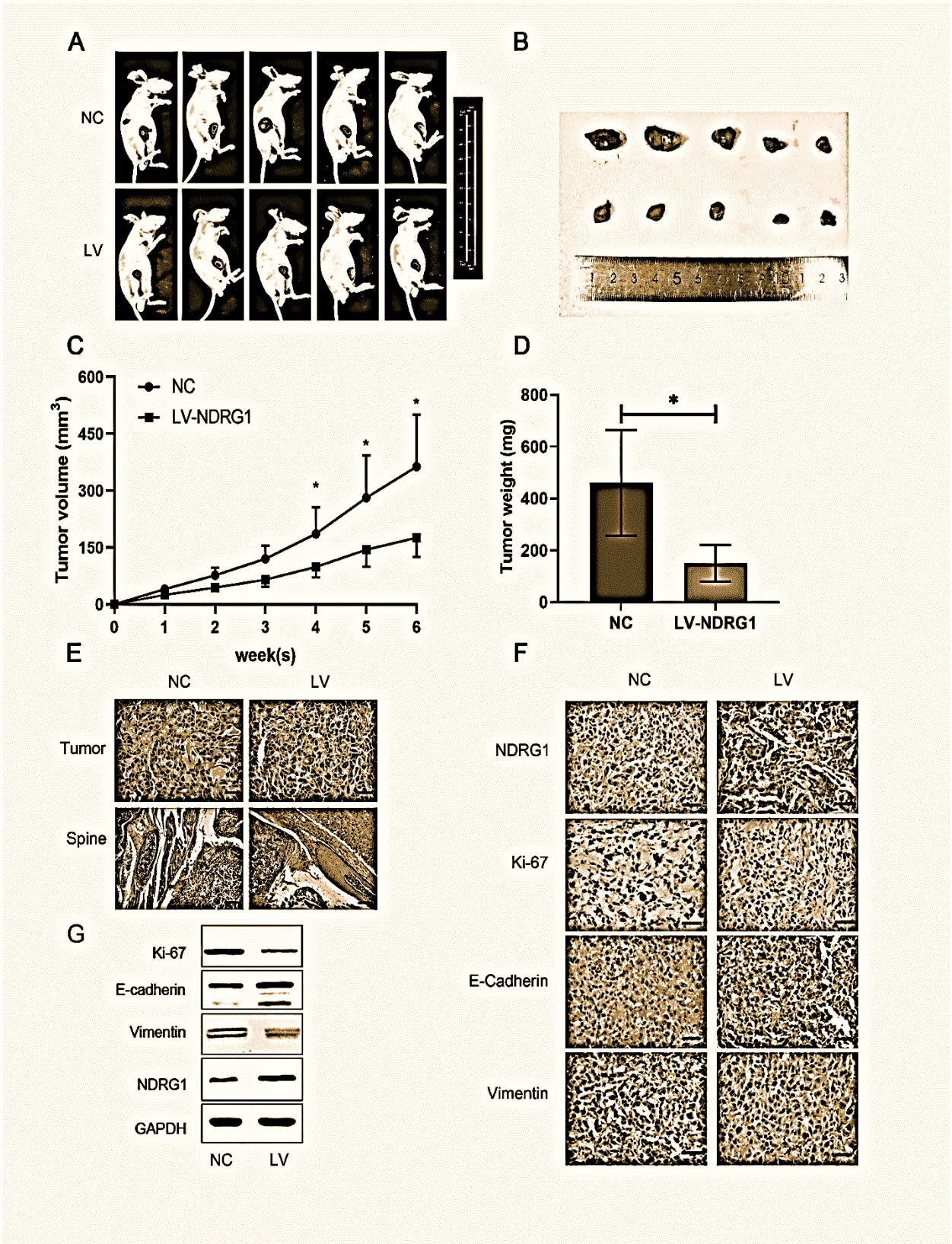

Fig. 7. Elevating *NDRG1* inhibits prostate cancer metastasis *in vivo*. A) Images of the tumors in the subcutaneous mouse model; B) images of subcutaneous tumors formed by the DU145

cells; C,D) growth curves and weight analyses of subcutaneous tumors formed by DU145 cells, *P < 0.05; E) hematoxylin & eosin staining show tumor *in situ* and one distant metastasis lesion near the spine, the red arrow indicated the metastatic tumor area, scale bar 25 μm (tumor),100 μm (spine); F) immunohistochemical staining of a tumor *in situ*, scale bar 25 μm; G) western blot of proteins changed in tumor tissues. The error bars in the bar plots indicate standard deviations.