Original Article
Diosgenin inhibits the expression of NEDD4 in prostate cancer cells

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Abstract: Prostate cancer is the second most common malignancy among men and causes a myriad of health problem for males that are diagnosed with the cancer. Although the 5-year relative survival rate of prostate cancer patients has been significantly increased due to prostate-specific antigen testing and treatment advances, patients that develop metastatic castrate-resistant prostate cancer continue to have poor survival rates. Thus, it is critical to discover new therapeutics to treat prostate cancer. Diosgenin is a steroidal saponin from Trigonella foenum grae-cum, which has been previously identified to exert anti-tumor properties. Neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) is an E3 ligase that degrades multiple different proteins, and plays an oncogenic role in human cancer. In this study, we explore the molecular mechanism by which diosgenin mediates anti-tumor effects in prostate cancer cells. We found that diosgenin treatment led to cell growth inhibition, apoptosis and cell cycle arrest. Notably, we found that diosgenin inhibited the expression of NEDD4 in prostate cancer cells. Furthermore, overexpression of NEDD4 overcame the diosgenin-mediated anti-tumor activity, while downregulation of NEDD4 promoted the diosgenin-induced anti-cancer function in prostate cancer cells. Our findings indicate that diosgenin is a potential new inhibitor of NEDD4 in prostate cancer cells.

Keywords: NEDD4, prostate cancer, diosgenin, growth, invasion

Introduction

Among men worldwide, prostate cancer is the second leading cancer diagnosis and causes a significant public health problem [1]. Almost 1.3 million new cases of prostate cancer and 358,989 deaths are projected to occur globally [1]. In addition, 164,690 new cases of prostate cancer were projected to be diagnosed in the United States in 2018, which would make it the most common cancer type affecting males in the United States [2]. This year, 29,430 deaths from prostate cancer are expected to occur in the United States, which among men is second only behind to lung cancer [2]. Earlier diagnoses due to widespread PSA (prostate specific antigen) testing combined with treatment advances have contributed to a significant increase in the 5-year relative survival rate of prostate cancer patients [3]. Currently, the therapeutic strategy for prostate cancer includes surgery, radiation, chemotherapy, and hormonal ablation therapy [4]. However, patients with prostate cancer can develop mCRPC (metastatic castrate-resistant prostate cancer) due to androgen deprivation treatment resistance, which has poor survival rates [5]. Therefore, it is necessary to identify new therapeutics to treat prostate cancer.

Diosgenin is a steroidal saponin that is commonly isolated from Trigonella foenum grae-cum, and has been identified to exert multiple anti-tumor properties [6]. For example, diosgenin inhibits cell proliferation, and induces apoptosis, and causes cell cycle arrest. Mechanistically, diosgenin upregulates p53 and caspase-3 activity, and it releases apoptosis inducing factor (AIF) in human cancer cells [7]. Studies also show that diosgenin treatment leads to cell cycle arrest and apoptosis due to activation of cPLA2 (calcium-sensitive cyto-

solic phospholipase A2) and COX-2 (cyclooxygenase-2) overexpression in erythroleukemia cells [8, 9]. Moreover, the effect of diosgenin on breast cancer cells was evaluated in an electrochemical study, which revealed that diosgenin effectively inhibits viability and proliferation [10]. Diosgenin also inhibits pAkt expression and Akt kinase activity, which subsequently downregulates downstream Akt targets, such as NF-κB (nuclear factor kappaB), Bcl-2 (B-cell lymphoma 2), Survivin and XIAP (X-linked inhibitor of apoptosis protein) in breast cancer cells [11]. Diosgenin induces apoptosis due to HMG (3-hydroxy-3-methylglutaryl) CoA suppression in human colon carcinoma cells [12]. In these same cells, diosgenin induces apoptosis via COX-2 (cyclooxygenase 2) and LOX-5 (5-lipoxygenase) regulation [13]. Diosgenin also inhibits cell proliferation through autophagy and apoptosis cascades that are downstream of PI3K (phosphoinositide 3-kinase)/Akt/mTOR (mammalian target of rapamycin complex) inhibition in prostate cancer cells [14]. Furthermore, diosgenin retards cell migration and invasion by reducing the expression of MMP-2 (matrix metalloproteinase-2) and MMP-9 in prostate cancer cells [15]. Although prior reports have identified a function for diosgenin in human cancer cells, including prostate cancer cells, the underlying molecular mechanisms have not been fully elucidated.

In the current study, we explored the molecular basis of diosgenin-mediated tumor suppression in prostate cancer cells. We found that diosgenin exposure induced cell growth inhibition, apoptosis, and cell cycle arrest. Moreover, we found that the expression of NEDD4 in prostate cancer cells was inhibited by diosgenin. Furthermore, overexpression of NEDD4 overcame the anti-tumor activity in prostate cancer cells that was mediated by diosgenin. NEDD4 downregulation promoted diosgenin-induced anti-cancer effects. Our findings indicate that diosgenin could be a new therapeutic inhibitor of NEDD4 in prostate cancer cells.

Materials and methods

Cell culture

Human PC-3 prostate cancer cells were purchased from the Chinese Academy of Science (Shanghai, China) and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified atmosphere with 5% CO_2.

Reagents

Diosgenin, MTT (3-(4, 5-dimethylthiazol-2- yl)-2, 5-diphenyltetrazolium bromide), and the anti-tubulin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorescein (FITC)-Annexin V Apoptosis Detection Kit was purchased from Beyotime Biotechnology (Shanghai, China). Calcein-AM was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Anti-NEDD4 (#2740), anti-p73 (#14620), TAZ (#4883), LATS1 (#3477), and anti-pAkt (#4060) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies were purchased from Thermo Fisher Scientific.

Cell proliferation assay

PC-3 cells (5×10^3 cells/well) were seeded in 96-well plates and cultured overnight. Then, the cells were treated with 0, 25, 50, 75, or 100 µM of diosgenin. After 48 and 72 h of diosgenin exposure, MTT assay was performed to measure PC-3 cell proliferation as described previously [16].

Cell apoptosis analysis

PC-3 cells (1×10^5 cells/well) were seeded in 6-well plates and cultured overnight. Then, the cells were treated with 0, 50 or 75 µM of diosgenin for 48 h. Cells were then collected and apoptosis was analyzed using Flow cytometric with the Annexin V-FITC/PI Kit, as described previously [16].

Cell cycle assay

PC-3 cells (1×10^5 cells/well) were seeded in 6-well plates and cultured overnight. Cells were then treated with different concentrations of diosgenin for 48 h. Flow cytometry was used to analyze the cell cycle stage, as describe previously [16].

Wound healing assay

PC-3 cells were incubated in 6-well plates until cells were more than 90% confluent. A scratch wound was then made in the cell monolayer using a sterile pipette tips. After 20 h, the wound area was photographed with an inverted

microscope and analyzed, as reported previously [16].

Transwell cell invasion assay

The treated PC-3 cells were cultured in 24-well Transwell chambers (8 µm pore size, Corning, NY, USA) that had a Matrigel coating. The FBS-free RPMI medium was added in the upper chamber of the inserts, and complete RPMI medium was added in the lower chamber. After 20 h, the invasive cells that had attached to the lower membrane surface were stained with Calcein-AM for 20 min and counted under a light microscope.

Transfection

PC-3 cells were seeded in six-well plates and transfected with NEDD4 siRNA (GenePharma, Shanghai, China), or control siRNA or NEDD4 cDNA construct for 48 hours using Lipofectamine 3000 following the manufacture's instruments.

Western blot analysis

PC-3 cells (1×10^6 cells/well) were seeded in 6-well plates and cultured. After treatment with diosgenin or NEDD4 cDNA constructs transfection or NEDD4 siRNA, or combinations for 72 h, cells were harvested and lysed in lysis buffer. The sample protein concentrations were measured with the BCA protein assay. The protein samples were separated by SDS-PAGE and transferred onto a PVDF membrane. The western blotting analysis was completed as described previously [16].

Statistical analysis

The data are represented as the mean ± SD (standard deviation) and were analyzed with GraphPad Prism 5.0 (Graph Pad Software, La Jolla, CA). ANOVA was used to compare groups and $P < 0.05$ was considered statistically significant.

Results

Diosgenin inhibits cell growth in PC-3 cells

To investigate whether diosgenin could inhibit the cell growth in prostate cancer cells, the MTT assay was used to evaluate PC-3 cell proliferation after diosgenin exposure. PC-3 cells were treated with 0, 25, 50, 75, or 100 µM diosgenin for 48 h and 72 h. We found that diosgenin treatment inhibited cell growth in a dose-dependent manner (**Figure 1A**). Specifically, a 50 µM diosgenin treatment for 72 h induced approximately 50% cell growth inhibition (**Figure 1A**). Moreover, exposure to 75 µM diosgenin for 72 h induced 70% cell growth inhibition (**Figure 1A**). These findings demonstrated that diosgenin inhibited cell growth in prostate cancer cells. On the basis of these data, 50 µM and 75 µM diosgenin treatments were used for the remaining experiments.

Diosgenin induces cell apoptosis in PC-3 cells

In order to determine the impact of diosgenin on apoptosis death in prostate cancer cells, flow cytometry analysis was performed following diosgenin treatment. PC-3 cells were exposed to 50 µM or 75 µm of diosgenin for 48 h, following by double staining with Annexin V-FITC and PI. We found that diosgenin treatment induced cell apoptosis (**Figure 1B**). Indeed, 50 µM and 75 µM of diosgenin increased the number of apoptotic cells from 3.25% in control group to 8.72% and 18.81%, respectively (**Figure 1B**). Our data suggest that diosgenin promotes cell apoptosis in prostate cancer cells.

Diosgenin induces G2/M cell cycle arrest

Next, we investigate the effects of diosgenin on the cell cycle in prostate cancer cells. PC-3 cells were exposed to different concentrations of diosgenin for 48 h followed by labeling with PI. Our data indicated that diosgenin induced cell cycle arrest at G2/M phase. More specifically, 50 µM and 75 µM diosgenin treatment increased the G2/M phase cell population from 12.84% in the control group to 17.21% and 29.67%, respectively (**Figure 1C**). These results indicate that diosgenin exposure led to G2/M cell cycle arrest in prostate cancer cells, which could contribute to diosgenin-induced cell growth inhibition.

Diosgenin decreases cell migration and invasion

To explore the inhibitory effects of diosgenin on prostate cancer cell migration in prostate cancer cells, a wound healing assay was used to
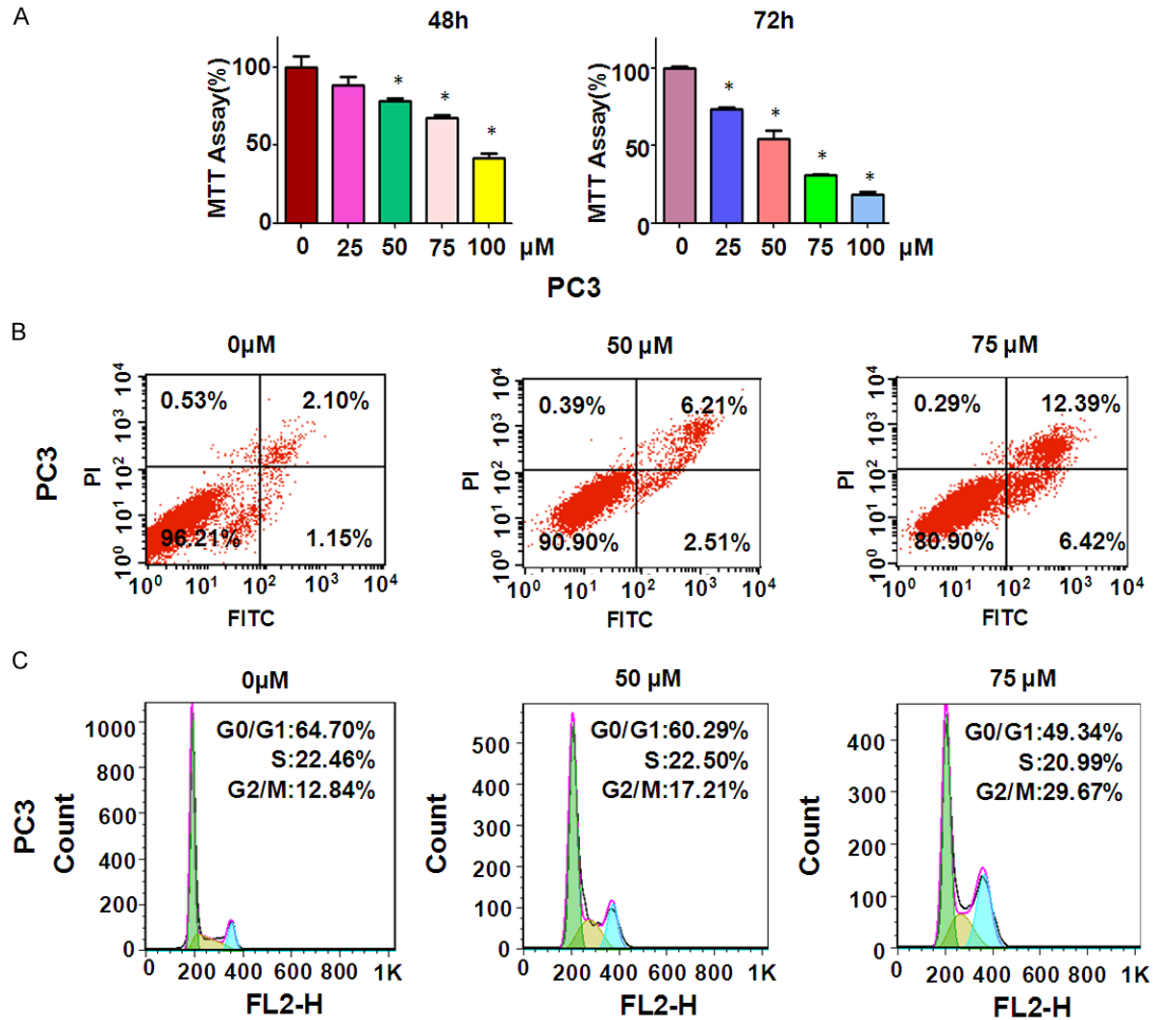
Figure 1. Diosgenin inhibits cell growth and induces apoptosis and cell cycle arrest. A: PC-3 cells were seeded in 96-well plates and cultured overnight, followed by treatment with 0, 25, 50, 75, or 100 μM of diosgenin. After treatment with diosgenin for 48 h and 72 h, the MTT assay was carried out to measure cell growth. *P < 0.05, when compared to the control group. B: PC-3 cells (1×10^{5} cells/well) were seeded in 6-well platesand cultured overnight, following by treatment with 50 μM and 75 μM diosgenin for 48 h. Annexin V-FITC/PI staining and flow cytometry were used to measure apoptotic cell death. C: Diosgenin-induced PC-3 cell cycle arrest was measured using PI staining and flow cytometry.

detect PC-3 cell motilitys after diosgenin exposure. Our data revealed that diosgenin inhibited cell migration in a dose-dependent manner (**Figure 2A**). Next, we evaluated cell invasion in PC-3 cells after diosgenin treatment using a Transwell assay. We observed that diosgenin exposure led to decreased cell invasion in PC-3 cells (**Figure 2B**). Moreover, cell invasion was impeded in a concentration-dependent manner (**Figure 2B**). Our data indicate that diosgenin could impede cell migration and invasion in prostate cancer cells.

Diosgenin inhibits NEDD4 expression

NEDD4 is a known oncoprotein in human cancer. Therefore, we investigated the role of diosgenin on NEDD4 expression in prostate cancer cells. Using western blotting, the data indicated that diosgenin exposure inhibits NEDD4 expression in PC-3 cells (**Figure 2C**). Moreover, the expression of two downstream targets, p73 and p-Akt, was also regulated by the diosgenin treatment (**Figure 2C**). Specifically, expression of p73 was significantly increased in PC-3 cells
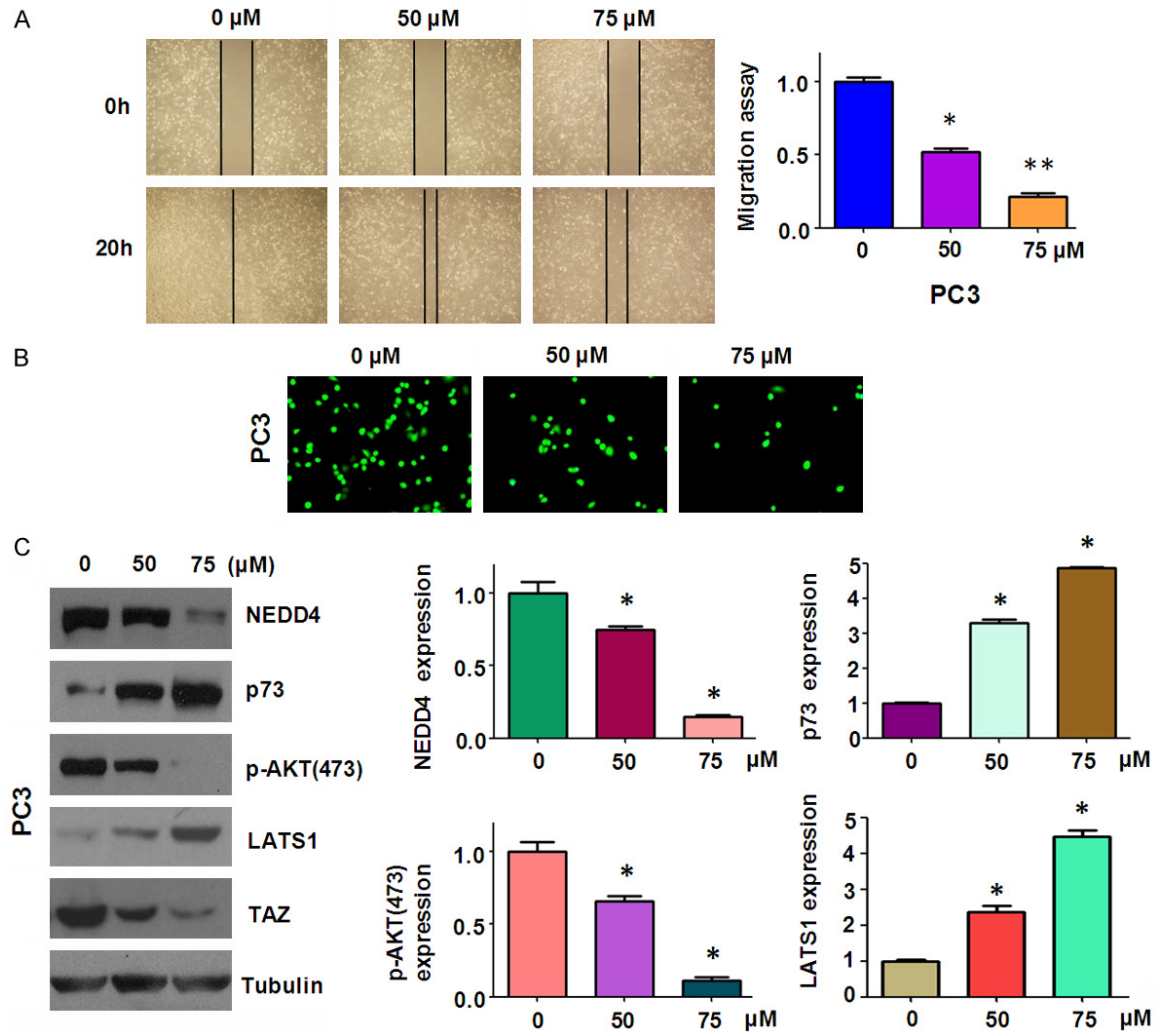
Figure 2. Diosgenin inhibits cell migration, invasion, and NEDD4 expression. A: Left panel: The inhibitory effects of diosgenin on PC-3 cell migration were analyzed with a wound healing assay in PC-3 cells after 20 h diosgenin treatment. Right panel: Quantification of the data presented in the left panel. *P < 0.05, **P < 0.01, when compared to the control group. B: Left panel: The inhibitory effect of diosgenin on cell invasion was measured with a Transwell assay. C: The expression of NEDD4, p73, LATS1, TAZ, and pAkt was measured with Western blotting analysis in PC-3 cells after exposure to disogenin for 72 h. *P < 0.05, **P < 0.01, when compared to the control group.

after exposure to diosgenin (**Figure 2C**). Conversely, p-Akt levels were remarkably decreased in diosgenin-treated PC-3 cells (**Figure 2C**). We also measured the expression of LATS1 and TAZ in PC-3 cells after diosgenin treatment. We found that diosgenin increased LATS1 and inhibited TAZ expression (**Figure 2C**). Collectively, diosgenin inhibited the expression of NEDD4 in prostate cancer cells.

Overexpression of NEDD4 rescues diosgenin-induced cell growth inhibition and apoptosis

To further confirm that diosgenin exerted its anti-tumor activity via inhibition of NEDD4 ex-

pression in prostate cancer, PC-3 cells were transfected with a NEDD4 cDNA construct or an empty vector. The results from western blotting indicated that NEDD4 expression was increased after PC-3 cells were transfected with NEDD4 cDNA vector (**Figure 3A**). Notably, NEDD4 cDNA transfection abolished NEDD4 inhibition that was induced by the diosgenin treatments (**Figure 3A**). Furthermore, overexpression of NEDD4 decreased p73 and LATS1 expression, and increased p-Akt expression (**Figure 3A**, **3B**). NEDD4 overexpression also abrogated diosgenin-mediated p73 and LATS1 inhibition and p-Akt activation in PC-3 cells (**Figure 3A**, **3B**). Our MTT assay results indicat-
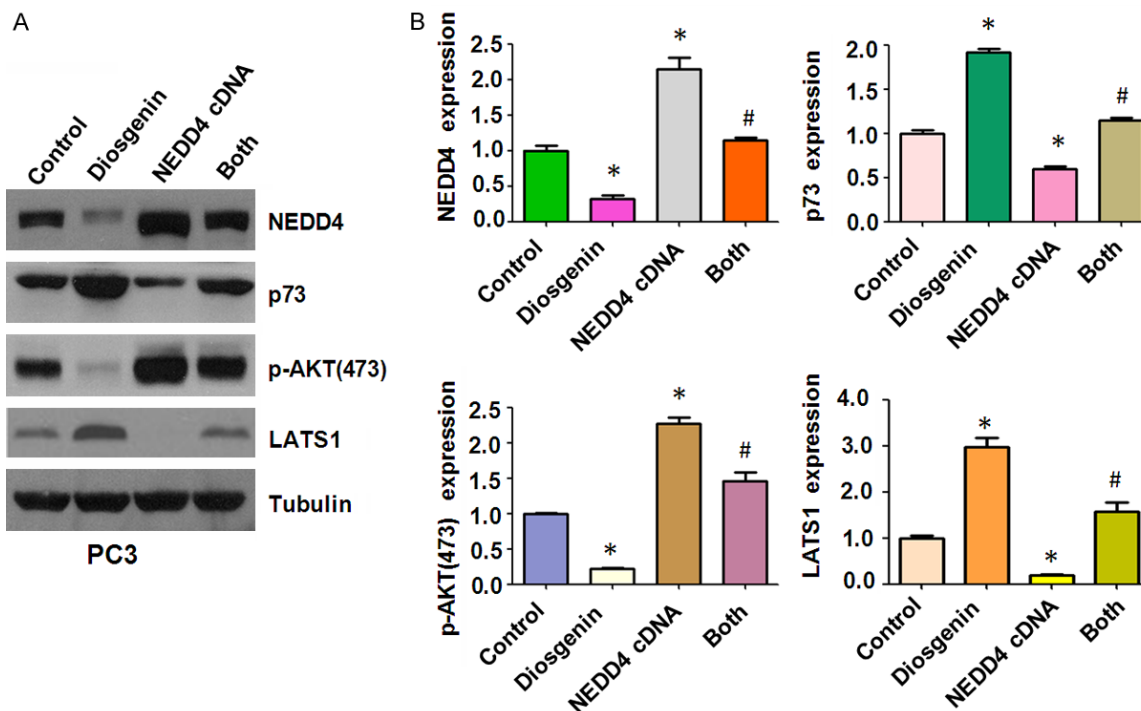
Figure 3. NEDD4 overexpression rescues diosgenin-inhibited NEDD4 levels. (A) The expression of NEDD4, p73, LATS1, and pAkt was determined by Western blotting analysis in PC-3 cells after exposure to diosgenin in combination with NEDD4 cDNA transfection. (B) Quantification of the data presented in (A). *P < 0.05, when compared to the control group; #P < 0.05, when compared to diosgenin treatment alone or NEDD4 cDNA transfection alone.

ed that overexpression of NEDD4 enhanced cell growth in PC-3 cells (**Figure 4A**). Consistent with these data, NEDD4 overexpression abolished cell growth inhibition that was induced by diosgenin treatment (**Figure 4A**). Overexpression of NEDD4 also inhibited cell apoptosis, and abolished diosgenin-induced cell apoptosis in PC-3 cells (**Figure 4B**). Diosgenin exhibits anti-tumor effects including apoptosis through down-regulation of NEDD4 in prostate cancer cells.

Overexpression of NEDD4 abolishes diosgenin-induced motility inhibition

To determine the underlying mechanism of impaired cell motility induced by diosgenin, wound healing assay was performed to measure in PC-3 cell migration after diosgenin exposure and in combination with NEDD4 cDNA transfection. We found that overexpression of NEDD4 enhanced migration of PC-3 cells (**Figure 4C**). In addition, NEDD4 upregulation abolished diosgenin-induced inhibition of cell migration in PC-3 cells (**Figure 4C**). Our Transwell assay showed that NEDD4 overexpression promoted an invasive phenotype in PC-3 cells, and

also abrogated diosgenin-mediated suppression of cell invasion (**Figure 4D**). These results suggest that diosgenin inhibits cell motility inhibition partly by down-regulation of NEDD4 in prostate cancer.

Downregulation of NEDD4 enhanced diosgenin-induced tumor suppressive activity

To further investigate the role of NEDD4 in diosgenin-mediated anti-tumor effects, NEDD4 siRNA was transfected into PC-3 cells that were also treated with diosgenin. We found that NEDD4 siRNA transfection inhibited NEDD4 expression in PC-3 cells (**Figure 5A**, **5B**). Notably, the inhibition of NEDD4 expression was more pronounced when PC-3 cells were subjected to the combined NEDD4 siRNA and diosgenin treatment when compared to NEDD4 siRNA alone or diosgenin treatment alone (**Figure 5A**, **5B**). Moreover, NEDD4 siRNA transfection plus diosgenin exposure in PC-3 cells downregulated pAkt to a greater extent than either NEDD4 siRNA alone or diosgenin exposure alone (**Figure 5A**, **5B**). Consistent with these data, p73 and LATS1 expression was significantly increased with the combined NEDD4 siRNA transfec-
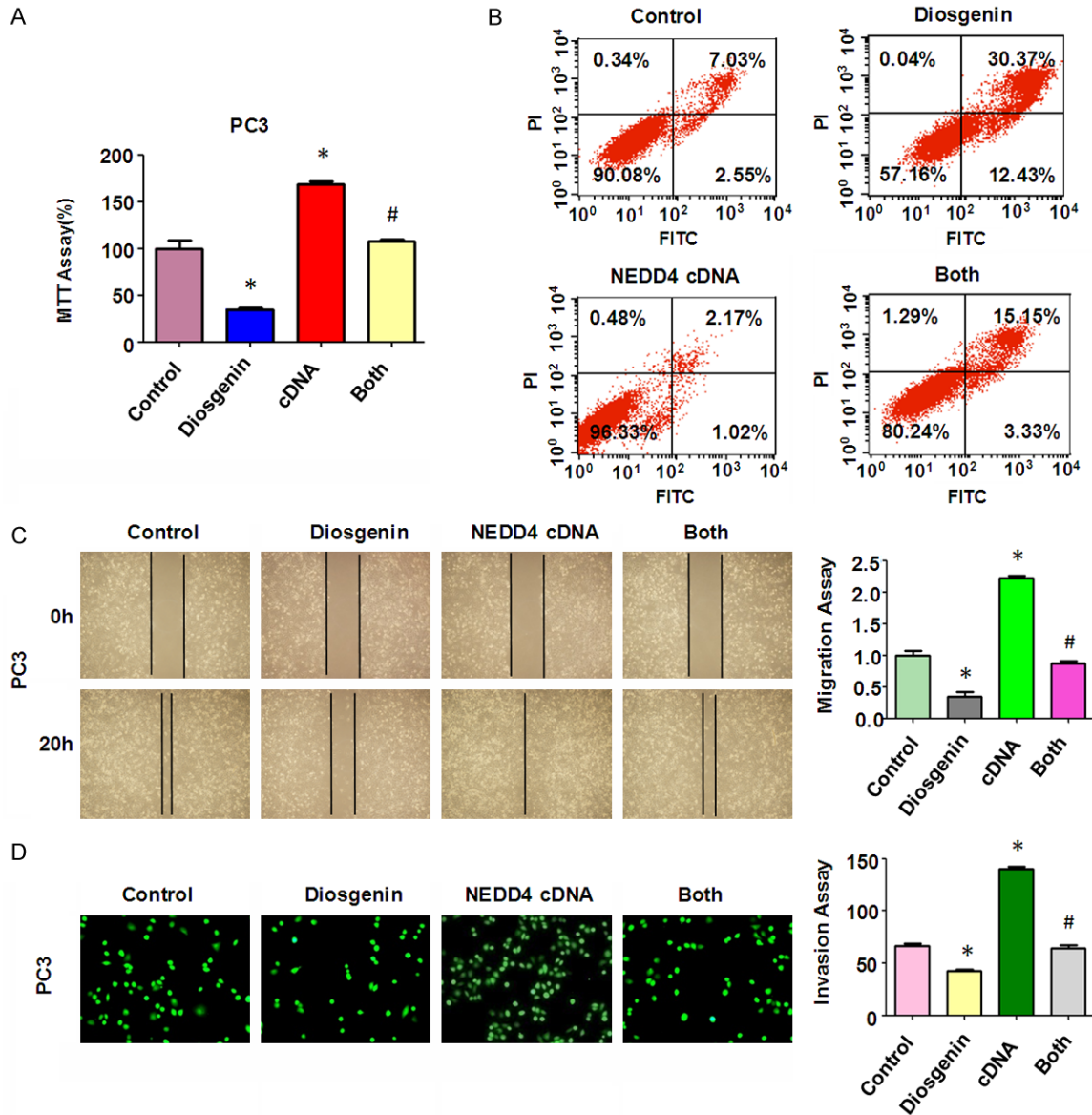
Figure 4. NEDD4 overexpression abrogates diosgenin-induced anti-tumor activity. A: The MTT assay was used to measure the growth of PC-3 cells after diosgenin exposure in combination with NEDD4 cDNA transfection. B: Cell apoptosis was detected with Annexin V-FITC/PI staining and flow cytometry in PC-3 cells after diosgenin exposure in combination with NEDD4 cDNA transfection. C: A wound healing assay was used to measure the migration of PC-3 cells after diosgenin exposure in combination with NEDD4 cDNA transfection. D: A Transwell chamber invasion assay was used to detect PC-3 cell invasiveness after diosgenin exposure in combination with NEDD4 cDNA transfection. *P < 0.05, when compared to the control group; #P < 0.05, when compared to diosgenin exposure alone or NEDD4 cDNA transfection alone.

tion and diosgenin exposure in PC-3 cells (**Figure 5A, 5B**). Moreover, MTT data indicated that downregulation of NEDD4 with siRNA transfection inhibited cell growth, and enhanced diosgenin-induced cell growth suppression (**Figure 6A**).

We also found that downregulation of NEDD4 induced cell apoptosis in PC-3 cells (**Figure 6B**).

Diosgenin exposure in combination with NEDD4 siRNA transfection increased cell apoptosis to a greater degree in PC-3 cells (**Figure 6B**). Wound healing assay results indicated that NEDD4 downregulation decreased cell migration, and promoted the inhibitory effects of cell migration induced by diosgenin exposure in PC-3 cells (**Figure 6C**). Finally, the results from Transwell invasion assay showed that down-
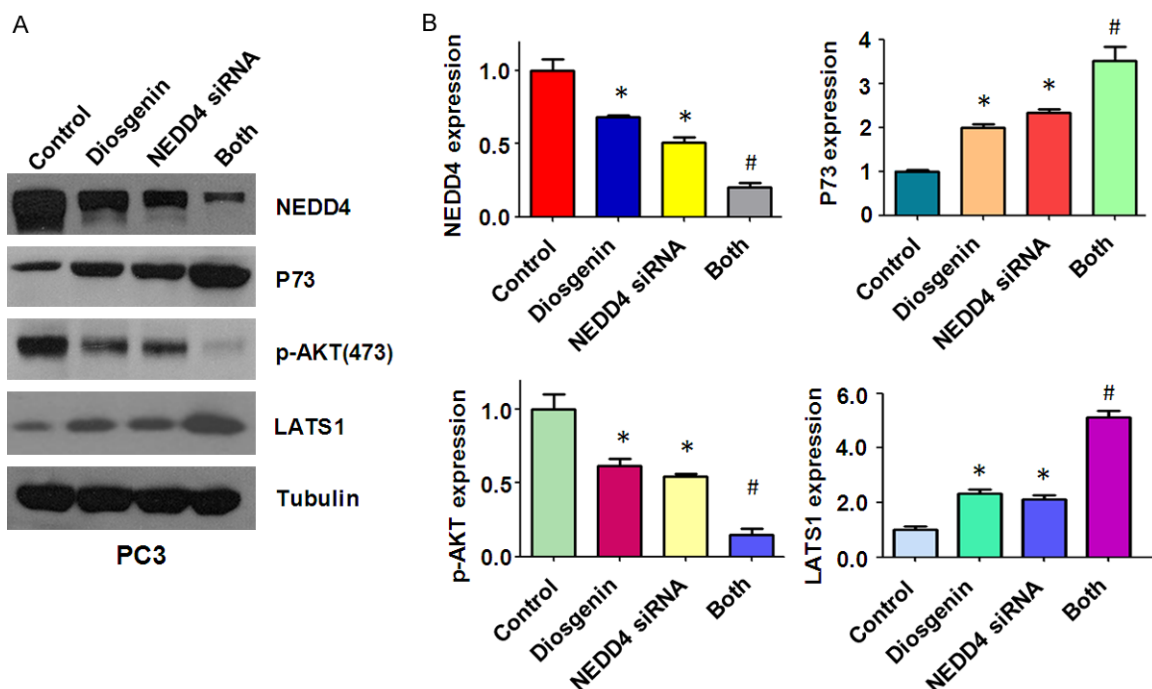
Figure 5. NEDD4 siRNA enhances diosgenin-inhibited NEDD4 level. (A) The expression of NEDD4, p73, LATS1, and pAkt was determined by Western blotting analysis in PC-3 cells after diosgenin exposure in combination with NEDD4 siRNA transfection. (B) Quantification of the data presented in (A). *P < 0.05, when compared with control; #P < 0.05, when compared to diosgenin treatment alone or NEDD4 siRNA transfection alone.

regulation of NEDD4 impeded cell invasion, and that the inhibition was greatest when NEDD4 siRNA transfection and diosgenin treatment were combined (**Figure 6D**). Collectively, these data demonstrate that diosgenin exerts its tumor suppressive effects in prostate cancer through downregulation of NEDD4.

Discussion

Mounting evidence suggests that diosgenin has anti-tumor activity in various types of human cancers [17]. For example, diosgenin inhibits cancer stem-like cells by attenuating the Wnt/β-catenin signaling pathway in breast cancer cells [18]. In addition, diosgenin inhibits cell growth and motility in hepatocellular carcinoma by regulating TAZ expression [19]. Jiang et al reported that diosgenin triggers ROS (reactive oxygen species)-dependent autophagy and cytotoxicity through mTOR pathway regulation in chronic myeloid leukemia cells [20]. Multiple other studies indicate that diosgenin suppresses cell growth in a variety of human cancer cells [17]. Our MTT assay data clearly demonstrated the anti-proliferative of diosgenin on prostate cancer cells by diosgenin. Several studies have revealed that diosgenin induces apoptosis in

multiple human cancer cells [21-23]. Our results support this clam and demonstrated that diosgenin stimulated cell apoptosis in prostate cancer cells.

Li et al report that diosgenin induced G2/M phase cell cycle arrest in human hepatocellular carcinoma cells [21]. Consistent with this finding, our study revealed that diosgenin treatment led to G2/M phase arrest in prostate cancer cells. Emerging evidence suggests that diosgenin inhibits migration and invasion in human cancer cells [15, 19, 24]. Diosgenin exhibits anti-invasion effects on gastric cancer cells via regulation of E-cadherin, integrin α5 and integrin β6 [25]. Diosgenin has anti-metastatic effects because it acts upstream of multiple signaling pathways including MMPs, ERK (extracellular signal-regulated kinase), JNK, PI3K/Akt, and NF-κB in prostate cancer [15]. Chang et al report that diosgenin abolishes HGF-induced cell invasion in prostate cancer by suppressing Mdm2 and vimentin due to p-Akt and mTOR downregulation [26]. Consistent with these prior studies, our data showed that diosgenin inhibited cell motility in prostate cancer cells. Therefore, diosgenin also exhibits anti-tumor properties in prostate cancer.
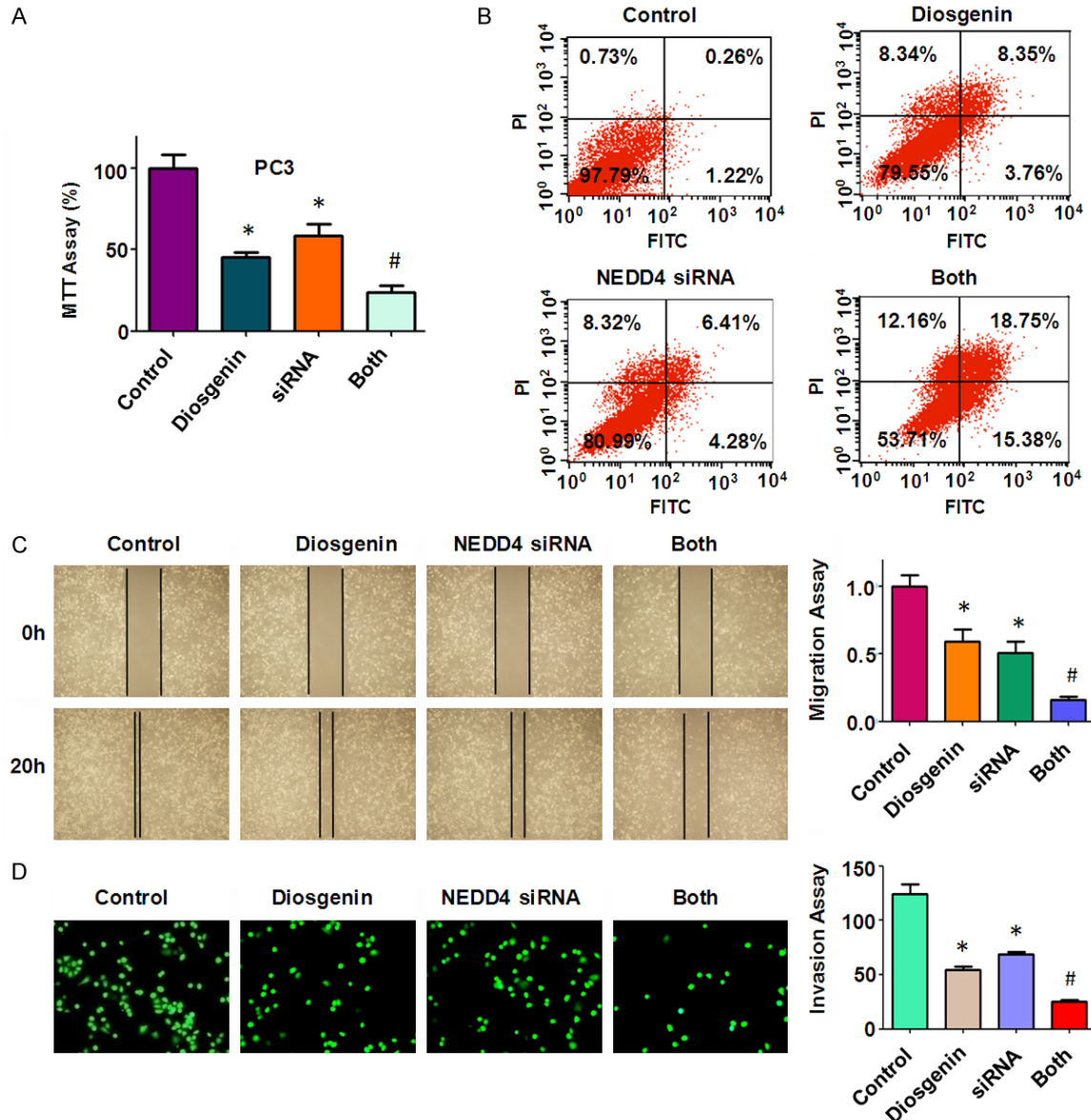
Figure 6. NEDD4 downregulation promotes diosgenin-induced anti-tumor activity. A: The MTT assay was used to measure the cell growth in PC-3 cells after diosgenin exposure in combination with NEDD4 siRNA transfection. B: Apoptosis in PC-3 cells was detected by Annexin V-FITC/PI staining and flow cytometry after diosgenin exposure in combination with NEDD4 siRNA transfection. C: A wound healing assay was used to measure PC-3 cell migration after diosgenin exposure in combination with NEDD4 siRNA transfection. D: A Transwell invasion assay was used to evaluate cell invasiveness in PC-3 cells after diosgenin exposure plus NEDD4 siRNA transfection. *P < 0.05, when compared to the control group; #P < 0.05, when compared to diosgenin treatment alone or NEDD4 siRNA transfection alone.

NEDD4 (Neural precursor cell expressed developmentally down-regulated protein 4) is an E3 ligase involved with the degradation of multiple proteins, and therefore, regulates several cellular processes [27, 28]. Recent studies validate that NEDD4 is an oncoprotein in a variety of human cancers including prostate cancer [29-33]. Overexpression of NEDD4 occurs in various types of human cancers, indicating that

NEDD4 has an oncogenic role in promoting cancer progression [27]. In this study, we found that overexpression of NEDD4 increased cell growth, migration, and invasion, whereas downregulation of NEDD4 decreased cell growth and motility in prostate cancer cells. This reinforces an oncoprotein role for NEDD4 in prostate cancer. Thus, targeting NEDD4 could be useful for the treatment of prostate cancer.

Multiple inhibitors of NEDD4 including natural compounds have been identified. For example, indole-3-carbinol disrupts NEDD4-mediated PTEN degradation, leading to decreased melanoma cell proliferations [34]. I3C analogues were also reported to inhibit NEDD4 ubiquitin ligase activity, to inhibit melanoma cell proliferation [35]. Curcumin decreases NEDD4 expression in glioma cells and pancreatic cancer [36, 37]. Our study provides evidence that diosgenin is a potential inhibitor of NEDD4 in prostate cancer cells. Future studies are needed to determine the therapeutic potentials of diosgenin in prostate cancer including animal models and clinical trial.

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Disclosure of conflict of interest

None.

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