

Targeting of *NHERF1* through RNA interference inhibits the proliferation and migration of metastatic prostate cancer cells

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Abstract. The present study aimed to investigate the effects of Na⁺/H⁺ exchanger regulatory factor 1 (*NHERF1*) gene knockdown, using short-hairpin RNA (shRNA), on the malignant behaviors of prostate cancer cells. A pSuper.puro *NHERF1* shRNA vector was transfected into PC-3M prostate cancer cells using Lipofectamine 2000. Stable cell lines were obtained and *NHERF1* knockdown was verified through western blot analysis. MTT assays were then used to measure PC-3M cell proliferation; in addition, cell migration was assessed using a wound healing assay. Flow cytometry was employed in order to determine the effects of *NHERF1* knockdown on apoptosis. Expression levels of apoptotic pathway proteins B cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein were then determined by western blot analysis. The results demonstrated that shRNA knockdown of *NHERF1* significantly suppressed the proliferation of PC-3M cells by >50%. In addition, knockdown of *NHERF1* significantly inhibited the migration of PC-3M cells. PC-3M cells harboring *NHERF1* shRNA exhibited significantly increased apoptosis, with an ~4-fold increase compared with

that of the parental PC-3M cells and cells transfected with an empty vector. Furthermore, the results revealed that knockdown of *NHERF1* reduced the protein expression of Bcl-2, although the expression of Bax was unaltered. In conclusion, *NHERF1* knockdown using shRNA inhibited the proliferation and migration of PC-3M cells and promoted apoptosis, highlighting the role of *NHERF1* in prostate cancer progression.

Introduction

Prostate cancer is the most prevalent type of tumor of the male urinary system. In the United States, prostate cancer is the second leading cause of cancer in men, the incidence of which is rising in China as well as in Western countries (1). In general, prostate cancer is a slow-growing disease, with the onset of symptoms occurring in advanced-stage disease, following tumor discovery (2). The initial treatment for prostate cancer is usually hormone therapy; however, when prostate tumors progress into an androgen-independent state, treatment becomes difficult (1).

Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1) was identified to be a multifunctional scaffolding protein that has various functions in numerous types of cancer through its interactions with oncogenic or tumor-suppressor proteins (3). In breast cancer, NHERF1 acts as a tumor suppressor protein, which is involved in influencing signal transduction and modulating the expression of phosphatase and tensin homolog (PTEN) to regulate the malignant phenotype (4). However, NHERF1 has been shown to have oncogenic functions in glioma and other types of cancer (5). While the specific role of NHERF1 in prostate cancer remains to be elucidated, studies have shown that the NHERF1 protein was overexpressed in tumor specimens *in situ* compared with normal prostate tissues (6,7). Therefore, further studies are required in order to fully elucidate the role of NHERF1 in prostate cancer.

The present study aimed to determine the effects of *NHERF1* knockdown using short-hairpin RNA (shRNA) in

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PC-3M cells, a prostate cancer cell line exhibiting abnormally high expression of *NHERF1*.

Materials and methods

Bacterial strains, cell lines and plasmids. The bacterial strain *Escherichia coli* DH5 α was purchased from Beijing CoWin Biotech Co., Ltd. (Beijing, China). The pSuper.puro *NHERF1* shRNA plasmid and negative control plasmid (pSuper.puro luciferase shRNA) were a gift from Professor Junfang Zheng (Capital Medical University, Beijing, China), originally constructed by Dr Margaret J. Wheelock (University of Nebraska Medical Center, Omaha, NE, USA). PC-3M prostate cancer cells were purchased from the Cell Resource Center of Beijing Xiehe (Beijing, China).

Reagents. RPMI 1640 medium, fetal calf serum and Lipofectamine 2000 were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Rabbit anti-human *NHERF1* (cat no. ab133599) and Bcl-2-associated X protein (Bax; cat no. ab32503) monoclonal antibodies, and rabbit anti-human GAPDH (cat no. ab181602) polyclonal antibody were purchased from Abcam (Cambridge, UK). Rabbit anti-human B cell lymphoma-2 (Bcl-2; cat no. ZA-0536) monoclonal antibody was purchased from Beijing Zhongshan GoldenBridge Biotechnology Co., Ltd. (Beijing, China), and goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (cat no. CW0103) was purchased from Beijing CoWin Biotech Co., Ltd. MTT was purchased from Sigma-Aldrich (St. Louis, MO, USA). The Annexin V-FITC Apoptosis Detection kit and Propidium Staining Cycle Detection kit were obtained from BestBio Biotechnology Co., Ltd. (Shanghai, China). All other reagents were of analytic grade.

Conversion and amplification of plasmid. The interference plasmid pSuper.puro harboring *NHERF1* shRNA and the negative control plasmid pSuper.puro harboring luciferase shRNA were transformed into competent *E. coli* DH5 α cells. Briefly, competent *E. coli* DH5 α (100 μ l) and plasmid (2 μ l) were added to Eppendorf tubes, and placed in ice for 30 min. Next, the Eppendorf tubes were incubated at 42°C for 90 sec and then placed back in ice for 2 min. Lysogeny broth (LB) media without antibiotic (800 μ l; Thermo Fisher Scientific, Waltham, MA, USA) was added and the transformed DH5 α cells were incubated at 37°C for 30 min with agitation. Transformed DH5 α (50 μ l) were spread on LB culture plates (Corning Incorporated, Corning, NY, USA) containing ampicillin and cultured at 37°C for 12–16 h. Positive colonies were selected following cultivation of the bacteria on LB plates (Beijing CoWin Biotech Co., Ltd.) containing 100 μ g/ml ampicillin (Sigma-Aldrich); the plasmid was then extracted and purified following overnight cultivation of the bacteria.

Cell culture and the establishment of a stable expression system. PC-3M cells were cultured in RPMI 1640 medium containing 10% fetal calf serum and 1% penicillin-streptomycin (Sigma-Aldrich) at 37°C in an incubator with 5% CO₂. Cells were transfected with 2.5 μ g plasmid using Lipofectamine 2000, according to the manufacturer's instructions. Stably transfected cells were selected using 2 μ g/ml puromycin

(Sigma-Aldrich) for 3–4 weeks at 37°C in an incubator with 5% CO₂. Clones were maintained in culture medium containing 1 μ g/ml puromycin. Media were replaced every 3 days.

Detection of cell growth. Cells stably expressing *NHERF1* shRNA or negative control shRNA were plated at a density of 5x10³ cells/well onto 96-well plates at 37°C in an incubator with 5% CO₂. Cell proliferation was then assessed every 24 h for 96 h using MTT assays according to standard protocols (8). For each sample at each time-point, six wells were analyzed. The experiment was repeated three times.

Western blotting. Cells were collected and lysed in radioimmunoprecipitation buffer (Beijing CoWin Biotech Co., Ltd., Beijing, China) in the presence of protease inhibitors for 20 min to extract total protein from cells stably expressing *NHERF1* shRNA or control cells, and protein levels were quantified using bicinchoninic acid assays (Beijing CoWin Biotech Co., Ltd.). Subsequently, 50 μ g protein from each sample was loaded onto 8% sodium dodecyl sulfate (SDS) polyacrylamide gels and subjected to SDS-polyacrylamide gel electrophoresis (PAGE; Beijing CoWin Biotech Co., Ltd.). Protein was then transferred to nitrocellulose membranes (Sigma-Aldrich), which were then blocked with bovine serum albumin blocking buffer (Invitrogen Life Technologies) for 1 h. Membranes were then incubated with primary antibodies targeting *NHERF1* (1:1,000 dilution), Bcl-2 (1:1,000 dilution), Bax (1:200 dilution) or GAPDH (1:1,000 dilution) overnight at 4°C, followed by incubation with secondary antibodies conjugated with HRP (1:3,000 dilution) for 1 h at room temperature. Detection was facilitated using an enhanced chemiluminescence kit and images were analyzed using ImageJ software (version 1.62; National Institute of Health, Bethesda, MD, USA).

Wound healing assay. Cells were plated at a density of 2x10⁵ cells/well onto six-well plates and grown to 80% confluence. The monolayer of cells was scratched with a 200- μ l pipette tip to create a wound and an image was captured immediately (0 h). Migration was then observed every 12 h and images of the closing wound were captured at each time-point with a digital camera connected to a phase-contrast microscope (CKX41; 10x objective lens; Olympus, Tokyo, Japan). The same visual field was used throughout the experiment. Image-Pro Plus software (version 6.0; Media Cybernetics, Rockville, MD, USA) was used to facilitate the calculation of the migration rate as follows: Migration rate = [(relative distance recorded at 0 h - relative distance recorded at 48 h) / relative distance recorded at 0 h] x 100%. The experiment was repeated three times.

Analysis of cell apoptosis. Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) flow cytometry was used to detect the rate of apoptosis. Cells were collected, washed with phosphate-buffer saline (PBS) and resuspended in 400 μ l buffer (BestBio Biotechnology Co., Ltd.). Subsequently, 5 μ l Annexin V-FITC (BestBio Biotechnology Co., Ltd.) was added, cells were mixed and the solution was incubated for 15 min in the dark at room temperature. PI (10 μ l; BestBio Biotechnology Co., Ltd.) was then added and the samples

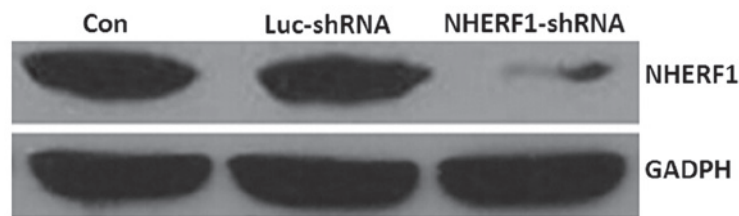


Figure 1. Expression of NHERF1 in PC-3M cells transfected with *NHERF1*-targeting shRNA. Western blot analysis was used to detect NHERF1 expression in Con, Luc-shRNA and *NHERF1*-shRNA cells. GAPDH was used as a loading control. NHERF1, Na⁺/H⁺ exchanger regulatory factor 1; shRNA, short-hairpin RNA; Con, untransfected cells; Luc-shRNA, cells stably transfected with luciferase shRNA; *NHERF1*-shRNA, cells stably transfected with *NHERF1* shRNA.

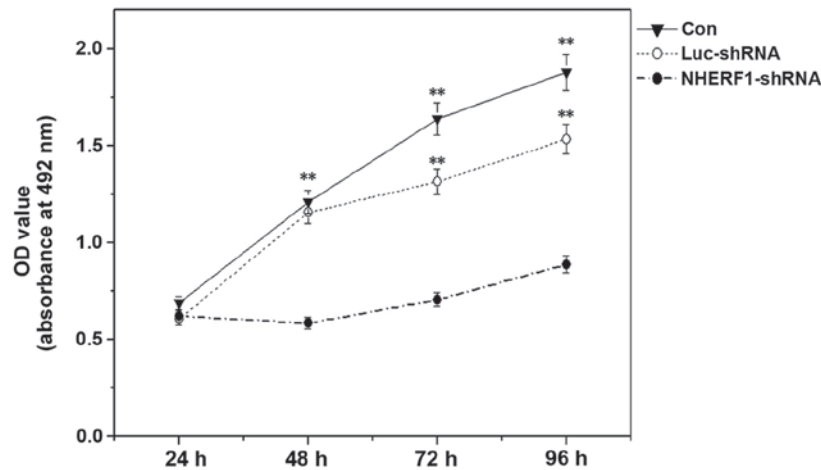


Figure 2. Growth curves for PC-3M cells stably expressing *NHERF1* shRNA. Cell proliferation was measured every 24 h up to 96 h in Con, Luc-shRNA and *NHERF1*-shRNA cells using an MTT assay. **P<0.01 vs. Con and Luc-shRNA. NHERF1, Na⁺/H⁺ exchanger regulatory factor 1; shRNA, short-hairpin RNA; OD, optical density; Con, untransfected cells; Luc-shRNA, cells stably transfected with luciferase shRNA; *NHERF1*-shRNA, cells stably transfected with *NHERF1* shRNA.

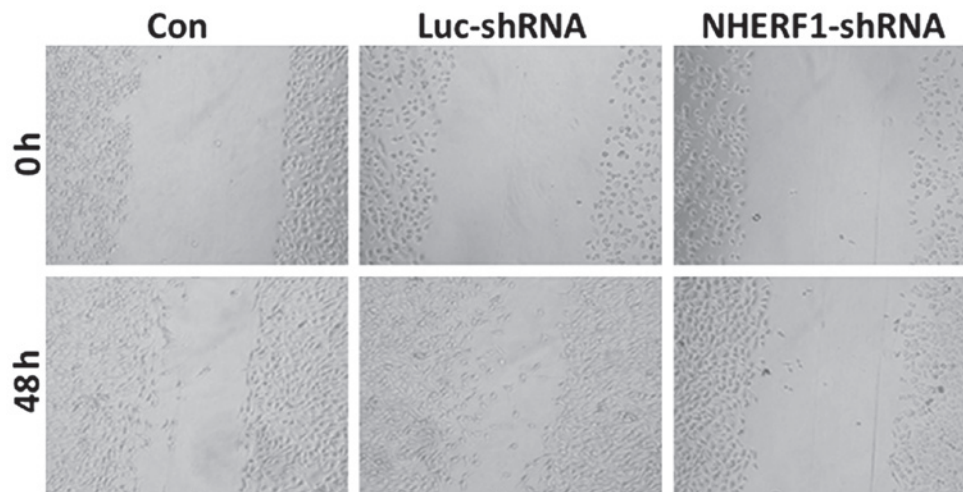


Figure 3. Migration of PC-3M cells transfected with *NHERF1* shRNA. Wound healing assays were performed using Con, Luc-shRNA and *NHERF1*-shRNA cells in order to determine the rate of migration following 48 h. NHERF1, Na⁺/H⁺ exchanger regulatory factor 1; shRNA, short-hairpin RNA; Con, untransfected cells; Luc-shRNA, cells stably transfected with luciferase shRNA; *NHERF1*-shRNA, cells stably transfected with *NHERF1* shRNA.

were incubated in the dark at room temperature for 5 min. Cells were then analyzed using flow cytometry within 1 h (Accuri C6; BD Biosciences, Franklin Lakes, NJ, USA).

Analysis of cell cycle. Cultured cells were removed with trypsin and fixed with 70% ethanol at 4°C overnight. Subsequently,

they were stained with PI (20 µg/ml PI, 200 µg/ml DNase-free RNase A and 0.1% Triton X-100, prepared freshly in PBS). The cellular DNA content was analyzed using a FACSCalibur machine (Becton Dickinson, San Jose, CA, USA). Data were analyzed using CellQuest software (version 3.0; Becton Dickinson.)

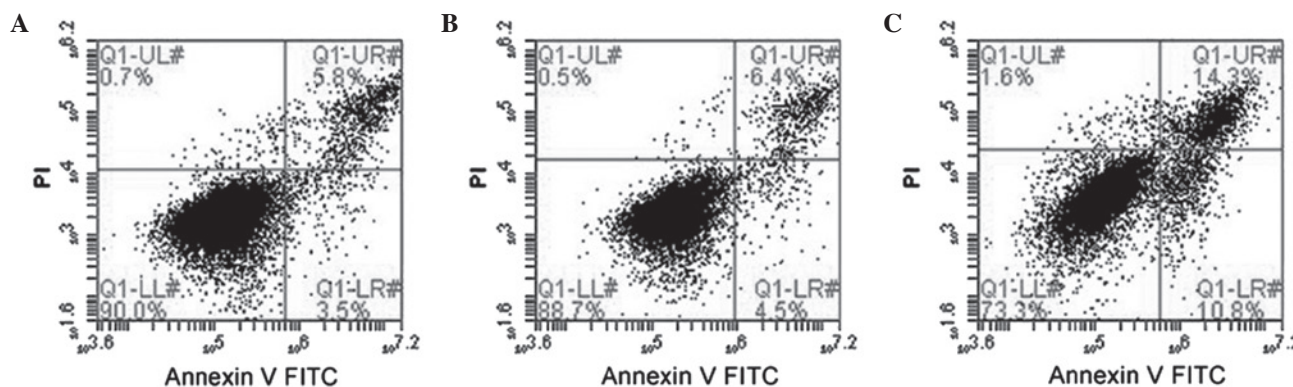


Figure 4. Flow cytometry analysis of apoptosis in PC-3M cells. Double staining with Annexin V-FITC and PI was used to assess apoptosis in (A) untransfected cells, (B) cells transfected with luciferase shRNA and (C) cells transfected with *NHERF1* shRNA. FITC, fluorescein isothiocyanate; PI, propidium iodide; *NHERF1*, Na⁺/H⁺ exchanger regulatory factor 1; shRNA, short-hairpin RNA.

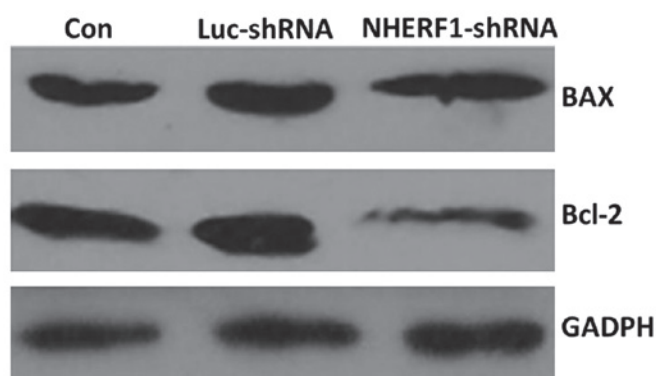


Figure 5. Expression of Bcl-2 and Bax in PC-3M cells transfected with *NHERF1* shRNA. Western blot analysis was performed to determine Bcl-2 and Bax protein expression in Con, Luc-shRNA and *NHERF1*-shRNA cells. Data are representative of three identical experiments. Bcl-2, B cell lymphoma 2; Bax, Bcl-2-associated X protein; *NHERF1*, Na⁺/H⁺ exchanger regulatory factor 1; shRNA, short-hairpin RNA; Con, untransfected cells; Luc-shRNA, cells stably transfected with luciferase shRNA; *NHERF1*-shRNA, cells stably transfected with *NHERF1* shRNA.

Statistical analysis. All experiments were repeated at least three times. SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA) was used to analyze the results. Growth curves were analyzed using a repeated-measures analysis of variance with Fisher's least significant difference post-hoc tests and apoptosis was analyzed using independent sample t-tests. $P < 0.05$ was considered to indicate a statistically significant difference between values.

Results

Analysis of *NHERF1* knockdown in stably transfected PC-3M cells. As shown in Fig. 1, *NHERF1* expression was successfully knocked down in *NHERF1* shRNA-transfected cells compared with untransfected cells and cells transfected with the negative control plasmid. These cells were used in subsequent experiments as *NHERF1* knockdown cells.

Influence of *NHERF1* knockdown on the proliferation capacity of PC-3M cells. The ability of *NHERF1* to modulate the proliferation of prostate cancer cells was analyzed using an MTT assay. The results indicated that *NHERF1* knockdown

significantly inhibited the proliferation capacity of PC-3M cells by $>50\%$ at all time-points following 24 h of incubation, as compared with control cells ($P < 0.01$) (Fig. 2). No significant differences were observed between untransfected cells and luciferase shRNA-transfected cells.

Influence of *NHERF1* knockdown on the migration capacity of PC-3M cells. At 48 h following wounding, there were no significant differences in wound healing rates between untransfected cells and cells transfected with luciferase shRNA (Fig. 3). However, cells transfected with *NHERF1* shRNA exhibited a significant reduction in migration capacity ($P < 0.05$), with the wound size measuring 94–98% of the wound size at 0 h (Fig. 3). Thus, these data demonstrated that knockdown of *NHERF1* significantly inhibited the migration capacity of PC-3M prostate cancer cells.

Influence of *NHERF1* knockdown on apoptosis in PC-3M cells. Representative results of flow cytometric analysis are shown in Fig. 4. In general, the rates of cell death were low ($<5\%$). However, cells stably expressing *NHERF1* shRNA exhibited a significant increase in apoptosis by ~ 4 -fold compared with cells transfected with luciferase shRNA (11.98 ± 3.28 vs. $2.55 \pm 0.92\%$, respectively; $P < 0.001$); untransfected cells exhibited $3.5 \pm 0.96\%$ apoptosis compared with the luciferase shRNA-transfected cells ($P > 0.05$). These data indicated that reduced *NHERF1* expression substantially increased apoptosis in PC-3M cells.

Influence of *NHERF1* knockdown on cell cycle in PC-3M cells. Next, the effect of *NHERF1* knockdown on the cell cycle was analyzed. Transfection with *NHERF1* shRNA significantly increased the sub-G1 fraction (the fraction of apoptotic cells) compared with the luciferase shRNA-transfected and parental control cells (22, 10 and 8% in *NHERF1* shRNA-transfected, luciferase shRNA-transfected and parental cells, respectively; data not shown). Collectively, these results suggest that *NHERF1* is important in cell growth, as well as cell cycle progression, in a subset of prostate cancer cell lines.

Influence of *NHERF1* knockdown on the expression of apoptosis-associated proteins in PC-3M cells. As shown in Fig. 5, the expression of Bax, a pro-apoptotic protein, was unchanged

following transfection with *NHERF1* shRNA, while the expression of Bcl-2, an anti-apoptotic protein, was markedly decreased ($P < 0.05$). These data supported the results of flow cytometry analysis indicating that apoptosis was increased following knockdown of *NHERF1*.

Discussion

The present study examined the effects of *NHERF1* knockdown on the malignant phenotype in PC-3M prostate cancer cells. The results demonstrated that *NHERF1* knockdown inhibited proliferation and migration, while increasing apoptosis. Therefore, *NHERF1* may represent a novel oncogene in prostate cancer.

NHERF1 has been reported to be involved in the development and occurrence of cancer. Of note, abnormalities in *NHERF1* expression have been demonstrated to be associated with the occurrence, development and metastasis of certain types of cancer (4,9,10). While the details of these mechanisms remain to be fully elucidated, *NHERF1* was reported to form a protein complex with epidermal growth factor receptor (EGFR) and neurofibromatosis type 2 (NF2) at intercellular adherens junctions (11), thereby mediating the internalization and signal transduction of EGFR in order to regulate various oncogenic processes. In addition, *NHERF1* was reported to interact with platelet-derived growth factor receptor (PDGFR) to form a transduction complex with NF2 (12); it was suggested that this complex functions to block the signaling associated with PDGFR, thereby affecting apoptosis during the epithelial-to-mesenchymal transition and promoting cancer development and metastasis (13). In addition, *NHERF1* was reported to interact with several other specific growth factor receptors that have important functions in proliferation, invasion and angiogenesis (14,15). Therefore, *NHERF1* was thought to directly or indirectly affect the development and progression of cancer (5).

The expression level of *NHERF1* in prostate cancer varies depending on the pathological pattern and cancer phase (6). This may be explained by the observation that different cell systems and tissues have different genetic backgrounds. In addition, in samples where *NHERF1* is expressed at similar levels, *NHERF1* may combine with different proteins and thus exhibit different functions in samples with varying genetic backgrounds. In the prostate cancer cell system adopted in the present study (PC-3M cells), *NHERF1* exhibited high endogenous expression, which was reduced through stable transfection with a *NHERF1*-targeting shRNA. The results indicated that inhibition of *NHERF1* expression attenuated the proliferation and migration rates of PC-3M cells. Of note, major changes in cell cycle distribution were not observed (data not shown), although changes were detected in the height of the sub-G1 peak in flow cytometry analysis, which prompted the investigation of apoptosis in *NHERF1*-knockdown cells. It was then demonstrated that knockdown of *NHERF1* increased apoptosis, thereby contributing to the observed inhibition of cell proliferation. Cell apoptosis, proliferation and differentiation are all basic, vital functions of cells, which are closely associated; imbalances in these functions have been associated with tumor development (16,17). In general, apoptosis has a negative regulatory role in cancer cells and may inhibit tumor growth (18). Consistent with the increase in apoptosis observed in the present study, inhibition of Bcl-2

expression in *NHERF1* knockdown cells was detected. Bcl-2 is an anti-apoptotic protein; thus, inhibition of Bcl-2 expression may promote apoptosis. Therefore, in the present study, data from western blot analysis of apoptosis-associated proteins supported the flow cytometry results, demonstrating the role of *NHERF1* in modulating the cell proliferation and apoptosis of prostate cancer cells.

In conclusion, the results of the present study demonstrated that *NHERF1* was involved in regulating malignant biological behaviors, including proliferation and migration, in PC-3M cells. In addition, *NHERF1* knockdown was demonstrated to promote apoptosis through inhibition of Bcl-2 expression. Therefore, these results indicated that *NHERF1* acts as an oncogenic protein in prostate cancer and may be a potential therapeutic target for the treatment of prostate cancer. Although these data support the role of *NHERF1* in complexes with growth factor receptors and ligands or in influencing the expression and stability of β -catenin (19), further studies with other associated transmembrane ligands are required.

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