ERK5-regulated RERG expression promotes cancer progression in prostatic carcinoma

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Abstract. Big mitogen-activated protein kinase 1 [also named extracellular signal-regulated kinase (ERK) 5] is activated by mitogens and oncogenic signals and is strongly implicated in tumorigenesis. Our previous investigation indicated that ERK5 can induce prostatic carcinoma cell proliferation by promoting entry into the S phase of the cell cycle. In the present study, microarray and western blot analysis revealed that ERK5 can inhibit Ras-like oestrogen-regulated growth inhibitor (RERG) protein expression and that the inhibition of RERG expression promotes prostatic carcinoma cell proliferation and migration. In addition, pathological analysis indicated that the RERG expression level was associated with the malignancy of prostatic carcinoma. Furthermore, an apoptotic assay and western blot analysis demonstrated that the downregulation of RERG expression inhibits apoptosis by regulating the protein expression levels of B cell lymphoma-2 and c-Myc. Moreover, a luciferase activity assay indicated that the nuclear factor-κB pathway is associated with RERG-mediated apoptosis in prostatic carcinoma. Therefore, these data suggested that ERK5-regulated RERG expression plays a role in the progression of prostatic carcinoma, indicating that RERG may be a potential biomarker for the prognosis of patients with prostatic carcinoma.

Introduction

Prostatic carcinoma is one of the most common malignancies worldwide, affecting 1 in 9 men >65 years of age (1-3). According to the statistical analysis, the percentage of patients with prostatic carcinoma among men aged 65 years or older will rise to 19.6% (4). Currently, there are no effective treatments for advanced-stage prostatic carcinoma, and it is the second leading cause of cancer-associated mortality in men (5,6). Therefore, the identification of novel endogenous factors responsible for proliferation, migration and invasion will facilitate understanding of the progression of prostatic carcinoma, as well as the development of novel approaches for its diagnosis and therapy.

There are four mitogen-activated protein kinase (MAPK) pathways in mammalian cells: The extracellular signal-regulated kinase (ERK) 1/2, Janus kinase, p38 and ERK5 pathways. Activation of the MAPK pathway results in the phosphorylation of the downstream mediators, including substrates, transcription factors, protein kinases, and enzymes, to regulate cell proliferation, differentiation, apoptosis, and migration, as well as tumourigenesis, tumour invasion and migration and chemoresistance (7,8). The ERK5 pathway was the most recently identified and is the least studied mammalian MAPK cascade. The ERK5 pathway is usually activated by mitogens and oncogenic signals and has been demonstrated to be involved in tumourigenesis. Deregulated ERK5 signalling has been associated with properties of human malignancies, including the chemoresistance of breast tumour cells (9), the uncontrolled proliferation of erb-b2 receptor tyrosine kinase 2-overexpressing carcinomas (10), the metastatic potential of prostatic carcinoma cells (11) and tumour-associated angiogenesis (12). Additionally, our previous study indicated that ERK5 pathway activation induces cell proliferation and regulates the cell cycle in prostatic carcinoma cells (13).

Ras-like oestrogen-regulated growth inhibitor (RERG) was initially identified as a potential tumour suppressor gene and is regulated by oestrogen in breast tumours (14,15). RERG is widely expressed in multiple normal tissues, whereas RERG expression is inhibited in tumours of the breast, kidney, ovary and colon (15,16). Previous studies have indicated that RERG expression may be associated with cell proliferation and distant metastasis in breast cancer and nasopharyngeal carcinoma (17,18), suggesting that it has potential as a prognostic biomarker in malignant tumours. Similarly, several reports have revealed the presence of significantly hypermethylated RERG in breast cancer and colorectal adenocarcinoma carcinoma (19,20). Although this evidence has been demonstrated to be associated with cell proliferation and tumour metastasis, its biological significance for the progression of prostatic carcinoma remains elusive.

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In the present study, mRNA microarray analysis demonstrated RERG expression inhibition in EGF-treated prostatic carcinoma cells. Further investigations revealed that the expression level of RERG protein was associated with malignancy in patients with prostatic carcinoma. In addition, the results indicated that the inhibition of RERG promoted cell proliferation by inhibiting NF-κB-mediated apoptosis. Furthermore, the overexpression of RERG protein suppressed tumour metastasis in prostatic carcinoma cells. It was additionally demonstrated that matrix metallopeptidase (MMP)-2 and MMP-9 were associated with RERG-induced tumour metastasis in this process. Taken together, the findings highlighted the importance of ERK5-regulated RERG expression in prostatic carcinoma progression.

Materials and methods

Cell culture, antibodies and reagents. PC-3 prostatic carcinoma cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured at 37˚C in a humidified environment containing 5% CO_2. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1:100 penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.).

Anti-mouse ERK5 antibody (dilution 1:2,000; cat. no. sc-398015) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-rabbit RERG antibody (1:2,000 for western blotting; 1:200 for immunohistochemistry; cat. no. 10687-1-AP) was purchased from ProteinTech Group, Inc. (Rosemone, IL, USA). Anti-MMP-2 antibody (dilution 1:2,000; cat. no. ab92536), anti-MMP-9 antibody (dilution 1:2,000; cat. no. ab137867), anti-B cell lymphoma (Bcl)-2 antibody (dilution 1:2,000; cat. no. ab196495), and anti-c-Myc antibody (dilution 1:2,000; cat. no. ab39688) were purchased from Abcam (Cambridge, UK; dilution 1:2,000). Anti-GAPDH antibody (dilution 1:4,000; cat. no. 100242-MM05) was purchased from Sino Biological, Inc. (Beijing, China). All of the secondary antibodies, including goat anti-mouse IgG antibody-(cat. no. 715-035-003) and goat anti-rabbit IgG antibody (cat. no. 111-035-045)-conjugated horseradish peroxidase (HRP), were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA; dilution 1:4,000). XMD8-92, an ERK5 inhibitor that specifically inhibits the phosphorylation of ERK5, but not the phosphorylation of ERK1/2, was purchased from Selleck Chemicals (Houston, TX, USA). EGF was obtained from Sino Biological, Inc. The final concentration of the XMD8-92 and EGF treatment was 5 μM and 0.5 ng/ml, respectively. The cells were serum starved overnight followed by treatment with XMD8-92 as indicated for 1 h, and/or stimulated with EGF for 20 min, as previously described (21).

Plasmid construction. The human RERG open-reading frame was amplified by RT-polymerase chain reaction (PCR) from the total RNA of PC-3 cells. The primer sequences of RERG are presented in Table I. The RERG cDNA were amplified by PCR and inserted into the *EcoRI*/*XbaI* sites of the pCDNA3.1(+) eukaryotic expression vector (Invitrogen; Thermo Fisher Scientific, Inc.) with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). To construct pSpCas9-RERGtarget-2A-GFP, the RERG sgRNA oligo was designed in http://crispr.mit.edu, phosphorylated, ligated, and inserted in the pSpCas9-2A-GFP vector (Addgene, Cambridge, MA, USA) after *BbsI* digestion (Thermo Scientific, Inc.).

Overexpression and knockdown of RERG expression. To establish PC-3 cells with stable RERG protein overexpression or inhibition, PC-3 cells were transfected with pCDNA3.1(+)-RERG, pSpCas9-RERGtarget-2A-GFP recombinant plasmid, or pCDNA3.1(+) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Briefly, for transfection, $1x10^6$ cells were seeded in a 6-well plate. A total of 5 μl Lipofectamine® 3000, and 5 μg pCDNA3.1(+)-RERG or pSpCas9-RERGtarget-2A-GFP were diluted in 200 μl Opti-MEM medium (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 30 min, respectively. The mixture was then added and incubated in the cell culture medium for 48 h. For the selection of RERG-overexpressing PC-3 cells, cells were transfected with pCDNA3.1(+)-RERG and selected by G-418 treatment for three weeks. For the selection of cells with silenced RERG expression, pSpCas9-RERG-target-2A-GFP-transfected PC-3 cells were sorted by flow cytometry on a FACSAria II system (BD Biosciences, San Jose, CA, USA), and the selected fluorescently labelled cells were then cultured in 6-well plates. PC-3 cells were also transfected with pCDNA3.1(+) and selected using G-418, as vector control cells.

Western blot analysis. To prepare protein extracts, the cells were washed with PBS three times. Following centrifugation, the harvested cells were resuspended and protein extracted using lysis buffer (Pierce; Thermo Fisher Scientific, Inc.) for 30 min on ice. The lysates were centrifuged at 16,000 x g for 10 min at 4˚C. The supernatants of the lysates were mixed with SDS sample buffer and boiled for 10 min. The samples (~100 μg of each lane) were separated on a 10% SDS polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% (w/v) dry skim milk for 1 h at room temperature, and then blotted with the corresponding antibody in PBST buffer (0.1% Tween-20 in PBS) under gentle shaking at room temperature for 2 h. After being washed with PBST three times, the membranes were incubated with the indicated secondary antibody for 2 h at room temperature. The signals were detected using a SuperSignal West Pico Substrate kit (Pierce; Thermo Fisher Scientific, Inc.). The signals were measured by fluorescence intensity with ImageJ software (version no. 1.8.0; National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed using a SYBR PrimeScript RT-PCR kit (Takara Bio, Inc., Otsu, Japan) on a Rotor-Gene 6000 Real-Time Genetic Analyser (Qiagen, Valencia, CA, USA), and quantified according to the previously described method (22). The

Table I. Primers for recombinant plasmid construction and RT-qPCR assay.

A, Plasmid construction	
Primer name	Sequence (5'-3')
RERG	
F	CCCGAATTCATGGCTAAAAGT
R	CCCTCTAGACTAAGTACTGATTTT
RERG gRNA	
F	CACCTAGTGAGATTTCTGACCAAA
R	GTGGTCGATGATGAAGTTGTTTCC

B, RT-qPCR	
Primer name	Sequence (5'-3')
RERG	
F	CAACCATCGATGATGAAGTTG
R	TCAGCTTTGTTTCCAACCAAG
MMP-2	
F	AGAAGTTCTTTGGACTGCCCC
R	CAGGTGTGTAGCCAATGATCC
MMP-9	
F	TGTACCGCTATGGTTACACTC
R	GGCAGGGACAGTTGCTTCT
GAPDH	
F	TGCACCACCAACTGCTTAGC
R	GGCATGGACTGTGGTCATGAG

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; RERG, Ras-like oestrogen-regulated growth inhibitor; MMP, matrix metallopeptidase; F, forward; R, reverse.

primer sequences of RERG, MMP-2, MMP-9, and GAPDH are presented in Table I. The PCR protocol included a denaturation programme (95˚C for 2 min), followed by 40 cycles of an amplification and quantification programme (95˚C for 5 sec and 55-57˚C for 30 sec) and a melting curve programme (55-95˚C, with a 0.5˚C increment each cycle). Each sample was replicated three times.

Immunohistochemistry (IHC). A paraffin-embedded tissue microarray (Alenabio, Xi'an, China), which contains 58 prostatic carcinoma specimens and 6 normal prostate tissues, was dewaxed with xylene and rehydrated in descending concentrations of ethanol. The slide was blocked with Immuol Staining blocking buffer (cat. no. P0102; Beyotime Institute of Biotechnology, Shanghai, China) for 60 min at room temperature, and then was incubated with anti-RERG antibody (dilution 1:200; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. After washing three times in PBST buffer for 10 min, the slide was processed according to the instructions of the Super Sensitive Polymer HRP Detection System/DAB kit (Thermo Fisher Scientific, Inc.) and counterstained with haematoxylin for 15 min at room temperature. The intensity of the immunostaining (1, weak; 2, moderate, and 3, intense) and the percentage of positive cells (0, <0.5%; 1, 5-35%; 2, 26-50%; 3, 51-75%; 4, >75%) were assessed in at least 5 high-power fields under a light microscope (Nanjing Jiangnan Inc., Nanjing, China). All values for each sample were multiplied to obtain a final score, and the tissues with scores <4 and ≥4 were determined to have low and high expression, respectively.

Cell proliferation assay. Cell counting kit (CCK)-8 assay was performed to examine PC-3 cell proliferation. The cells were seeded at $2x10^3$ cells/well in phenol red-free DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS in a 96-well plate. The cell growth rate was determined by CCK-8 assay (Beyotime Institute of Biotechnology). A total of 10 μl of CCK-8 working solution was added to each well on days 1-5, followed by incubation for 2 h at 37˚C, and the absorbance at a wavelength of 450 nm was measured using a model 3550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Colony formation assay. A total of $5x10^2$ cells were added to a 24-well plate. All of the cells were then cultured for 2 weeks with fresh medium every three days. Cells were washed with PBS for three times, and stained with 0.1% crystal violet for 10 min at room temperature, and destained with PBS three times. Colonies were counted using ImageJ software for each well, and each condition was tested in triplicate.

Transwell assay. A 24-well micropore polycarbonate membrane filter with 8-μm pores (Corning Inc., Corning, NY, USA) was coated with 10 μl of Matrigel (BD Biosciences). Then, 200 μl of cell suspension ($1x10^4$/ml) was seeded into the top chamber in serum-free DMEM medium. The bottom chamber was filled with DMEM supplemented with 10% FBS. After 24 h of incubation, the membranes were fixed and stained by 0.1% crystal violet for 15 min at room temperature, and the cells on the upper surface were then removed with a cotton swab. The invasive cells on the lower surface were observed and counted under a phase-contrast microscope. Each condition was tested with five replicates.

Wound healing assay. Cell monolayers in all groups were wounded using a 200-μl pipette tip in 6-well plates and then incubated in DMEM supplemented with 10% FBS. The wound width was imaged at 0, 12, 24, or 36 h post-scratching under a phase-contrast microscope.

Apoptosis assay. Hoechst 33342 staining (Beyotime Institute of Biotechnology) was performed to examine apoptosis. In brief, cells were seeded on coverslips in 24-well plates and cultured for 24 h; then, the DMEM was removed, and the cells were washed three times. Cells in all of the groups were incubated in Hoechst labelling solution (2 μg/ml) for 20 min at room temperature before being washed with PBS three times. The cells were then observed under a ZEISS Observer A1 fluorescence microscope at 350 nm.

Nuclear factor (NF)-κB luciferase activity assay. In total, $5x10^4$ cells were seeded into 24-well plates and cultured for

24 h. Cells in the different groups were transfected with 0.3 μg of pNF-κB-Luc plasmid (Agilent Technologies, Inc., Santa Clara, CA, USA) with Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 48 h after transfection, the cells were lysed in lysis buffer. The luciferase activity was determined using a luciferase assay kit (Promega Corp., Madison, WI, USA). Each experiment was repeated at least three times.

Statistical analysis. Data are presented as the mean ± standard error of the mean. The data were analysed using SPSS software, version 14.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0. (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analyses were performed by analysis of variance using Dunnett's multiple comparison test, or Students t-test. $P<0.05$ was considered to indicate a statistically significant difference. Each experiment was replicated at least three times.

Table II. Characteristics of patients with prostatic carcinoma.

Characteristic	Adenocarcinoma (n=58) (%)	Normal tissues (n=6)
Age, years		
≥60	52 (90)	0
<60	6 (10)	6
Grades		
1-2	35 (60)	-
3-4	23 (40)	-
Stages		
I+II	33 (57)	-
III+IV	25 (43)	-

Results

Activation of the ERK5 pathway induces the inhibition of RERG expression in prostate tumour cells. Our previous study indicated that activation of the ERK5 pathway induces prostatic carcinoma cell proliferation (13). To further investigate the function of ERK5 in the progression of prostatic carcinoma, the present study activated or inhibited the ERK5 pathway with treatments with EGF or XMD8-92, respectively. Fig. 1 revealed that phosphorylated ERK5 was markedly upregulated in EGF-treated PC-3 cells, indicating that the ERK5 pathway can be activated by EGF treatment (21). The results indicated that EGF treatment can activate the ERK5 pathway, which induces more phosphorylated ERK5 in total ERK5 protein. Thereafter, EGF- and XMD8-92-treated PC-3 cells were harvested and examined by microarray analysis (Sangon Biotech, Shanghai, China). Fig. 1B revealed the regulation of various genes in EGF- and XMD8-92-treated PC-3 cells. Notably, it was noted that RERG expression was significantly upregulated in XMD8-92-treated PC-3 cells, but inhibited in EGF-treated PC-3 cells, compared with control, which was also confirmed by western blot analysis (Fig. 1C). Similarly, RT-PCR also demonstrated that RERG was regulated by ERK5 signalling at the transcriptional level (Fig. 1D). Therefore, the results suggested that RERG may be associated with ERK5-mediated cell proliferation in prostate tumour cells.

RERG expression is associated with malignancy in prostatic carcinoma. To investigate the role of RERG expression in prostatic carcinoma, the present study then analysed the expression level of RERG in the specimens of tissue microarray, which contained 58 prostatic carcinoma specimens and 6 normal prostate tissues. The characteristics of the patients with prostatic carcinoma are presented in Table II. Immunohistochemistry revealed that RERG expression was lower in advanced prostatic carcinoma tissues, whereas RERG protein expression was higher in normal prostate tissues and less malignant tumour tissues (Fig. 2A), suggesting that the loss of RERG expression may result in cancer progression. In addition, the results indicated that the expression level of RERG was negatively associated with malignancy in prostatic carcinoma (Fig. 2B), which is consistent with previous reports regarding other tumours (16-18).

Inhibition of RERG expression promotes prostate cancer cell proliferation. The aforementioned results indicated that RERG expression may be involved in the malignancy of prostatic carcinoma. It was expected that RERG expression may play a role in tumourigenesis in prostatic carcinoma. To confirm our hypothesis, the present study stably overexpressed and knocked down RERG expression in PC-3 cells. RT-qPCR and western blot analysis demonstrated the overexpression and knockdown of RERG mRNA and protein in PC-3 cells (data not shown and Fig. 3A, respectively). Additionally, cell proliferation assays demonstrated that the inhibition of RERG expression promoted cell proliferation compared with RERG overexpression and the control (empty vector; Fig. 3B). Moreover, the colony formation assay indicated that the colony number was much greater in RERG-inhibited PC-3 cells compared with RERG-overexpressing or empty-vector-transfected PC-3 cells (Fig. 3C and D), suggesting that RERG expression is associated with prostate cancer cell proliferation.

RERG expression regulates tumour invasion and migration by inhibiting MMP-2 and MMP-9 expression in prostate cancer cells. As RERG has been reported to be associated with tumour metastasis in breast cancer, the present study then examined whether the regulation of RERG expression was associated with tumour invasion and metastasis in prostatic carcinoma. The invasion and migration of RERG-overexpressing, RERG-inhibited, and empty-vector-transfected PC-3 cells was examined. The *in vitro* wound healing assay demonstrated that RERG-inhibited PC-3 cells migrated into the scratched area more rapidly than RERG-overexpressing or empty-vector-transfected PC-3 cells at 12, 24, and 36 h (Fig. 4A and B). Further transwell studies demonstrated that RERG-inhibited PC-3 cells displayed an increased invasive capacity compared with RERG-overexpressing and empty-vector-transfected PC-3 cells (Fig. 4C and D).

MMP-2 and MMP-9 have been reported to be associated with the invasion and migration of many malignant tumours (23). Therefore, the present study examined the

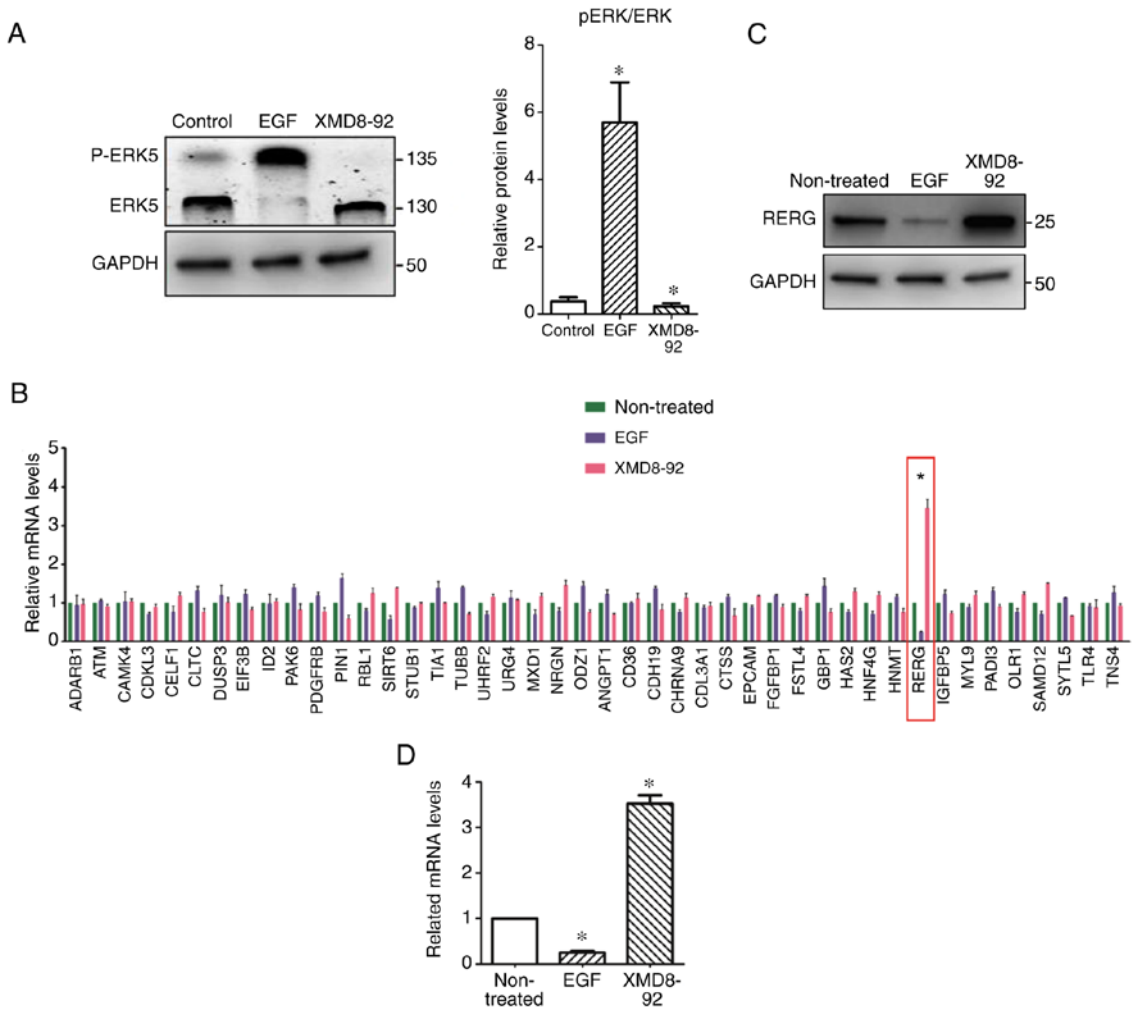


Figure 1. Inhibition of the ERK5 signalling pathway induces the upregulation of RERG protein in prostatic carcinoma cells. (A) Activation and inhibition of the ERK5 pathway in EGF- and XMD8-92-treated PC-3 cells, respectively. (B) Microarray assay of EGF- and XMD8-92-treated PC-3 cells. *P<0.05 vs. non-treated group. (C) Western blot analysis and (D) reverse transcription-quantitative polymerase chain reaction assay indicating regulation of RERG expression in EGF- and XMD8-92-treated PC-3 cells, respectively. *P<0.05 vs. non-treated group. Non-treated, non-treated PC-3 cells; ERK, extracellular signal-regulated kinase; RERG, Ras-like oestrogen-regulated growth inhibitor; t-ERK5, total ERK5, p-ERK5, phosphorylated ERK5.

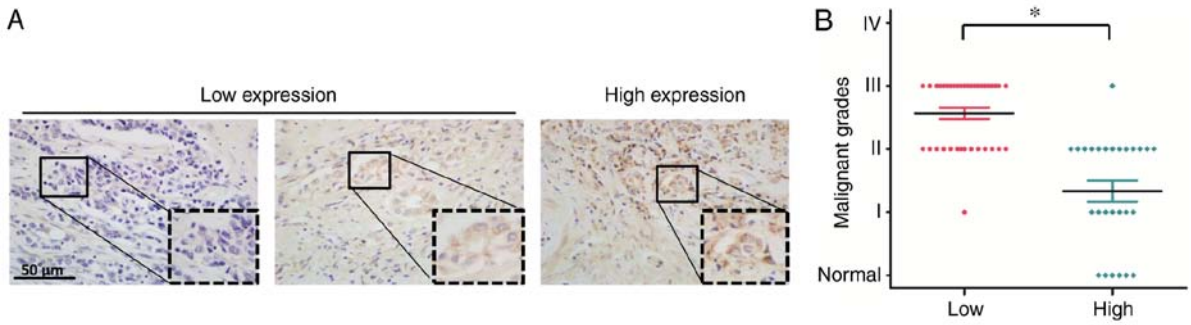
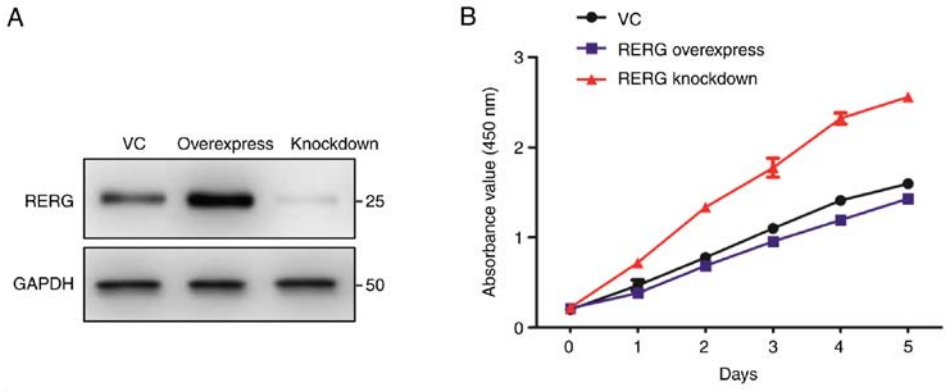


Figure 2. Association between RERG expression and malignancy in prostatic carcinoma and normal tissues. (A) Representative images of RERG expression in prostatic carcinoma and normal tissues. There are two phenotypes in the advanced-stage prostatic carcinoma tissues with lower RERG expression. The images at a higher magnification show details of RERG expression in prostatic carcinoma and normal tissues (magnification, x200 or x400). (B) Pathological analysis of the association between RERG expression and malignancy in prostatic carcinoma and normal tissues. *P<0.05. RERG, Ras-like oestrogen-regulated growth inhibitor.

function of MMP-2 and MMP-9 in RERG-associated invasion and migration in prostatic carcinoma. The expression levels of MMP-2 and MMP-9 in RERG-overexpressing, RERG-inhibited, and empty-vector-transfected PC-3 cells were determined. Notably, western blot analysis revealed that the expression levels of MMP-2 and MMP-9 were inhibited in RERG-overexpressing PC-3 cells, but upregulated in RERG-inhibited PC-3 cells (Fig. 4E). Therefore, the results



D

Group	Total colonies
Control	145.4 ± 28.3
RERG overexpress	137.2 ± 34.7
RERG knockdown	332.1 ± 49.5*

*P<0.01 vs. control

Figure 3. Inhibition of RERG expression induces prostatic carcinoma cell proliferation. (A) RERG expression levels in RERG-overexpressing, RERG-inhibited, and empty-vector-transfected (VC group) PC-3 cells. (B) Proliferation of RERG-overexpressing, RERG-inhibited, and empty-vector-transfected (VC group) PC-3 cells. (C) Representative images and (D) statistical analysis of colony formation in RERG-overexpressing, RERG-inhibited, and empty-vector-transfected (VC group) PC-3 cells, respectively. *P<0.01 vs. VC. RERG, Ras-like oestrogen-regulated growth inhibitor; VC, pcDNA3.1-transfected PC-3 cells.

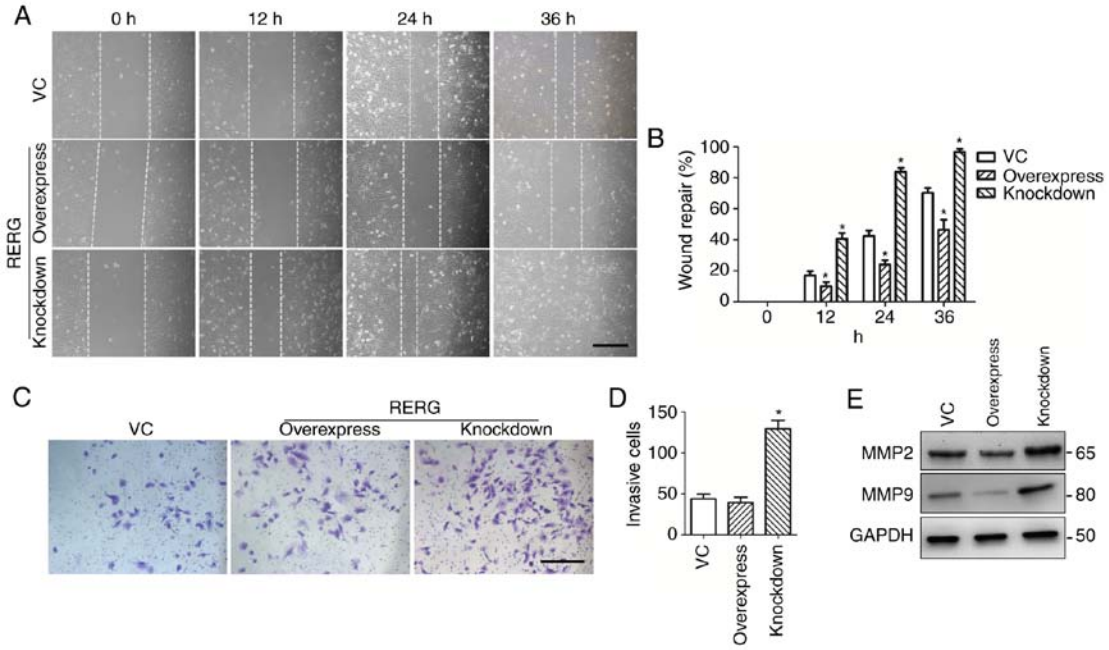


Figure 4. Effects of RERG on tumour invasion and migration in RERG-overexpressing, RERG-inhibited, and empty-vector-transfected PC-3 cells. (A and B) The migration of RERG-overexpressing, RERG-inhibited, and empty-vector-transfected (VC group) PC-3 cells was measured by wound-healing assay at 0, 12, 24, and 36 h. (C and D) Cell invasion was determined by transwell assay in RERG-overexpressing, RERG-inhibited, and empty-vector-transfected (VC group) PC-3 cells at 24 h. (E) MMP-2 and MMP-9 protein expression levels in RERG-overexpressing, RERG-inhibited, and empty-vector-transfected (VC group) PC-3 cells. *P<0.05 vs. VC. VC, pcDNA3.1-transfected PC-3 cells; RERG, Ras-like oestrogen-regulated growth inhibitor; MMP, MMP, matrix metallopeptidase. Scale bar, 200 μm.

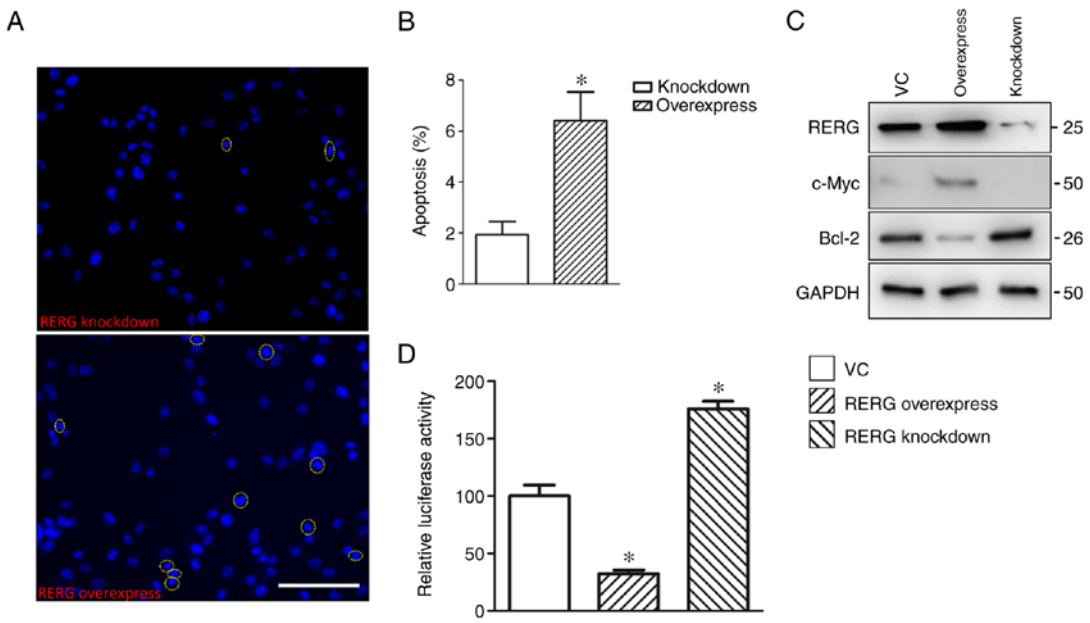


Figure 5. NF-κB-mediated apoptosis and RERG-regulated proliferation of RERG-overexpressing, RERG-inhibited, and empty-vector-transfected (VC group) PC-3 cells. (A) Representative images of Hoechst-stained RERG-overexpressing and RERG-inhibited PC-3 cells. (B) Percentage of apoptotic cells among RERG-overexpressing and RERG-inhibited PC-3 cells. (C) Expression levels of RERG, c-Myc, and Bcl-2 in RERG-overexpressing, RERG-inhibited, and empty-vector-transfected (VC group) PC-3 cells. (D) NF-κB activity in RERG-overexpressing, RERG-inhibited, and empty-vector-transfected (VC group) PC-3 cells. *P<0.05 vs. VC. VC, pcDNA3.1-transfected PC-3 cells; RERG, Ras-like oestrogen-regulated growth inhibitor; Bcl-2, B cell lymphoma-2. Scale bar, 200 μm.

indicated that MMP-2 and MMP-9 play important roles in RERG-associated invasion and migration in prostatic carcinoma.

NF-κB-mediated apoptosis is involved in RERG-regulated prostate cancer cell proliferation. As apoptosis plays important roles in the regulation of cell proliferation in various malignant tumours, it was expected that RERG may promote cell proliferation by inhibiting apoptosis in prostatic carcinoma cells. To clarify the mechanism of RERG function in prostatic carcinoma cell proliferation, Hoechst staining was performed to confirm whether the regulation of RERG expression was associated with the induction of cell apoptosis in RERG-overexpressing or RERG-inhibited PC-3 cells. The results indicated that cell apoptosis was increased in RERG-overexpressing PC-3 cells, suggesting that the regulation of RERG expression was involved in the apoptosis of prostatic carcinoma cells (Fig. 5A and B).

To further illustrate the possible mechanism of RERG-induced apoptosis in prostatic carcinoma cells, the present study then determined the protein expression levels of c-Myc and Bcl-2, which are key factors in apoptosis (24,25), in RERG-overexpressing, RERG-inhibited, and empty-vector-transfected PC-3 cells. Notably, it was revealed that the expression level of Bcl-2 was significantly inhibited, whereas that of c-Myc was upregulated in RERG-overexpressing PC-3 cells compared with empty-vector-transfected PC-3 cells (Fig. 5C). Bcl-2 is an anti-apoptotic factor that is also a regulator of the NF-κB signalling pathway (26). Inactivation of the NF-κB pathway can also induce apoptosis in malignant tumours (27,28). Thus, NF-κB activation in RERG-overexpressing, RERG-inhibited, and empty-vector-transfected PC-3 cells was investigated. As expected, the results indicated that RERG overexpression blocked NF-κB signalling pathway activation (Fig. 5D), which suggested that NF-κB-mediated apoptosis may be involved in RERG-regulated cell proliferation in prostatic carcinoma.

Discussion

As an MAPK family member, ERK5 is essential for cell proliferation and differentiation (29). Kato *et al* (30) observed that ERK5 can promote cell survival and inhibit apoptosis (30). In addition, the ERK5 pathway is essential for vascular development and proliferation (31) and plays a key role in the maintenance of the functions and integrity of endothelial cells (29). Activation of the ERK5 signalling pathway can cause disordered cell cycle regulation, which subsequently results in cell proliferation and tumourigenesis in malignant cancers (13,32). Furthermore, several studies have found high ERK5 expression in breast cancer, squamous cell carcinoma, and prostatic carcinoma, and its expression is associated with malignancy and prognosis in these tumours (33-36), suggesting that the ERK5 signalling pathway may be a potential target for the treatment of patients with malignant cancer.

RERG is a member of the Ras GTPase superfamily, which plays important roles in cell growth, proliferation, survival and differentiation. Unlike the functions of most Ras superfamily members, RERG is an inhibitor of tumourigenesis. Recent studies have demonstrated that RERG expression is associated with the ERK signalling pathway (18,37). In the present study, it was demonstrated that the EGF-activated ERK5 pathway induced the inhibition of RERG expression, whereas treatment with XMD8-92, which is a specific inhibitor of the ERK5 pathway, promoted the expression of RERG protein in prostatic carcinoma cells. Additionally, RERG expression has been

reported to be involved in activation of the Ras/MEK/ERK pathway (14). Therefore, it was expected that ERK5 activation may be a key factor in the Ras/MEK/ERK signalling axis in this process.

ERK5 can be activated by a series of stimulators, including mitogens and growth factors. Phosphorylated ERK5 can translocate from the cytoplasm to the nucleus and subsequently regulate the activity of transcriptional factors to regulate cell proliferation and differentiation. Previous studies have indicated that ERK5 can promote cell proliferation by regulating cell cycle progression in breast cancer and prostatic carcinoma cells (13,38). Herein, the results indicated that activation of the ERK5 pathway inhibited apoptosis to promote cell proliferation by regulating RERG expression in prostatic carcinoma cells. As a tumour suppressor gene, high RERG expression is associated with the expression of a series of genes that define an oestrogen receptor-positive breast tumour subtype and are associated with not only a slow rate of tumour cell proliferation but also a favourable prognosis in cancer patients (14). Moreover, it was demonstrated that the loss of RERG expression regulated the expression of Bcl-2 and c-Myc expression in PC-3 cells, which may be associated with RERG-mediated cell apoptosis. The results also indicated that NF-κB activation may be associated with RERG-induced apoptosis in prostatic carcinoma cells. A previous study demonstrated that the NF-κB/miRNA21/Bcl-2 signalling axis inhibits apoptosis in macrophages (26). Yuan *et al* (39) found that ANXA1 inhibits miRNA-196a in a negative feedback loop through NF-κB and c-Myc to reduce breast cancer cell proliferation (39). Therefore, it was expected that the ERK5/RERG/Bcl-2/NF-κB axis inhibited cell proliferation by inducing apoptosis in prostatic carcinoma cells.

The wound-healing assay indicated that the inhibition of RERG expression induced PC-3 cell migration. To further confirm the possibility that increased/decreased wound healing did not result from increased/decreased cell proliferation but from an increased invasive capability induced by the inhibition of RERG expression in PC-3 cells, a transwell assay, which is commonly used to study the migratory response of endothelial cells, was performed to examine the effects of RERG on PC-3 cell invasion. It was noticed that the number of invasive cells among RERG-inhibited PC-3 cells was much greater than that among RERG-overexpressing PC-3 cells in the transwell assay within only 12 h, which could hardly have been affected by cell proliferation, suggesting that the increased invasive capability of RERG-inhibited PC-3 cells was not a result of RERG-induced proliferation. Notably, the migration and invasion assay indicated that the regulation of RERG expression was also associated with PC-3 cell invasion and migration, and either MMP-2 or MMP-9 was downregulated in RERG-overexpressing PC-3 cells. A previous report indicated that the inhibition of NF-κB activity results in the downregulation of downstream target genes, such as MMPs (40). Therefore, it was hypothesized that XMD8-92 inhibited ERK5 phosphorylation, which inactivated the ERK5 signalling pathway and upregulated RERG expression in prostatic carcinoma cells. Increasing RERG expression subsequently significantly inhibited NF-κB transcriptional activity, which suppressed the expression of MMPs and finally inhibited tumour cell invasion and migration in prostatic carcinoma. However, further investigation, especially into the association underlying RERG, NF-κB and MMPs, will be required to fully clarify the mechanisms underlying RERG-regulated tumour cell invasion and migration in prostatic carcinoma.

In conclusion, the present data provided0. evidence indicating involvement of the ERK5/RERG/NF-κB axis in prostatic carcinoma cell proliferation, invasion and migration. Our findings reveal that the ERK5/RERG/NF-κB axis might be a potential for the targeted treatment of patients with prostatic carcinoma.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author's contributions

LZ conceived and designed the experiments and revised the paper; YX performed the experiments, analysed the data and wrote the paper; HH, SC and HD performed the experiments. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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