

Proprotein convertase subtilisin/kexin type 6 activates the extracellular signal-regulated kinase 1/2 and Wnt family member 3A pathways and promotes *in vitro* proliferation, migration and invasion of breast cancer MDA-MB-231 cells

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Abstract. Breast cancer progression results from the acquisition of genetic and epigenetic alterations that promote tumor cell proliferation and survival. Proprotein convertase subtilisin/kexin type 6 (PCSK6) is a proteinase that regulates the proteolytic activity of various precursor proteins as well as protein maturation. PCSK6 also influences cancer cell proliferation, invasion and migration. Therefore, to investigate the effects of PCSK6 in breast cancer, human breast cancer MDA-MB-231 cells were treated with recombinant human PCSK6 *in vitro*. Treatment with recombinant PCSK6 significantly increased the proliferation, invasion and migration abilities of MDA-MB-231 cells. In addition, PCSK6 treatment reduced cell cycle arrest and prevented apoptosis of MDA-MB-231 cells. This provides further support for the hypothesis that PCSK6 serves a role in promoting tumor cell proliferation. PCSK6 treatment also increased the expression of phosphorylated extracellular signal-regulated kinase 1/2 and Wnt family member 3A, suggesting that these pathways are activated by PCSK6. The results of the present study suggested that PCSK6 may promote the proliferation of breast cancer MDA-MB-231 cells by disturbing cell cycle arrest via the mitogen-activated protein kinase pathway. Therefore, PCSK6 may be a potential therapeutic target for breast cancer.

Introduction

Breast cancer is a major cause of mortality in women worldwide (1). The multistep process of breast cancer progression results from acquisition of genetic and epigenetic alterations in oncogenes and tumor suppressor genes, which confer growth and/or survival advantages to mammary cells. Subsequent molecular alterations may convert premalignant cells into malignant cells with invasive and metastatic abilities (2). These acquired capabilities, namely cell proliferation, tissue invasion and adhesion, are essential for malignant growth and progression (2).

Proprotein convertase subtilisin/kexin type 6 (PCSK6) is a neuroendocrine-specific mammalian subtilisin-associated endoprotease and a calcium-dependent serine proteinase (3). PCSK6 is able to cleave precursor proteins at basic residues within the following motif, (K/R)-(X)_n-(K/R)_↓, where n=0, 2, 4 or 6 and X refers to any amino acid except Cys (3). A total of 8 isoforms of PCSK6 have been reported: PCSK6A-I, A-II, B, C, CS, D, E-I and E-II (4). These isoforms are distributed throughout various human tissues and function in the secretory pathway (5). PCSK6 serves a pivotal role in the activation of proteins in the extracellular matrix (ECM) (4). The ECM contains numerous active proteins, including growth factors, cell adhesion molecules and oncogene products (5), which are important in the occurrence and progression of cancer. Previous studies have indicated that PCSK6 serves an important role in enhancing the progression of prostate cancer (6) and ovarian cancer (7). Furthermore, PCSK6 inhibition decreases skin cancer cell proliferation, tumorigenesis and metastasis (8).

The role of PCSK6 in cancer progression renders it an attractive target for cancer treatment. A previous study indicated that the proprotein convertase inhibitor, α 1-PDX, and the PCSK6 pro-segment, ppPCSK6, may increase breast cancer cell motility, migration and invasion (9). In addition, our previous study demonstrated that short interfering RNA inhibition of PCSK6 reduces proliferation, invasion and migration abilities in MDA-MB-231 cells arrested in G₀/G₁ phase (10).

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Therefore, the present study aimed to further investigate the direct effects and mechanisms of PCSK6 on breast cancer cells.

Materials and methods

Cell culture. The human breast cancer MDA-MB-231 cell line was obtained from the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare, Chicago, IL, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂.

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) with protease inhibitors (PMSF; Beyotime Institute of Biotechnology) on ice for 30 min. The cell lysates were centrifuged at 12,000 \times g for 30 min at 4°C. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol. A total of 25 μ g protein was separated by 10% SDS-PAGE, transferred into polyvinylidene difluoride membranes (GE Healthcare, Chicago, IL, USA), and blocked at room temperature for 30 min in 10 mM Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) and 5% (w/v) skimmed milk.

The membranes were incubated overnight at 4°C with the following primary antibodies at a dilution of 1:1,000: signal transducer and activator of transcription 3 (STAT3; cat. no. 30835), phosphorylated STAT3 (p-STAT3; cat. no. 9134), Wnt family member 3A (WNT3A; cat. no. 2721), extracellular signal-regulated kinase (ERK; p44; cat. no. 4695) and p-ERK (cat. no. 4370; Cell Signaling Technology, Inc., Danvers, MA, USA), as well as GAPDH (cat. no. sc47724; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequent to washing with TBST, the membranes were incubated with mouse anti-rabbit IgG (no conjugate; cat. no. 3677; Cell Signaling Technology, Inc.) as a secondary antibody at a dilution of 1:2,000 for 1 h at 37°C. The proteins were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The densitometric analyses were performed using ImageJ software (version k1.45; National Institutes of Health, Bethesda, MD, USA). All experiments were repeated \geq 3 times.

Cell proliferation assay. MDA-MB-231 cells were seeded onto 96-well culture plates and cultured to 80% confluence. Cultured cells were stimulated with recombinant human PCSK6 (rHuPCSK6) at 1, 10, 50, 100 or 150 ng/ml. Cells with only PBS were used as a control. Following incubation for 24, 48 and 72 h, 20 μ l 5 mg/ml MTT was added to each well, and the cultures were incubated for 4 h at 37°C. The MTT solution was removed and 150 μ l dimethyl sulfoxide (DMSO) was added to dissolve the formazan products at room temperature for 10 min. Absorbance was measured in triplicate at 490 nm using a spectrophotometer. Cell

proliferation was plotted over 72 h relative to each starting value at 24 h. Stimulation was apparent at 150 ng/ml rHuPCSK6 with no cytotoxicity; therefore, this concentration was used in subsequent experiments. All experiments were repeated \geq 3 times.

Cell invasion assay. Cell invasion ability was analyzed using a Transwell apparatus (BD Biosciences, Franklin Lakes, NJ, USA). MDA-MB-231 cells were plated at a density of 3×10^4 cells/well and incubated at 37°C with 150 ng/ml rHuPCSK6 in the upper chamber of the Transwell apparatus for 8 h. The upper and lower chambers were incubated with DMEM without FBS for 12 h, then DMEM containing 20% FBS was placed in the lower chamber for 48 h. Cells remaining in the upper chamber were removed with cotton swabs, and the cells which had invaded the lower chamber were stained with 10% Giemsa for 10 min at room temperature (cat no. G1015; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The number of invasive cells were counted in 5 random fields using a light microscope (Nikon Corporation, Tokyo, Japan; magnification, \times 100).

Wound-healing assay. To assess the migratory ability of MDA-MB-231 cells, they were plated onto 24-well plates and cultured at 37°C to 80% confluence. The cell layers were scratched linearly using a cell scraper, followed by treatment with 150 ng/ml rHuPCSK6. After 24 h, the number of cells that migrated over the scratched line was calculated under a light microscope (magnification, \times 100; Nikon Corporation). The mean of random 5 fields was calculated. The experiment was repeated \geq 3 times.

Cell cycle analysis. Cells were plated onto Costar 6-well culture plates (Corning Incorporated, Corning, NY, USA) at 1.0×10^6 cells/well and treated with 150 ng/ml rHuPCSK6. After 24 h, the cells were harvested by trypsinization, washed twice with phosphate-buffered saline (PBS), and fixed overnight with 70% ethanol at 4°C. The fixed cells were rinsed and resuspended in PBS, and stained for 30 min at 37°C with 1 ml 0.05 mg/ml propidium iodide solution containing 10 μ g/ml RNase (Beijing Dingguo Biotechnology, Beijing, China). Cells were detected with a Coulter Epics XL flow cytometer, and DNA content was analyzed using Beckman Coulter EXPO32 ADC software (version 1.2B; both Beckman Coulter, Inc., Brea, CA, USA). The experiments were repeated 3 times in triplicate.

Apoptosis assay. MDA-MB-231 cells were cultured in 2% FBS in the presence or absence of 150 ng/ml rHuPCSK6 for 24 h and stained using an Annexin V-fluorescein isothiocyanate apoptosis detection kit (BD Biosciences), according to the manufacturer's protocol. Cells were detected using a Coulter Epics XL flow cytometer, and DNA content was analyzed with Beckman Coulter EXPO32 ADC software (version 1.2B; both Beckman Coulter, Inc.). The experiments were repeated 3 times in triplicate.

Statistical analysis. All results were confirmed in \geq 3 independent experiments. Multiple comparisons were conducted using one-way analysis of variance followed by Bonferroni's

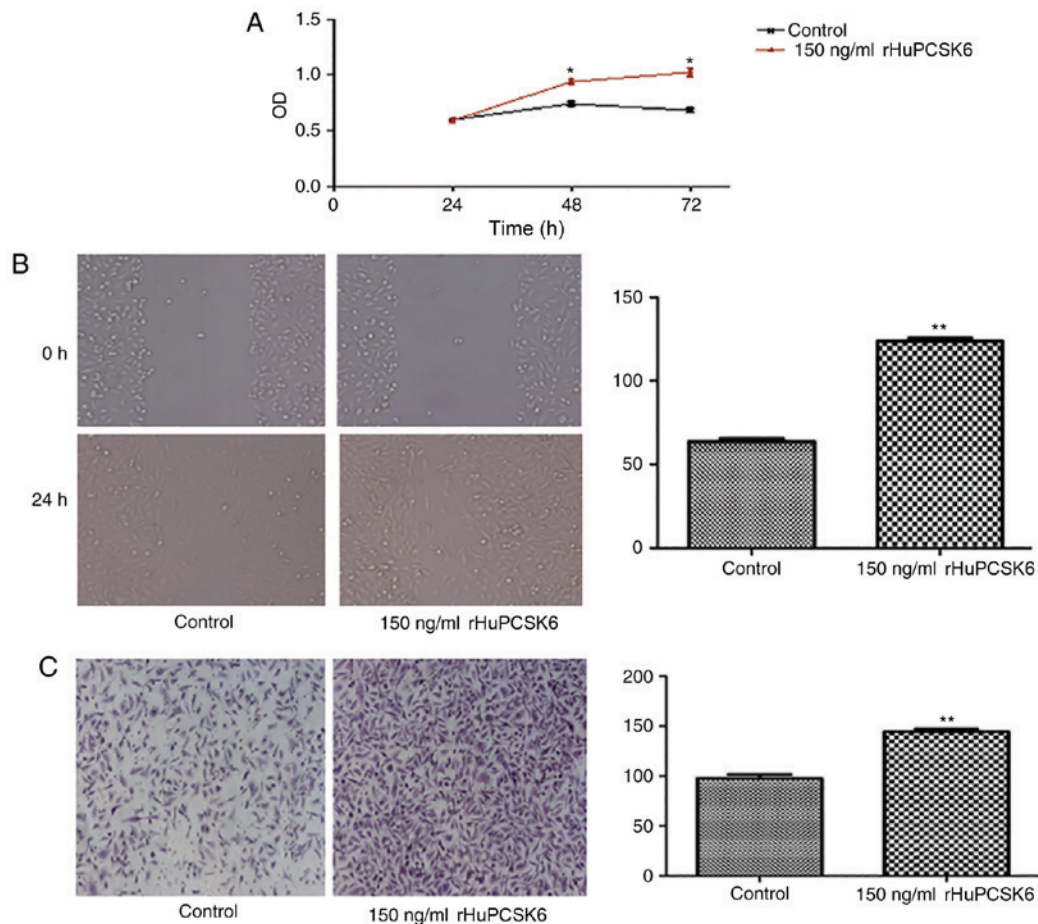


Figure 1. Effect of rHuPCSK6 on proliferation, migration and invasion of MDA-MB-231 cells. (A) MTT assay results demonstrate MDA-MB-231 cell proliferation at 24, 48 or 72 h after treatment with 150 ng/ml rHuPCSK6. Cells treated with vehicle only were used as control. (B) Representative images and quantification of wound healing assays measuring cell migration of non-proliferating MDA-MB-231 cells following wounding and control treatment or 150 ng/ml rHuPCSK6. (C) Representative images and quantification of Transwell assays revealing the mean number of MDA-MB-231 cells that invaded Transwell filters following control treatment or 150 ng/ml rHuPCSK6. * $P < 0.05$, ** $P < 0.01$ vs. control cells. The experiments were repeated ≥ 3 times. rHuPCSK6, recombinant human PCSK6; OD, optical density.

correction. The data are expressed as mean \pm standard error of the mean. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

rHuPCSK6 promotes the proliferation, migration and invasion of MDA-MB-231 cells. In our previous study, PCSK6 knockdown by RNA interference was demonstrated to reduce the proliferation, migration and invasion of MDA-MB-231 cells. Due to the fact that PCSK6 is a secreted protein, extracellular PCSK6 may also have an effect. MDA-MB-231 cells stimulated with 150 ng/ml rHuPCSK6 exhibited significantly increased proliferation compared with control cells ($P < 0.05$; Fig. 1A). In addition, wound-healing assays revealed that cells stimulated with rHuPCSK6 exhibited significantly increased migration capability compared with control cells ($P = 0.01$; Fig. 1B). Transwell assays indicated that cells stimulated with rHuPCSK6 had enhanced invasion capability compared with control cells ($P = 0.01$; Fig. 1C).

rHuPCSK6 reduces cell cycle arrest and prevents apoptosis of MDA-MB-231 cells. Flow cytometric analysis demonstrated that MDA-MB-231 cells stimulated with 150 ng/ml rHuPCSK6 had a significantly lower ratio of G_0/G_1 phase cells ($P < 0.001$) and a significantly higher ratio of S phase ($P < 0.01$) and G2/M phase ($P < 0.001$) cells compared with control cells (Fig. 2A). Furthermore, rHuPCSK6 treatment significantly reduced Annexin V-stained late apoptotic cells compared with control cells ($P = 0.02$; Fig. 2B).

rHuPCSK6 activates ERK1/2 and WNT3A pathways in MDA-MB-231 cells. ERK1/2, are catalytically activated by phosphorylation and are associated with cancer cell proliferation (11). To investigate the pathway by which PCSK6 directly or indirectly regulates cell proliferation, ERK1/2 activity was analyzed in MDA-MB-231 cells following stimulation with 150 ng/ml rHuPCSK6 for 30 min by western blotting. The western blots demonstrated that rHuPCSK6 treatment increased ERK1/2 phosphorylation in MDA-MB-231 cells (Fig. 3).

The WNT signaling pathway mediates biological processes, including cell adhesion, migration, proliferation, differentiation and survival (12). WNT3A is overexpressed in breast

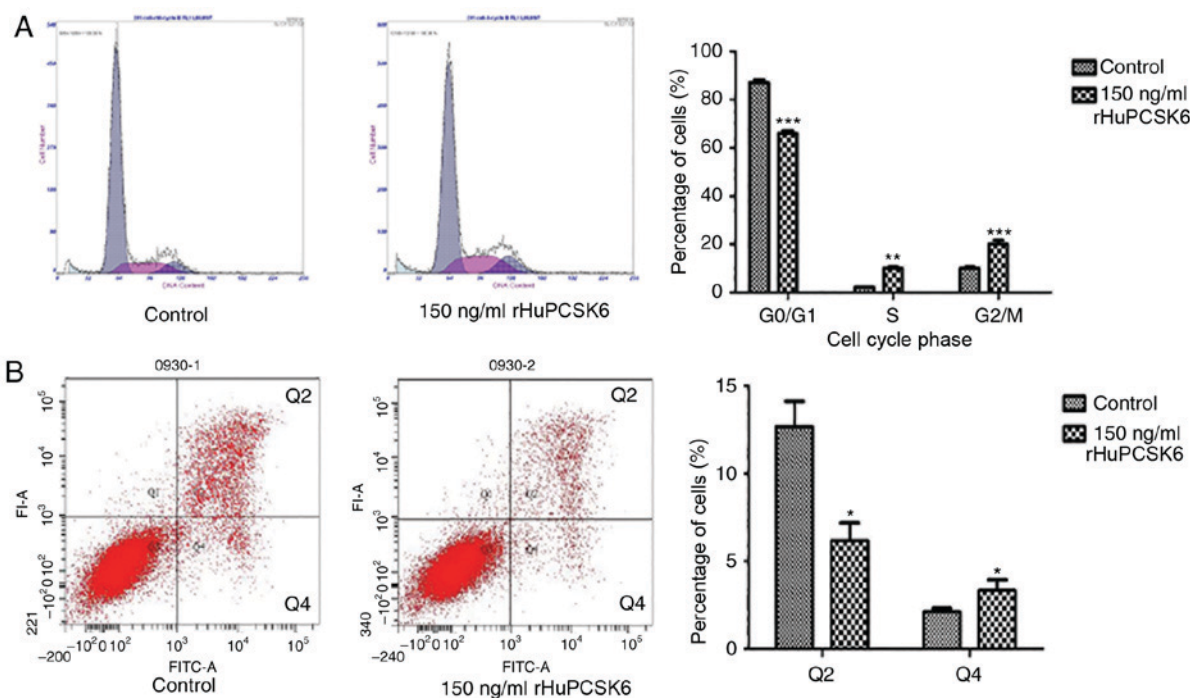


Figure 2. Effect of rHuPCSK6 on cell cycle distribution and apoptosis of MDA-MB-231 cells. (A) Representative graphs and quantification of flow cytometry results analyzing cell cycle distribution of propidium iodide-stained MDA-MB-231 cells. (B) Representative graphs and quantification of flow cytometry results analyzing apoptosis of MDA-MB-231 cells stained with Annexin V-FITC. Data are expressed as the mean \pm standard error of 3 independent experiments conducted in triplicate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control cells. rHuPCSK6, recombinant human PCSK6; FITC, fluorescein isothiocyanate.

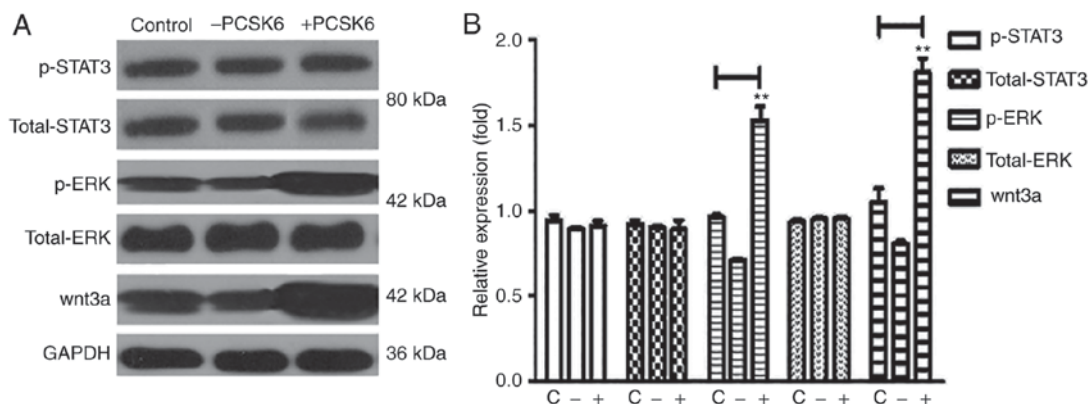


Figure 3. Effect of rHuPCSK6 on ERK1/2 and WNT3A pathways in MDA-MB-231 cells. (A) Representative western blots of activated ERK1/2, STAT3 and WNT3A in the lysates of control MDA-MB-231 cells or cells treated with 150 ng/ml rHuPCSK6. GAPDH was used as a loading control. The experiments were repeated 3 times. (B) Densitometric quantification of western blotting results. ** $P < 0.01$ vs. control. rHuPCSK6, recombinant human PCSK6; ERK1/2, extracellular signal-regulated kinase 1/2; STAT3, signal transducer and activator of transcription 3; WNT3A, Wnt family member 3A; p-, phosphorylated; C, control; -, without rhPCSK6; +, 150 ng/ml rhPCSK6.

cancer (13-15); therefore, the present study examined whether its expression may be influenced by PCSK6. Stimulation of MDA-MB-231 cells with rHuPCSK6 increased the expression of WNT3A (Fig. 3). Oncogenic STAT3 hyperactivation has been observed in breast cancer (16). However, activation of STAT3 by rHuPCSK6 treatment was not demonstrated in MDA-MB-231 cells in the present study.

Discussion

The critical role of proprotein convertases in proteolytic maturation of multiple precursor substrates implicated in

neoplasia makes them attractive targets for the development of potent and selective inhibitors of tumorigenesis (17,18). In recent years, the association between proprotein convertases and cancer has been extensively studied. PCSK6 is often overexpressed in breast cancer, and Lapierre *et al* (9) demonstrated that overexpression of the ppPCSK6 prosegment in MDA-MB-231 breast cancer cells significantly enhanced cell motility, migration and invasion abilities *in vitro*. However, the mechanistic role of PCSK6 in these processes remains unclear.

In our previous study (19), it was demonstrated that knock-down of PCSK6 expression in breast cancer MDA-MB-231

cells inhibited proliferation, invasion and migration abilities. As PCSK6 is a neuroendocrine-specific mammalian subtilisin-related endoprotease (20) that exhibits a C-terminal cysteine-rich region (10) and functions in the secretory pathway, it may serve important roles in the ECM. Therefore, in the present study, the effect of exogenous PCSK6 on MDA-MB-231 cells was investigated. The results of the present study were in line with those of previous studies using siRNA knockdown (19).

In breast cancer, increased cell proliferation and/or decreased apoptosis contribute to tumor cell hyperplasia, which contributes to breast cancer pathogenesis (21). In the present study, rHuPCSK6 stimulation of MDA-MB-231 cells was demonstrated to significantly promote cell proliferation, migration and invasion abilities, suggesting that PCSK6 may serve an important role in breast cancer hyperplasia. Due to the fact that rHuPCSK6 was isolated from wheat germ, there was no risk of lipopolysaccharide contamination. In our previous study (19), it was demonstrated that knockdown of PACE4 (PCSK6) is able to decrease the migration, invasion and proliferation abilities of MDA-MB-231 cells, which is consistent with the report by Lapierre *et al* (9). In the present study, it was indicated that stimulation with recombinant PCSK6 significantly increased the proliferation, invasion and migration abilities of breast cancer cells compared with control cells. PCSK6 was also indicated to reduce cell cycle arrest and prevent apoptosis of MDA-MB-231 cells, and increase the expression of the phosphorylated forms of ERK1/2 and WNT3A, compared with control cells. These results are consistent with our study on rheumatoid arthritis synovial fibroblasts and in prostate cancer (10), and a previous study by Lapierre *et al* (9), in which α 1-antitrypsin Portland (α 1-PDX) was used as an inhibitor of proprotein convertases, including PCSK6, and reduced the mRNA expression level of furin but increased that of PCSK6. In the cell migration assay, α 1-PDX was demonstrated to increase the migratory ability of MDA-MB-231 cells, suggesting that PCSK6 may induce the migration of MDA-MB-231 cells.

Overall, the results of the present study suggested that PCSK6 may promote the proliferation of MDA-MB-231 cells by disturbing cell cycle arrest through the mitogen-activated protein kinase pathway. The present study also demonstrates that rHuPCSK6-mediated activation of MDA-MB-231 cells likely occurs via the ERK1/2 and WNT3A pathways, as the level of phosphorylation of these proteins was increased following rHuPCSK6 treatment. ERK1/2 isoforms serve a major role in cell proliferation signaling (10). The main limitation of the present study was that only MDA-MB-231 cells were used. Further research should analyze the role of PCSK6 in other breast cancer cell lines. In summary, exogenous PCSK6 may exacerbate the progression of breast cancer through its effects on tumor cells. Furthermore, the ERK1/2 and WNT3A signaling pathways may mediate the stimulatory role of PCSK6 in breast cancer. Therefore, PCSK6 may be a potential therapeutic target of breast cancer.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PW and FW performed the experiments. LW analyzed the data. JP designed the study.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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