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CCDC3 Gene Regulates the Proliferation of Breast Cancer Cells

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We studied the effect of *CCDC3* on the viability of human breast cancer cell line MDA-MB-231. The levels of *CCDC3* mRNA and the corresponding protein in MDA-MB-231, MCF-7, T-47D, and HCC1937 cell lines were measured by reverse transcription quantitative real-time PCR and Western blotting. Since MDA-MB-231 cells had higher expression of mRNA *CCDC3* and *CCDC3* protein, we used this cell line for transfection with small interfering RNA by lentivirus. Cell Counting Kit-8 and clone formation assay were used to detect the effects of *CCDC3* knockdown on cell viability; flow cytometry was used to detect the effects of *CCDC3* knockdown on cell apoptosis and cell cycle. In MDA-MB-231 cell line, the CCDC3 protein level was significantly down-regulated after *CCDC3* knockdown in comparison with the control group ($p<0.05$). The cell viability and the number of clones in the *CCDC3* knockdown group were significantly reduced ($p<0.05$), while the apoptosis rate significantly increased ($p<0.05$). Thus, after *CCDC3* knockdown, cell viability is weakened in MDA-MB-231 cells, and cell apoptosis rate is increased. Therefore, *CCDC3* gene is promising as a new candidate target for BC treatment.

Key Words: *CCDC3; breast cancer; cell viability; apoptosis; recurrence*

Breast cancer (BC) is the most common malignant tumor and the main reason for the high mortality rate mortality rate due to oncological diseases in women [1,2]. According to the National Center for Health Statistics report, BC accounts for 30% of new cancer cases, and the mortality rate ~15% in 2021 [3]. BC is a heterogeneous disease with currently unclear pathogenesis. Despite great progress in the early detection and treatment of BC and improved survival rate, BC cells are prone to distant metastasis, such as lung, liver, bone, and brain metastasis. Many patients, especially triple-negative BC patients, often relapse due to organ metastasis. The 5-year survival rate of BC patients with distant metastasis is less than 20% [4]. If the pathological tissue is removed before the invasion and metastasis of cancer cells, the 10-year survival rate can be increased up to 98%. Therefore, early diagnosis and early treatment are important means to reduce BC-related mortality.

Coiled-coil domain containing 3 (*CCDC3*) is a newly discovered gene encoding fat/vessel-derived secretory protein (favine, NCBI nucleotide entry NM_028804) [5]. Human *CCDC3* encodes a 270 a.a. protein molecule with a molecular weight of 32 kDa. CCDC3 protein contains a coiled-coil domain in its C-terminal region, which is a structural feature of many proteins, especially transcription factor and motor proteins. Proteins containing helical domains usually interact with other helical proteins and participate in other protein–protein interactions [6]. Being a cell secretory factor, CCDC3 is highly expressed in the adipose tissue and endothelial cells [6]. In patients with abdominal obesity, the expression of CCDC3 is increased specifically in the visceral adipose tissue, but not in the subcutaneous adipose tissue. These results suggest that CCDC3 can be a new marker of visceral adipose tissue and can participate in the development of lipid metabolism disorders in obese patients. CCDC3, as a

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novel TAp63 target, acts as an endocrine molecule that targets hepatocytes and alleviates the pathological changes associated with steatosis induced by high-fat diet, and attenuates progression or deterioration of diseases associated with insulin resistance via an anti-lipogenesis effect in the liver [7].

Additional research [8] also showed that in cattle, pigs, and humans, *CCDC3* gene transcripts have the highest activity in the adipose tissue, suggesting that the protein encoded by *CCDC3* can play a functional role in the pathophysiology of obesity and atherosclerosis. As a cytokine secreted by vascular endothelial cells, CCDC3 can be involved in inhibition of the endothelial inflammatory response induced by TNF and NK-κB [9]. In studies on other types of tumors, low expression of *CCDC3* has been found to reduce the proliferation, migration, invasion, and epithelial—mesenchymal transition of cervical cancer cells. However, the effect of *CCDC3* on BC cells had not been reported.

The aim of this paper was to study the role of *CCDC3* gene and its protein in the maintenance of the viability of BC cell line MDA-MB-231.

MATERIALS AND METHODS

Cell culture. Human BC cell lines MDA-MB-231, MCF-7, T-47D, and HCC1937 were purchased from Shanghai Jikai Gene Chemical Technology Company. After thawing, the cells were placed in high glucose DMEM medium containing 10% fetal bovine serum (Ausbian) and cultured at 37°C and 5% CO_2 in a CO_2 incubator (Sanyo).

Vector construction and transfection. The specific small interfering (siRNAs) for negative control (NC, CON077) and *CCDC3* gene (CCDC3-RNAi (93113-1) for KD1 group: CGAACCAGAAACTCAGTGAGA, CCDC3-RNAi (93114-1) for KD2 group: CCTCACGGAGTCAATTTCCAA, and CCDC3-RNAi for KD3 group (93115-1) CGCATTTGGTAGAGTCTAAAT) were synthesized by Jikai Gene Chemical Technology Company. MDA-MB-231 cells were transfected with siRNAs or NC using lentivirus and cultured for 5 days. Three repetitions per group were performed. Fluorescence of transfected cells was assessed using an Olympus IX71 fluorescence microscope.

Reverse transcription quantitative real-time PCR (RT-qPCR). RNA was extracted from BC cell lines with TRIzol (Pufei Biotech Co., Ltd.) and reverse transcribed into cDNA using M-MLV reverse transcriptase. Then, we used SYBR Green qPCR Master Mix (Thermo Scientific) to perform qPCR on the LightCycler 480 II real-time PCR detection system (Roche). The primers were purchased from Ruibo Biotechnology Co., Ltd.: for *ACTB* — F: 5'-GCGTGACATTAAGGAGAAGC-3', R: 5'-CCACGTCACACTTCATGATGG-3' and for *CCDC3* — F: 5'-AGTCAATTTCCAAGATGCCA-3', R: 5'-CGAGGAGCACATGAGCCTAC-3'. The RT-qPCR reaction conditions were: pre-denaturation at 95°C for 30 sec, 40 cycles at 95°C for 5 sec, 56°C for 30 sec, 72°C for 15 sec. The relative expression of *CCDC3* and *ACTB* is calculated as $2^{-\Delta\Delta Ct}$.

Western blotting. The total protein was extracted with cell lysate RIPA (Bi Yuntian Biotech Co., Ltd.) and the protein expression was measured. The protein concentration was measured using BCA reagent (Bi Yuntian Biotech Co., Ltd.). The proteins were separated by SDS-PAGE electrophoresis using an SDS-PAGE protein electrophoresis instrument (Shanghai Tanon). The separated proteins were transferred to a membrane. The membrane was blocked with 5% skim milk in PBS with 0.1% Tween-20 for 1.5 h at room temperature and then incubated overnight at 4°C with primary antibodies to CCDC3 (1:1000; Genetex) and β-actin (1:5000; Santa Cruz). After that, the membrane was washed and incubated with secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:10,000, cat.# 7074, CST) and horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:10,000; cat.# 7076, CST) for 1 h at room temperature. The protein bands were visualized using Pierce ECL Western Blotting Substrate (Thermo Scientific) according to the manufacturer's protocol and analyzed using ImageJ software.

Cell viability assay. After 12-h transfection, the cells were digested with 0.25% trypsin (Gibco) to prepare a suspension and seeded in a 96-well plate. After culturing for 1, 2, 3, 4, and 5 days, 10 μl CCK-8 reagent was added (Japan Tongren Chemical Technology Co., Ltd.), and optical density (OD) was measured at 450 nm on a M2009PR microplate reader (Tecan Infinite).

Colony formation assay. The cells were inoculated into a 6-well plate, and after continuous culture for 14 days, the cells were fixed with 4% paraformaldehyde for 60 min, and stained with crystal violet for 10 min; cell clones were photographed and counted using inverted microscope (Shanghai Caikon Optical Instrument Co., Ltd.).

Apoptosis detection. Cell apoptosis was assessed using Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime Institute of Biotechnology) according to manufacturer's protocol. The apoptotic cells were counted by flow cytometry on a FACSCalibur (BD Biosciences) and analyzed using C6 Plus analysis software (BD).

Cell cycle analysis. The cells were collected and resuspended in a centrifuge tube. The cell cycle was analyzed on a FACSCalibur flow cytometer (BD) using PI-FACS Cell Cycle Detection Kit (Sigma) according to the manufacturer's instructions.

Statistical analysis. SPSS Statistics 25.0 software (IBM) was used for statistical analysis. The continuous variables conforming to the normal distribution were represented as *M*±*SD*. If the comparison between the two groups conformed to the normal distribution and met the homogeneity of variance, independent sample *t* test was used. The continuous variables not conforming to the normal distribution were represented as the Me (Q1; Q3). The comparison between the groups was conducted using Mann–Whitney *U* test. The relative expression of mRNA was analyzed by $2^{-\Delta\Delta Ct}$ method, and the relative expression of protein level was analyzed by gray value. The difference was considered significant at $p<0.05$.

RESULTS

The expression of *CCDC3* gene in BC cell lines. The results of RT-qPCR showed that the level of *CCDC3* expression in MDA-MB-231 was significantly higher than in other cell lines ($p<0.05$; Fig. 1, *a*). The results of Western blotting showed that the level of CCDC3 protein expression in MDA-MB-231 was also significantly higher than in other cell lines ($p<0.05$; Fig. 1, *b*). Therefore, all BC cell lines expressed the *CCDC3* gene, and the highest level was observed in MDA-MB-231 cells. In light of this, we choose MDA-MB-231 line for *CCDC3* knockdown.

The immunofluorescence results showed effective knockdown of the *CCDC3* gene in all groups of MDA-MB-231 (KD1, KD2, and KD3) in comparison with NC (Fig. 1, *c*). The RT-qPCR results showed that the KD3 group had the highest knockdown rate of *CCDC3* (94.4%) in comparison with the NC group. The results of Western blotting showed that the expression of CCDC3 protein in the KD3 group was significantly downregulated in comparison with the NC group ($p<0.001$; Fig. 1, *d*).

The effect of *CCDC3* gene knockdown on the proliferation, apoptosis, and cell cycle of MDA-MB-231 cells. CCK-8 analysis showed that the proliferation rate of the KD3 group was significantly reduced at each time point, and the inhibition of cell proliferation began to be significant on day 3, and

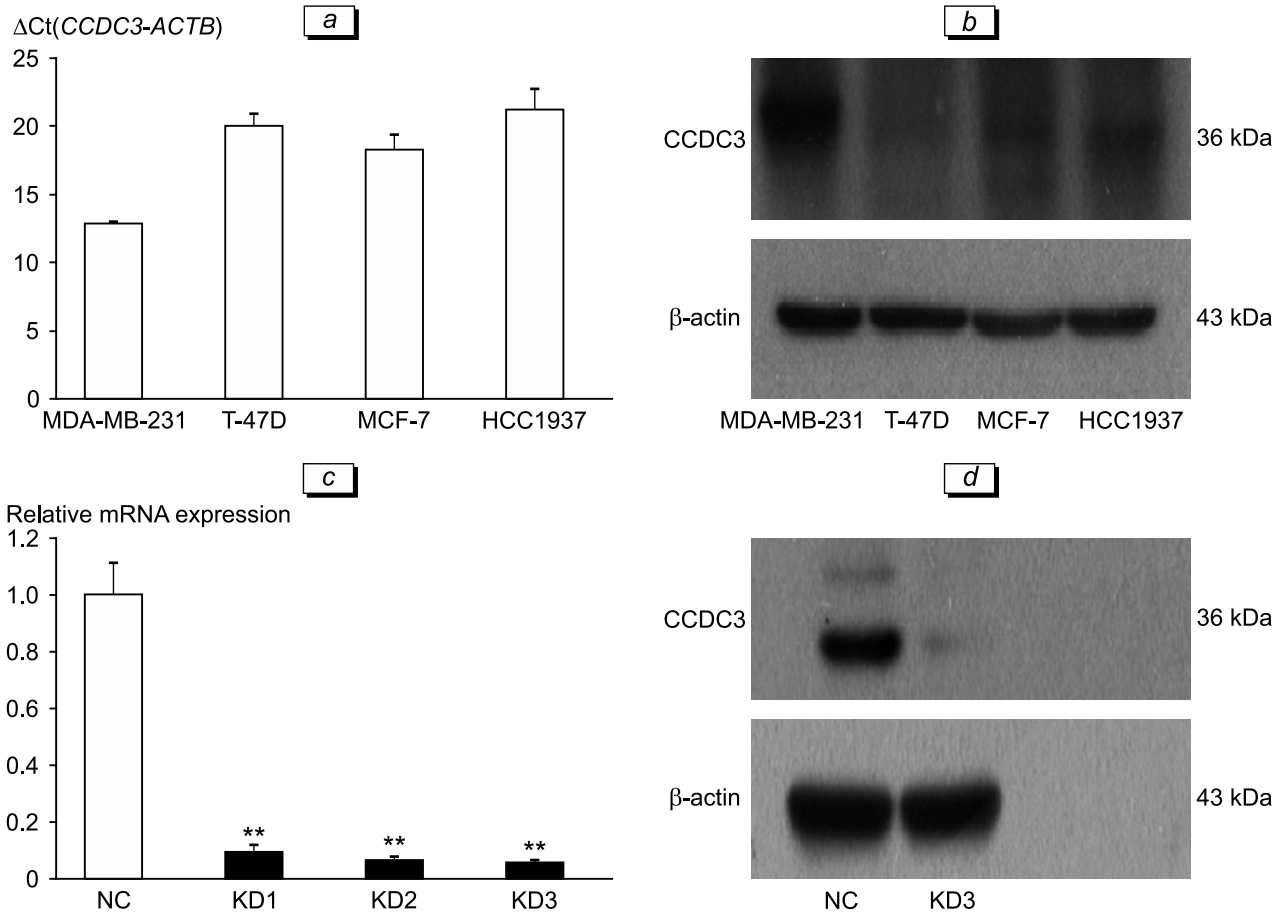

Fig. 1. The expression of *CCDC3* gene (*a*), *CCDC3* mRNA (*c*), and the corresponding protein (*b*, *d*) in intact MDA-MB-231, T-47D, MCF-7, and HCC1937 cell lines (*a*, *b*) or after *CCDC3* knockdown in MDA-MB-231 cells (KD1-3) (*c*, *d*). **$p<0.01$ in comparison with NC group.

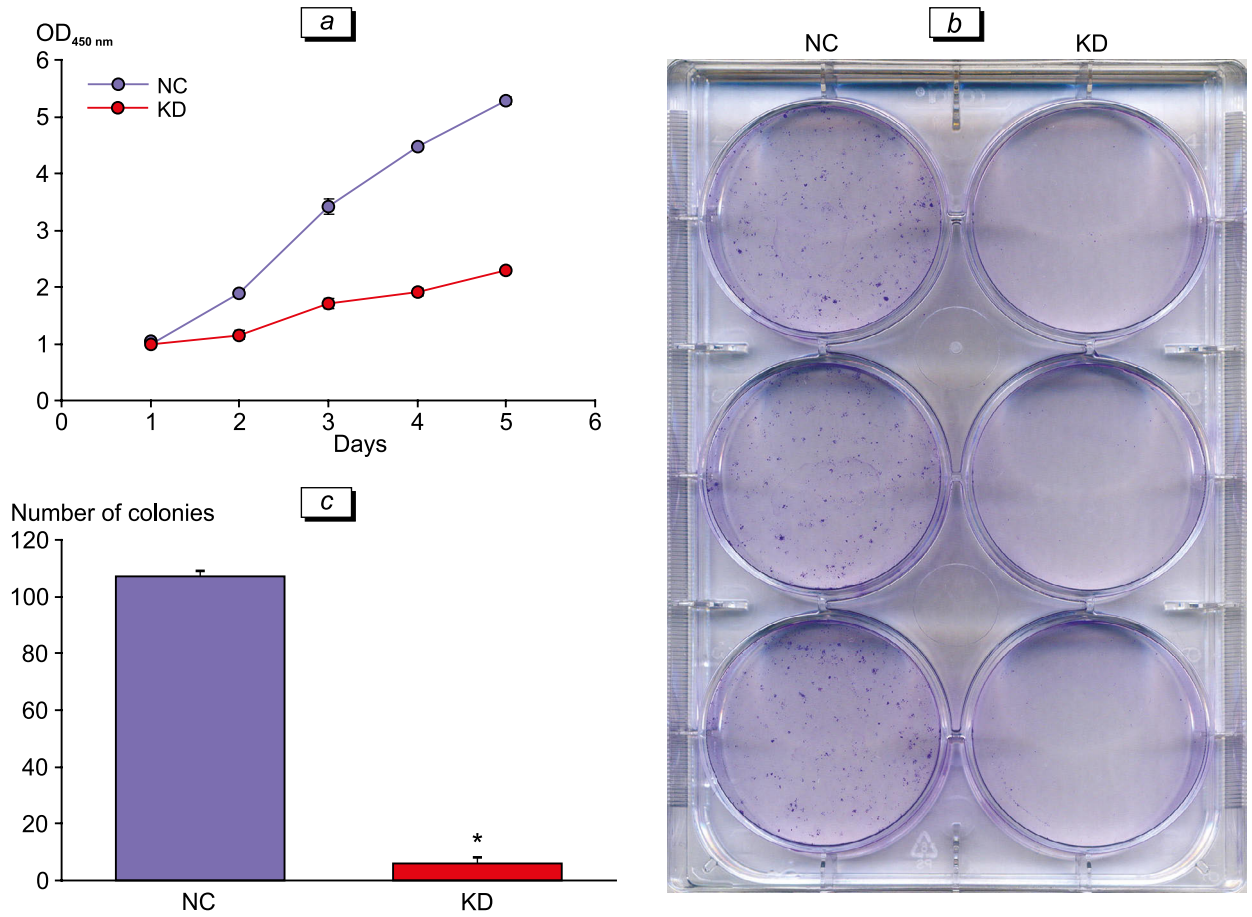

Fig. 2. The effect of *CCDC3* knockout on proliferation (*a*) and cloning ability (*b*, *c*) in normal and transfected MDA-MB-231 cells. *$p<0.05$ in comparison with NC group.

reached the peak on day 5 ($p<0.05$ in comparison with NC group; Fig. 2, *a*). At the same time, the results of the clone formation experiment also indicated that the number of cell clones in the KD3 group was significantly ($p<0.05$) reduced in comparison with the NC group (Fig. 2, *b*, *c*). Therefore, the *CCDC3* knockdown inhibited the proliferation and cloning ability in MDA-MB-231 cells.

The apoptosis rate of the KD3 group was significantly ($p<0.05$) increased in comparison with NC group, indicating that the knockout of *CCDC3* can promote apoptosis of BC cells (Fig. 3, *a*, *b*).

The cell cycle test results showed that the number of S-phase KD cells increased in comparison with that in the NC group, indicating that the *CCDC3* gene affected the cell cycle distribution of MDA-MB-231, and the *CCDC3* knockdown blocked the cells in the S-phase ($p<0.05$; Fig. 3, *c*).

Although clinical treatment of BC has made great progress in recent years, the comprehensive treatment mode, such as BC screening, early diagnosis, surgery, radiotherapy, chemotherapy, endocrine and immunotherapy can reduce the mortality of patients. However, there are still many BC patients who dies of recurrence and metastasis after BC surgery. Metastatic BC is the most common cause of death [10-14]. It has been reported that the 5-year survival rate of BC patients is 82.4%, while that of metastatic BC is only 21%. Therefore, we need to constantly explore the molecular mechanism of recurrence, metastasis, invasion, and proliferation of BC in order to search for potential intervention targets. Early diagnosis and effectively treatment BC cells before invasion and metastasis can greatly improve the survival rate of patients.

In BC, cancer cells continue to proliferate and inhibit apoptosis to complete the process of expanding the lesion, invading normal tissues, and further distant metastasis, making the patient's progress worsen. CCDC3 plays an important role in tumor cell proliferation [11], but its expression and role in BC are still unclear. This study first verified its expression in 4 main BC cells and found that the *CCDC3* gene was significantly highly expressed in BC cells with the highest level in MDA-MB-231 line. Further verification after *CCDC3* knockdown in MDA-MB-231 showed that the *CCDC3* downregulation significantly inhibited the

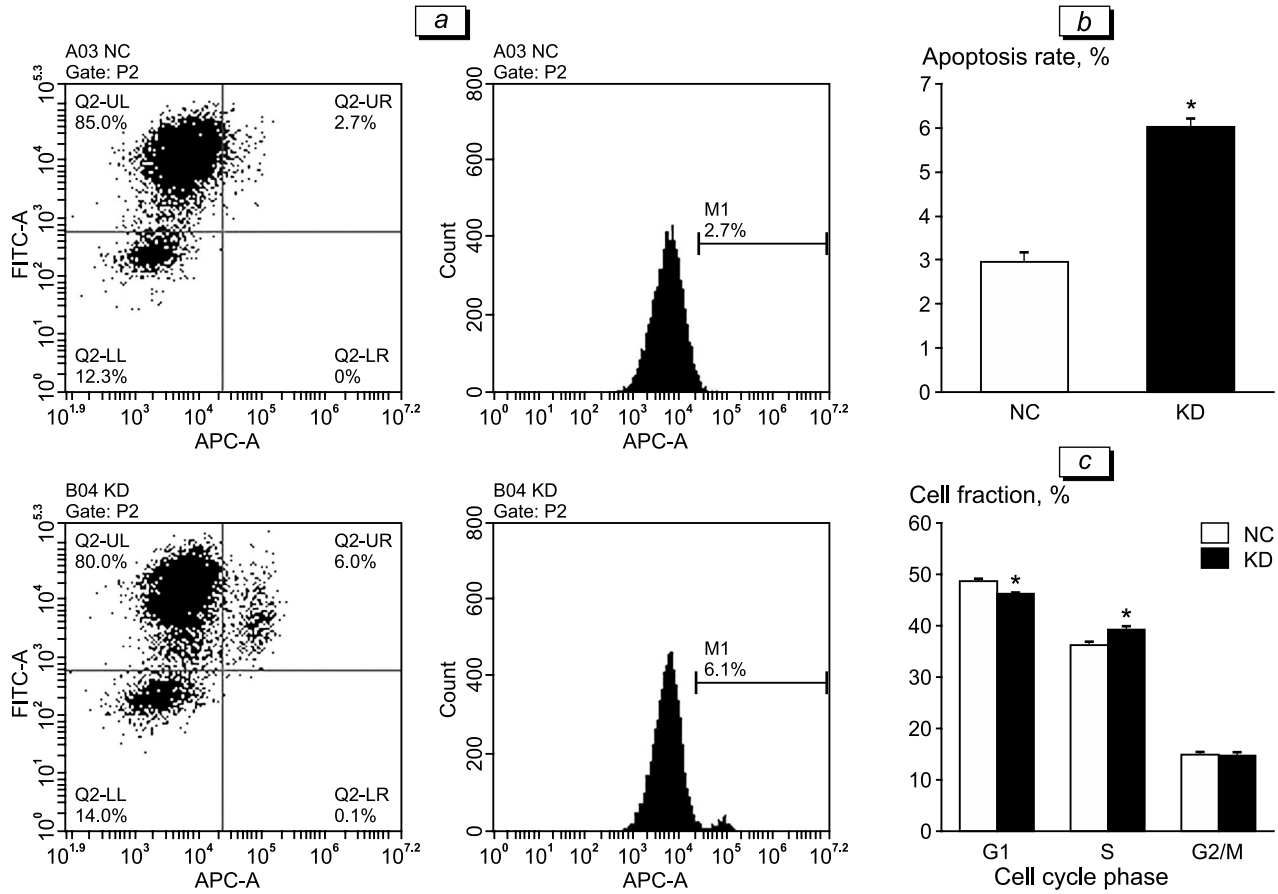

Fig. 3. The effect of *CCDC3* knockout on apoptosis rate (*a*, *b*) and the fraction of G1-, S- and G2/M-phase cells (*c*) in normal and transfected MDA-MB-231 cells. **p*<0.05 in comparison with NC group.

proliferation and cloning ability, promoted apoptosis, and affected the cell cycle in MDA-MB-231 cell line. This shows that, similar to reports in other types of tumors, *CCDC3* is highly expressed in BC and plays an important role in the proliferation of BC cells.

Although we have demonstrated the effect of *CCDC3* on the proliferation of BC, the role and specific molecular mechanisms of *CCDC3* in cell migration, invasion, and epithelial–mesenchymal transition after proliferation require further studies to clarify.

In conclusion, this study proves that *CCDC3* is highly expressed in MDA-MB-231 cell line, and *CCDC3* knockdown attenuates cell proliferation, increases the rate of apoptosis, and affects the cell cycle. Therefore, CCDC3 is promising as a new candidate target for therapeutic intervention in BC.

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