Original Article

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Lentivirus-mediated knockdown of CEP55 suppresses cell proliferation of breast cancer cells

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Summary **Centrosomal protein 55 (CEP55), as a microtubule-bundling protein, plays an important role in cell cycle regulation. CEP55 has been recognized recently in several human cancers. In this study, we first observed that the mRNA level of CEP55 is commonly up-regulated in breast cancer compared with their normal counterparts as demonstrated by data derived from Oncomine database. To further evaluate the functional role of CEP55 in breast cancer cells. Expression of CEP55 was efficiently knocked down using lentivirus-mediated RNA interference in human breast cancer cell line ZR-75-30, as evidenced by quantitative real-time PCR (qRT-PCR) and Western blot analysis. Further investigations revealed that CEP55 knockdown significantly inhibited cell proliferation and colony formation. Moreover, flow cytometer analysis indicated knockdown of CEP55 induced cell cycle arrested at G0/G1 phase and cell apoptosis. These findings suggest that CEP55 plays a crucial role in promoting breast cancer cell proliferation and it might be a potential therapeutic target in breast cancer.**

Keywords: Breast cancer, CEP55, RNA interference, cell proliferation

1. Introduction

Breast cancer is recognized as one of the most frequently diagnosed cancers in women in the worldwide (*1*), whose main characteristic is the uncontrollably proliferative ability of cancer cells (*2*). Although significant progress has been made in surgical techniques and early detection for breast cancer in recent years, higher recurrence and mortality rate remain to be major obstacles in the effective therapeutics for breast cancer (*3*). The environmental factors and genetic factors have been proved to be closely associated with breast cancer (*4*). However, the deep molecular mechanism underlying the growth and development of breast cancer remains elusive. Therefore, it is urgently needed to identify novel therapeutic targets, which might provide fundamental information for future new therapy for breast cancer.

Centrosomal protein 55 (CEP55) has been identified as a microtubule-bundling protein required for cytokinesis. It is located to the mitotic spindle during prometaphase and metaphase and recruited into the midbody during cytokinesis (*5,6*). In addition, CEP55 is a key regulator of cytokinesis essential for the midbody structure and vesicle trafficking (*6*). Accumulating evidences have shown CEP55 is highly expressed in several cancers including human colon cancer and lung cancer (*7-10*). Interestingly, CEP55 has been demonstrated to be involved in tumorigenesis of breast cancer (*11-13*). However, the functional role of CEP55 in breast cancer is still largely unknown. As our best knowledge, regulation of cell cycle is closely related with the occurrence and development of tumor (*14*). Cyclins are regulatory proteins involved in cell cycle process, which is associated with CEP55 expression levels (*7*). Related study indicates that knockdown of CEP55 induces cytokinesis failure and generates genomic instability, even down regulates tumor suppressor genes and activates oncogenes (*5*), which suggests CEP55 expression plays a tight role in cell division occurrence. Considering CEP55 is implicated as an oncogene in human lung cancer (*15*), it might be supposed to be involved in human breast cancer.

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In this study, we first determined the expression pattern of CEP55 in Oncomine database. Furthermore, we investigated the biological function of CEP55 in breast cancer cell growth. Our results might help to gain better understanding of the role of CEP55 in breast cancer growth and development.

2. Materials and Methods

2.1. *Analysis of Oncomine cancer gene microarray database*

To evaluate the expression level of CEP55 in breast cancer, publicly available Oncomine cancer microarray database (*www.oncomine.com*) was used to examine the expression of CEP55 in cancer tissue and determine the association of CEP55 with breast cancer outcomes. Richardson Breast 2 (*16*), Sorlie Breast 2 (*17*) and Ma Breast 4 (*18*) Ductal breast carcinoma were used to compare CEP55 expression levels between cancer and normal tissues. Ma Breast 4 (*18*), Karnoub Breast (*19*), Curtis Breast (*20*) and The Cancer Genome Atlas (TCGA, *http://tcga-data.nci.nih.gov/tcga/*) invasive breast carcinoma gene expression data were also used to compare CEP55 expression levels between cancer and normal tissues. The gene expression of CEP55 was compared between breast cancer tissues with normal breast tissues according to the standard procedures as previously described (*21*).

2.2. *Cell culture*

Human breast cancer cell lines ZR-75-30 and embryonic kidney cell line 293T (HEK293T) were purchased from the Cell Bank of Shanghai Institute of Cell Biology (Chinese Academy of Sciences, Shanghai, China). ZR-75-30 and HEK293T were cultured in RPMI-1640 (Hyclone SH30809.01B, Logan, UT, USA) and Dulbecco's modified Eagle's medium (DMEM, Hyclone SH30243.01B), respectively, supplemented with 10% fetal bovine serum (FBS, Biowest, Kansas City, MO, USA). The two lines were maintained in atmosphere of 5% CO_2 at 37°C.

2.3. *Construction of recombinant lentivirus and cell infection*

According the sequence of CEP55 (NM_001127182.1 and NM_018131.4) downloaded from the GEO database, two short hairpin RNAs (shRNA), including sequence 1 (5'-GCAGCGGGAAGTCTATGTAAAC TCGAGTTTACATAGACTTCCCGCTGCTTTTT-3') and sequence 2 (5'-CCCAAGTGCAATATACAGTAT CTCGAGATACTGTATATTGCACTTGGGTTTTT-3') were designed to knock down CEP55 expression. A scrambled shRNA (shCon, 5'- GCGGAGGGTTTGAA AGAATATCTCGAGATATTCTTTCAAACCCTCCGC TTTTTT-3') was used as control. Then these stem-loop-stem oligos were synthesized and inserted into pFH-L vector (Shanghai Hollybio, China) containing a green fluorescent protein (GFP) gene as a reporter gene. The recombined pFH-L vector was triple transfected into 80% confluent HEK293T cells with packing plasmids pVSVG-I and pCMVΔR8.92 (Shanghai Hollybio, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For lentivirus infection, ZR-72-30 cells were seeded into six-well plates and transfected with two CEP55 shRNAs and control shRNA (shCon), respectively and then correspondingly divided into four groups: CEP55 knockdown cells (KD-1 and KD-2), negative control cells (shCon) and blank control (Con). After 5 day infection, the infection efficiency was determined by counting the numbers of GFP-expressing cells under a fluorescence microscope (DMI4000B, Leica Microsystems, Germany).

2.4. *Knockdown efficiency determination*

To investigate the knockdown efficiency of CEP55, we determined the mRNA and protein levels of CEP55 in ZR-72-30 cells using quantitative real-time PCR (qRT-PCR) and Western blot analysis, respectively.

For qRT-PCR analysis, total RNA was extracted from each group cell with TRIZOL Reagent (Invitrogen, Grand island, NY, USA) according to the manufacturer's instructions. Reverse transcription was performed using SuperScript II RT 200 U/Ml (Invitrogen, Carlsbad, CA, USA). The β-actin was applied as an endogenous control. Primers were listed as follows: CEP55 (forward: 5'-AGCAGCAAGAAGAACAAACAAGGG-3' and reverse: 5'-AGTGACTAATGGCTCTGTGATGGC-3') and β-actin (forward: 5'-GTGGACATCCGCAAAGAC-3' and reverse: 5'-AAAGGGTGTAACGCAACTA-3'). The qRT-PCR reaction system contained 10 μL 2 × SYBR premix ex taq, 0.5 μL primers (2.5 μM), 5 μL cDNA and 4.5 μL ddH_2O. The qRT-PCR analysis was performed in triplicated on BioRad Connet Real-Time PCR platform (BioRad, Hercules, CA, USA) using the following reaction procedure: initial denaturation at 95°C for 1 min followed by 40 cycles (denaturation at 95°C for 5 s and annealing extension at 60°C for 20 s). The $2^{-\Delta\Delta Ct}$ method was used to normalize the expression of CEP55 mRNA to the expression of the β-actin.

For Western blot analysis, total protein of each group cell was extracted using ice-cold protein lysis buffer (10 mM EDTA, 100 mM Tris-Hcl (pH 6.8), 4% SDS and 10% Glycine). The protein concentration was determined by BCA protein assay. Total 30 μg proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Blots were blocked and then probed with rabbit anti-CEP55 (1:1,000 dilution, Proteintech Group, Inc,

no: 23891-1-AP) and rabbit anti-GAPDH (1:500,000 dilution, Proteintech Group, Inc, no: 10494-1-AP) overnight. After washing, the blots were incubated with HRP-conjugated goat anti-rabbit (1:5,000, Santa Cruz, SC-2054) as a second antibody for 20 min at room temperature. Blots were visualized by enhanced chemiluminescence (Millipore, Billerica, MA).

2.5. *Methylthiazol tetrazolium (MTT) assay*

After infection for 72 h, ZR-75-30 cells from different groups were seeded in a 96-well plate at a density of 3000 cells per well. MTT solution was added to the wells at different time points (1, 2, 3, 4 and 5 days) followed by incubation at 37°C for 4 h after lentivirus infection. Acidic isopropanol (10% SDS, 5% isopropanol and 0.01 mol/L HCl) was then added to stop the reaction. The optical density was measured with an enzyme-linked immunosorbent assay reader (Bio-Rad) at a wavelength of 595 nm.

2.6. *Colony formation assay*

After infection for 72 h, ZR-75-30 cells from different groups were plated into six-well plates and cultured for 6 days to form natural monolayer colony. Cells were fixed with methanol and then stained with 4% crystals purple according to previously description (*22*). Cell colonies (more than 50 cells per colony) were imaged and counted under the fluorescence microscopy.

2.7. *Flow cytometry assay*

After 6 day infection, ZR-75-30 cells from different groups were reseeded on 6-cm dishes and cultured for 6 days. For cell cycle analysis, cells were fixed in 70% ethanol and kept at 4°C for 30 minutes. The suspension was filtered and stained with propidium iodide at 4°C in the dark. The cells were then analyzed by FAC Scan (FACS) flow cytometer (Becton Dickinson, San Jose, CA, USA) according to the manufacturer's instruction. For cell apoptosis analysis, cells were dual stained with an Annexin V-APC and 7-AAD Apoptosis Detection kit (Keygen, Nanjing, China) according to the manufacturer's instruction. Cell apoptosis profiles were determined using FlowJo software (TreeStar, San Carlos, CA, USA).

2.8. *Statistical analysis*

All statistical analyses were performed using SPSS 19.0 software and GraphPad Prism 5.0. Data were expressed as mean ± standard deviation (SD) of three independent experiments. The difference between CEP55 knockdown group and non-knockdown group was evaluated using the Student's *t*-test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. *CEP55 mRNA expression was up-regulated in breast cancer*

We analyzed six independent microarray datasets from Oncomine database for CEP55 expression. As shown in Figure 1, the comparison showed the expression level of CEP55 was significantly up-regulated in various breast cancer types, compared to normal tissues ($p < 0.05$).

3.2. *Lentivirus-mediated RNA interference markedly suppressed CEP55 expression*

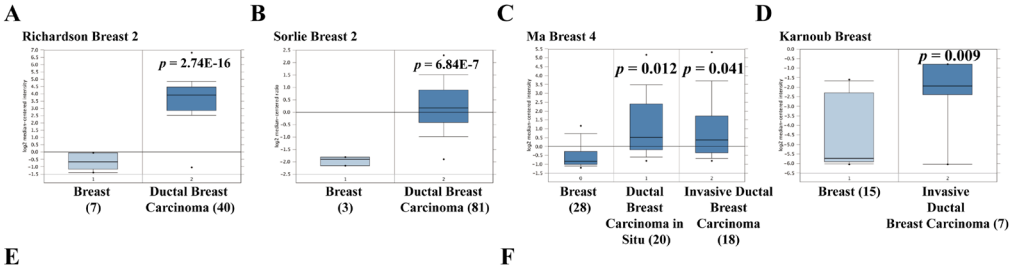
To investigate the role of CEP55 in breast cancer cells, two specific CEP55-targeting shRNA were used to infect ZR-75-30 cells. As shown in Figure 2A, efficiency of lentivirus infection was over 80%, as evidenced by GFP expression in ZR-75-30 cells from different groups. Furthermore, the efficiency of CEP55 knockdown was determined by qRT-PCR and Western blot analysis. As depicted in Figure 2B, endogenous CEP55 mRNA of ZR-75-30 cells was significantly reduced in KD-1 and KD-2 group ($p < 0.001$) compared with that in shCon and Con groups. Consistent with the results of qRT-PCR, the protein expression of CEP55 of ZR-75-30 cells in KD-1 and KD-2 group was noticeably depleted (Figure 2C). Collectively, the lentivirus system constructed could efficiently suppress CEP55 expression in breast cancer cells.

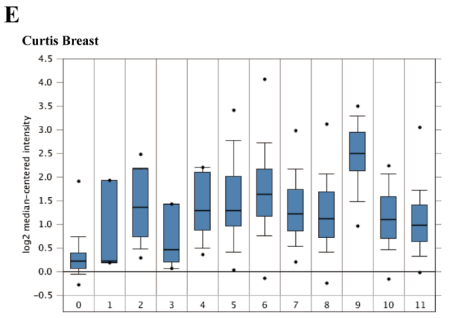
3.3. *Depletion of CEP55 obviously inhibited the proliferation of breast cancer cells*

To elucidate whether the knockdown of CEP55 related with the malignant phenotype of ZR-75-30 cells, MTT assay was performed to examine the cell proliferation in ZR-75-30 cells from different groups. As shown in Figure 3A, the proliferation rate of KD-1 and KD-2 cells was significantly decreased compared with the shCon and Con groups ($p < 0.001$), while there was no significant difference between shCon and Con groups. In addition, we tested the effect of CEP55 knockdown on the growth of ZR-75-30 cells by colony formation assay (Figure 3B). According to the result of monolayer culture, the number of surviving colonies of KD-1 was obviously reduced and the size of single colony was apparently fewer than that in shCon and Con groups (Figure 3C, $p < 0.001$). Taken together, CEP55 might be indispensable for ZR-75-30 cell proliferation.

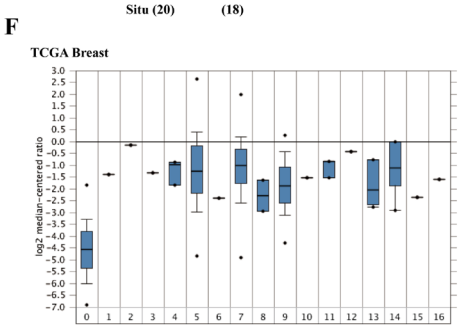
3.4. *Knockdown of CEP55 induced cell cycle arrest and apoptosis*

To further investigate the mechanism underlying CEP55 knockdown suppressed cell growth, the cell cycle distribution and apoptosis were detected in ZR-





No.	Subsets of breast tissues	Number of tissues	*P* value
0	Breast	144	-
1	Benign Breast Neoplasm	3	-
2	Breast Carcinoma	14	2.36E-5
3	Breast Phyllodes Tumor	5	-
4	Ductal Breast Carcinoma in Situ	10	-
5	Invasive Breast Carcinoma	21	3.90E-6
6	Invasive Ductal Breast Carcinoma	1556	2.30E-120
7	Invasive Ductal and Invasive Lobular Breast Carcinoma	90	5.78E-27
8	Invasive Lobular Breast Carcinoma	148	-
9	Medullary Breast Carcinoma	32	7.42E-19
10	Mucinous Breast Carcinoma	46	-
11	Tubular Breast Carcinoma	67	-



No.	Subsets of breast tissues	Number of tissues	*P* value
0	Breast	61	-
1	Apocrine Breast Carcinoma	1	-
2	Breast Large Cell Neuroendocrine Carcinoma	1	-
3	Ductal Breast Carcinoma	1	-
4	Intraductal Cribriform Breast Adenocarcinoma	3	-
5	Invasive Breast Carcinoma	76	9.18E-34
6	Invasive Cribriform Breast Carcinoma	1	-
7	Invasive Ductal Breast Carcinoma	392	9.88E-40
8	Invasive Ductal and Lobular Carcinoma	3	-
9	Invasive Lobular Breast Carcinoma	36	1.54E-19
10	Invasive Papillary Breast Carcinoma	1	-
11	Male Breast Carcinoma	3	1.05E-5
12	Metaplastic Breast Carcinoma	1	-
13	Mixed Lobular and Ductal Breast Carcinoma	7	2.17E-6
14	Mucinous Breast Carcinoma	4	-
15	Papillary Breast Carcinoma	1	-
16	Pleomorphic Breast Carcinoma	1	-

Figure 1. Microarray data extracted from Oncomine database. The expression of CEP55 gene obtained from **(A)** Richardson Breast 2 dataset, **(B)** Sorlie Breast 2 dataset, **(C)** Ma Breast 4 dataset, **(D)** Karnoub Breast dataset, **(E)** Curitis Breast dataset and **(F)** TCGA Breast dataset, respectively is shown as histograms.

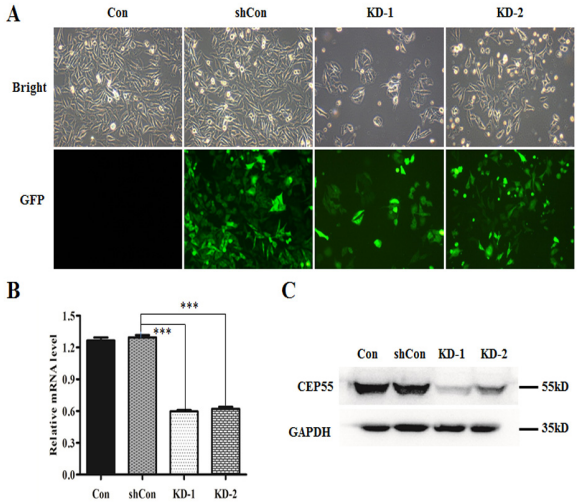


Figure 2. Knockdown of CEP55 expression by lentivirus-mediated shRNA in breast cancer cells, ZR-75-30. (A) Images recorded cells under bright and fluorescence microscopes after lentivirus infection at a magnification of ×100. Knockdown efficiency of CEP55 was determined by **(B)** qRT-PCR and **(C)** Western blot analysis. ***$p < 0.001$, compared to shCon and Con.

75-30 cells after lentivirus infection. As shown in Figure 4A, cells in three groups (Con, shCon and KD-1) were stained by PI and analyzed by FACS. Statistical analysis indicated that the percentage of cells in G0/G1 was significantly increased in the KD-1 group (81.02 ± 0.24%), compared with shCon (68.38 ± 0.26%) and Con (67.98 ± 0.37%) groups, accompanied by a decrease in the S phase (Figure 4B, $p < 0.001$). Additionally, more cells were obviously accumulated in the sub-G1 phase representing early apoptotic cells (Figure 4C, $p < 0.001$). As depicted in Figure 5A, Annexin V-APC vs 7-AAD plots from the gated cells showed the populations corresponding to viable (Annexin V-/7-AAD-), necrotic (Annexin V-/7-AAD+), early apoptotic (Annexin V+/7-AAD-) and late apoptotic (Annexin V+/7-AAD+) cells. Statistical analysis indicated both early apoptotic cells and late apoptotic cells were significantly increased in the KD-1 group, in contrast to shCon and Con groups (Figure 5B, $p < 0.001$). These results suggested that knockdown of CEP55 induced cell cycle arrest in G0/G1 phase and cell apoptosis.

4. Discussion

Human breast cancer is one of the most leading causes of diseases worldwide (*23*). What's more, it is the most common malignancy in female, leading to approximately

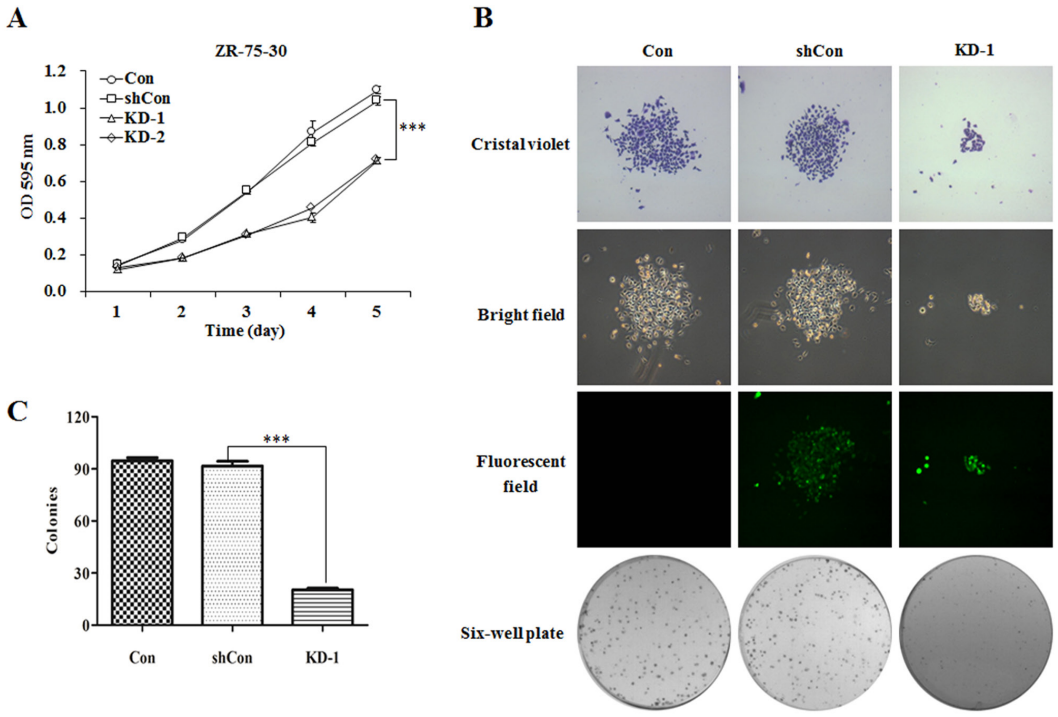


Figure 3. Knockdown of CEP55 suppressed the proliferation and colony formation capacity of ZR-75-30 cells. (A) Growth curves of ZR-75-30 cells with four different treatments (Con, shCon, KD-1 and KD-2) determined by MTT analysis. **(B)** Representative microscopic images of colonies stained by crystal violet and full vision of six-well plate under a microscope. **(C)** Statistical analysis of the colonies numbers in all three groups (Con, shCon, KD-1). ***$p < 0.001$, compared to shCon and Con.

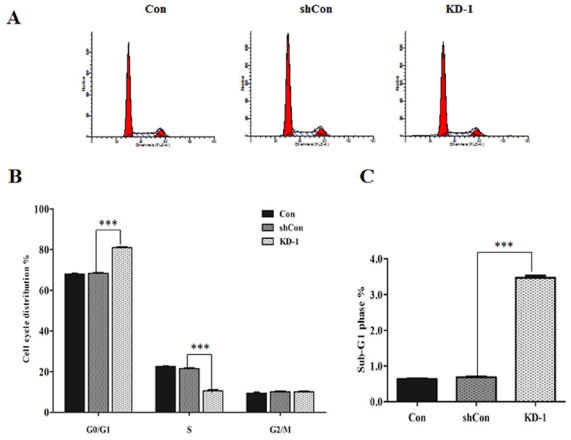


Figure 4. Effect of CEP55 silencing on cell cycle distribution of ZR-75-30 cells. (A) Cell cycle distribution of ZR-75-30 cells with three different treatments (Con, shCon, KD-1) was analyzed by flow cytometry using PI staining. **(B)** Statistical analysis of the percentages of ZR-75-30 cells in G0/G1, S and G2/M phases. **(C)** Statistical analysis of the percentage of ZR-75-30 cells in the sub-G1 phase. ***$p < 0.001$, compared to shCon and Con.

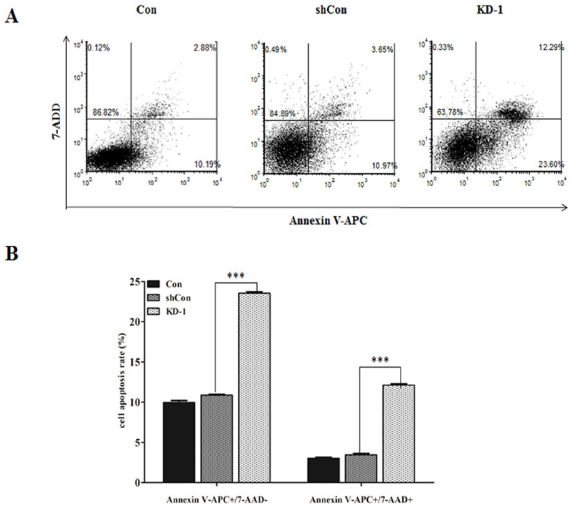


Figure 5. Effect of CEP55 silencing on apoptosis of ZR-75-30 cells. (A) Apoptosis of cells infected with lentivirus after Annexin-V/7-AAD staining. **(B)** Apoptotic cells including early-stage (Annexin V+/7-AAD-) and late-stage (Annexin V+/7-AAD+) apoptosis. ***$p < 0.001$, compared to shCon and Con.

40,000 deaths per year (*24*). More recently, CEP55 was found to be overexpressed in various kinds of tumor progression, including colon carcinoma (*25*) and gastric specimens (*15*). In this study, the data extracted from Oncomine database suggested CEP55 gene expression was significantly increased in breast cancer tissues compared to that in normal tissues. To further explore the role of CEP55 in breast cancer. Two different CEP55 shRNAs were designed to specifically block the endogenous expression of CEP55 in human breast cancer cell ZR-75-30. Further analysis found decreased CEP55 expression significantly inhibited cell growth and proliferation ability and induced cell cycle arrest and apoptosis.

Centrosome acts an essential component of cell cycle progression (*26*). It has been reported that the centrosome-associated proteins correlate with many kinds of diseases, including carcinoma (*27*). CEP55, as

member of the centrosome-associated protein family, is located in the centrosome during the interphase, dissociates from the centrosome in the M phase and condenses to the midbody during cytokinesis (*5*), which suggest that CEP55 plays a crucial role in cell cycle progression. In addition, previous studies have indicated that CEP55 plays an essential role in G2/M phase in hepatocellular carcinoma (*7*) and lung cancer cells (*15*). These studies are different from our results, which might be ascribed to the different cancers and cell lines. Flow cytometry also showed a noticeable accumulation of cells in the sub-G1 phase after CEP55 knockdown. Furthermore, Annexin V-APC/7-AAD double staining demonstrated that knockdown of CEP55 increased the apoptotic cells. Related reporter has indicated that CEP55 peptides might be useful as part of a therapeutic strategy for therapy-resistant breast cancer patients (*13*). Therefore, we could infer that the growth inhibition by CEP55 silencing in breast cancer was probably due to the induction of centrosome-related apoptosis.

In summary, this study demonstrated the crucial role of CEP55 in promoting breast cancer cell proliferation *in vitro*. However, it is necessary to investigate the specific mechanism through which CEP55 regulates the growth of breast cancer and further confirm whether or not CEP55 could act as a therapeutic target for breast cancer.

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