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Overexpressed ubiquitin ligase Cullin7 in breast cancer promotes cell proliferation and invasion via down-regulating p53

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ABSTRACT

Ubiquitin ligase Cullin7 has been identified as an oncogene in some malignant diseases such as choriocarcinoma and neuroblastoma. However, the role of Cullin7 in breast cancer carcinogenesis remains unclear. In this study, we compared Cullin7 protein levels in breast cancer tissues with normal breast tissues and identified significantly higher expression of Cullin7 protein in breast cancer specimens. By overexpressing Cullin7 in breast cancer cells HCC1937, we found that Cullin7 could promote cell growth and invasion in vitro. In contrast, the cell growth and invasion was inhibited by silencing Cullin7 in breast cancer cell BT474. Moreover, we demonstrated that Cullin7 promoted breast cancer cell proliferation and invasion via down-regulating p53 expression. Thus, our study provided evidence that Cullin7 functions as a novel oncogene in breast cancer and may be a potential therapeutic target for breast cancer management.

1. Introduction

Breast cancer is the most common cancer among women in the United States, other than skin cancer. It is the second leading cause of cancer death in women, after lung cancer [1,2]. Breast cancer is a heterogeneous disease, and breast cancer metastasis is a multistep process that results from genetic alterations, including the activation of oncogenes and the loss of function of tumor suppressors [2]. Though many targeted therapies have improved the survival of breast cancer patients, a proportion of patients will eventually die as a result of metastasis [1]. Therefore, there is an urgent need to identify novel genes and reveal the detailed mechanisms underlying breast cancer metastasis.

Cullin7 assembles a SCF-like E3 complex containing Skp1, the Fbw8 F-box protein, and ROC1 [3–5]. Cullin7 selectively interacts with the Skp1·Fbw8 heterodimer [3,6,7]. Recently, Cormier-Daire and colleagues have linked the 3-M syndrome, which is characterized by pre- and post-natal growth retardation, to Cullin7 germline mutations, a majority of which have been implicated for loss of the functional cullin domain [8]. In addition to the genetic evidence in mice, Cullin7 mutations have also been identified in 3-M syndrome and the Yakuts short stature syndrome, both of which are characterized by pre- and post-natal growth retardation but with relatively normal mental and endocrine functions [8,9]. Cullin7 may have additional functions that include transformation mediated by simian virus-40 (SV40) large T antigen, apoptosis, p53 regulation, and the degradation of cyclin D1 [3,10–12]. Taken together, the emerging genetic evidence has strongly suggested a pivotal role for the Cullin7 E3 ligase in growth control. However, whether or not Cullin7 functions in breast cancer development and metastasis remains unknown.

This study showed that Cullin7 expression was increased in breast cancer tissues and cell lines. In human breast cancer cells, proliferation, migration and invasion were significantly enhanced when Cullin7 was overexpressed. By contrast, these properties were inhibited when Cullin7 was knocked down. Moreover, we demonstrated that Cullin7 promoted breast cancer cell proliferation and invasion via down-regulating p53 expression. Thus, our study provided evidence that Cullin7 functions as a novel oncogene in breast cancer and may be a potential therapeutic target for breast cancer management.

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2. Materials and methods

2.1. Samples, cells, and antibodies

All the normal breast tissue samples and breast cancer tissue samples along with matched normal tissues used in this study were provided by the Department of General surgery of the Affiliated Hospital of Guangdong Medical College between 2011 and 2013. All experiments were approved by the ethics committee of the Affiliated Hospital of Guangdong Medical College and informed consent was obtained from all patients prior to specimen collection. The human breast cancer cell lines HCC1937, MDA-MB-468, MCF7, BT474, BT549, HCC70, normal human breast epithelial cell line MCF10A were obtained from American Type Culture Collection (Manassas, VA, USA). Cell culture was according to the manufacturer's protocol. Mouse monoclonal Cullin7, p53, p27, and p21 antibodies were purchased from Abcam. Mouse monoclonal β-actin antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA).

2.2. Plasmid construct and generation of stable cell lines

Human cDNA of Cullin7 was cloned as previously reported [12]. Full-length cDNA of Cullin7 was cloned into a HA tagged CMV10 vector, which was subsequently cloned into a pBabe.puro retroviral vector. Short hair-pin RNA (shRNA) targeting Cullin7 (5′-TGAGATCCTAGCTGAACTG-3′) was initially inserted into the Sal I and Xba I sites of pSuper plasmid, forming the pSuper shCullin7 plasmids.

Full-length cDNA of p53 was cloned into a 3XFLAG tagged CMV10 vector, which was subsequently cloned into a pBabe.puro retroviral vector. HCC1937 and BT474 cells were transfected with the pBabe or pBabe-Cullin7 plasmid and pSuper or pSuper-shCullin7 plasmid, respectively, using the Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Stable transfectants were obtained after selection with puromycin (Invitrogen; 10 μg/mL) for 2 weeks.

2.3. p53-specific siRNA inhibition

To knock down p53 expression in BT474-shCullin7 cells, the siRNA for p53 was from Santa Cruz Biotechnology (sc-29435). Cells were grown in dishes until they reached 75% confluence and transfected for 24 h with siRNA specific to p53 using the Lipofectamine 2000 transfection reagent, according to the manufacturer's instructions. P53 expression was then confirmed by Western blot analyses.

2.4. qRT-PCR

Total RNA was extracted from glioma tissues and cell lines using the TRIZOL reagent (Invitrogen). Reverse transcription was performed using the Thermoscript RT System (Invitrogen). qRT-PCR involved use of an ABI7000 sequence detector (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems). Levels of Cullin7 and p53 mRNA were expressed as a ratio to that of human GAPDH and measured by the CT method. This study used the following primers: Cullin7 (sense 5′- CCATCTCAGAGTCCCAACAC-3′ and antisense 5′-TTCAGCACCACGGCATAG-3′); p53 (sense 5′-GGCACTTTTGAAGATCATTTTCTC-3′ and antisense 5′- CTGTGTTGAGGGCAATGAG-3′), and GAPDH (sense 5′-TGCCTCCTGCACCACCAACT-3′ and antisense 5′-CCCGTTCAGCTCAGGGATGA-3′).

2.5. Western blot

Samples and cells were solubilized in radioimmunoprecipitation assay lysis buffer [50 mmol/LTris–HCl (pH7.4), 1%NP40, 0.25%Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonylfluoride, 1 mg/mL each of aprotinin, leupeptin, and pepstatin, 1 mmol/L Na_3VO_4, 1 mmol/L NaF]. The supernatants, which contained the whole-cell protein extracts, were obtained after centrifugation of the cell lysates at 10,000×*g* for 10 min at 4 °C. A total of 20 μg of protein samples were loaded on a sodium dodecyl sulfate-PAGE gel (5% stacking gel and 12% separating gel). These proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were initially probed with a primary antibody and then with a secondary antibody. The bound antibody was detected by enhanced chemiluminescence detection reagents (Amersham Bioscience, Piscataway, NJ, USA) according to the manufacturer's instructions. The band intensity was quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

2.6. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

MTT assay was conducted to determine the overexpression or knockdown effect of FOXC2 on glioma cell proliferation. A total of 5000 cells were seeded in 96-well plates. These cells were cultured in 100 μL of growth medium. At various time points, 20 μL of sterile MTT dye (5 mg/mL) was added and incubated at 37 °C for 4 h. The MTT solution was then replaced with DMSO (200 μL) and thoroughly mixed for 30 min. Spectrometric absorbance at 490 nm was measured using a microplate reader with a background subtraction at 660 nm.

2.7. Colony formation assay

Cells were seeded in triplicate at 500 cells/6 cm dish in complete medium. After 3 weeks of growth, these cells were fixed and stained with crystal violet stain, and visible colonies were counted according to cell numbers in each colony. All experiments were repeated at least three times. Plating efficiency was determined as the number of colonies formed divided by the total number of cells plated.

2.8. Wound healing assay

Equal numbers of different cells were seeded in six-well tissue culture plates. When 90% confluence was reached, a single wound was created by gently removing the attached cells using a sterile plastic pipette tip. Cell migration of the cells into the wounded area was observed and noted at different time points. Various migrations and extended protrusion of cells from the border of the wound were visualized and photo-documented using an inverted microscope.

2.9. Cell invasion and motility assay

Cell invasion was measured in Matrigel-coated Transwell inserts containing polycarbonate filters with 8 μm pores. The inserts were coated with 50 μl of 1 mg/mL Matrigel matrix according to the manufacturer's recommendations. Cells (2×10^5) in 200 μL of serum-free medium were plated in the upper chamber, whereas 600 μL of medium with 10% fatal bovine serum were added to the lower well. After 24 h incubation, the top cells were removed and the bottom cells were counted. The cells that migrated to the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet.

Motility assays were performed using Transwell membrane inserts containing polycarbonate filters with 8 μm pores. Methods used in cell migration assay were similar to those in the Matrigel invasion assay, except that the Transwell insert was not coated with Matrigel.

2.10. Immunohistochemistry

Surgically excised specimens were fixed with 10% neutral formalin, embedded in paraffin, and 4 μm-thick specimen sections were prepared. Immunostaining was performed using the avidin–biotin–peroxidase complex method. The sections were deparaffinized in xylene, rehydrated with graded alcohol, and boiled in 0.01 M citrate buffer for 2 min with an autoclave. Hydrogen peroxide was applied to block endogenous peroxide activity, and the sections were incubated with normal goat serum to reduce nonspecific binding. Tissue sections were incubated with Cullin7 rabbit polyclonal antibody (1:250 dilution). Mouse immunoglobulin was used as a negative control. Staining for both antibodies was performed at room temperature for 2 h. Biotinylated goat anti-mouse serum IgG was used as a secondary antibody. The sections were washed and incubated with streptavidin–biotin conjugated with horseradish peroxidase. The peroxidase reaction was developed with 3, 30-diaminobenzidine tetrahydrochloride. Two independent blinded investigators randomly examined all tumor slides. Five views were examined per slide, and 100 cells were observed per view at 400× magnification.

2.11. Statistical analysis

Experimental data are shown as mean ± S.D. A two-tailed Student's *t*-test was used to compare the results from the different treatment groups. Differences with $P < 0.05$ were considered statistically significant. SPSS/Win11.0 software (SPSS, Inc., Chicago, Illinois, USA) was used to analyze the data.

3. Results

3.1. Overexpressed of Cullin7 in human breast cancer tissues and breast cancer cell lines

We first compared the expression levels of Cullin7 in 16 breast cancer tissue samples to those in the adjacent normal tissues using western blot. Cullin7 protein levels were found to be increased in the tumor lesions compared with the matched normal tissue lesions in all of the samples (Fig. 1A). Cullin7 expression was also analyzed in more samples by immunohistochemistry (normal breast tissues = 26; breast cancer tissues = 39). Human normal breast tissues did not show immunostaining (Fig. 1B). Cullin7 protein, which was localized in the nuclei of tumor cells, was expressed in all human breast cancer samples. These results indicating significant overexpression of Cullin7 in the nuclei of breast cancer cells (Fig. 1C). qRT-PCR and Western blot also measured Cullin7 mRNA and protein expression levels in the normal breast epithelial cell line (MCF10A) and breast cancer cell lines (HCC1937, MDA-MB-468, MCF7, BT474, BT549, and HCC70), respectively (sFig. 1A and B). The results showed that MCA10A cells expressed low level of Cullin7, whereas breast cancer cell lines had the higher level of Cullin7 both in mRNA and protein levels.

3.2. Up-regulating Cullin7 promotes proliferation, migration, and invasion capacities of breast cancer cell in vitro

We used HCC1937 cell line, which is the lowest Cullin7 expression cell line in the 5 breast cancer cells (sFig. 1A and B),

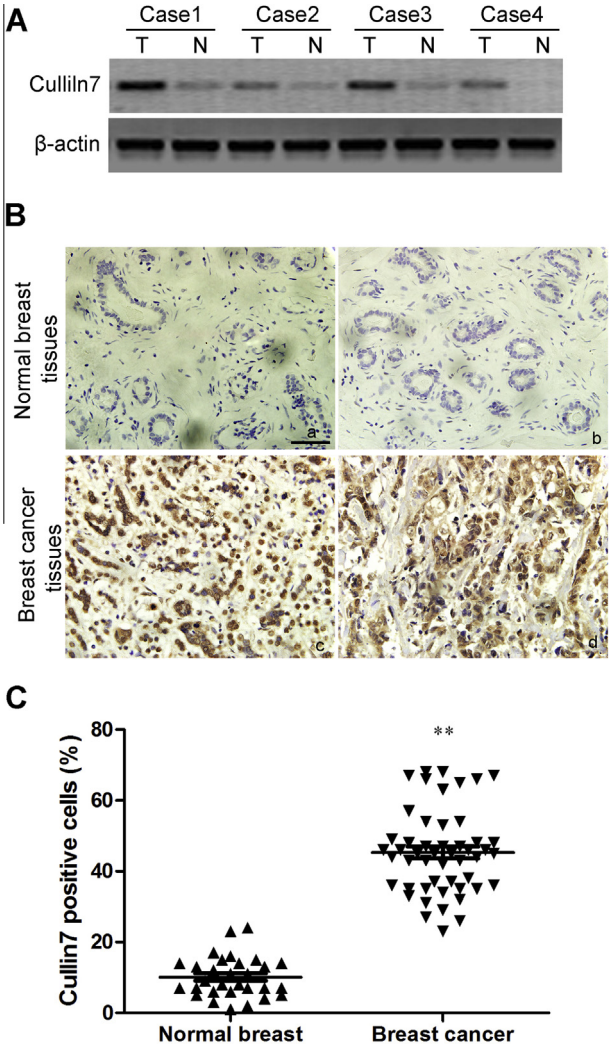

Fig. 1. Expression of Cullin7 in breast cancer tissues. (A) Cullin7 protein levels in tumor tissues and matched normal tissue lesions, as assessed using western blot analyses. (B) Immunohistochemistry analysis showed Cullin7 expression in paraffin-embedded sections form normal breast tissues (B: a, b) and breast cancer tissues (B: c, d). (C) Ratio of Cullin7 positive cells in normal breast and breast cancer tissues. Scale bars indicate 500 μm (B). $^{**}P < 0.01$ is based on Student's *t*-test. All results are from three or four independent experiments. Error bars indicate standard deviation.

to establish a stable cell line that constitutively overexpressed the Cullin7 protein with the aim of revealing the role that Cullin7 expression has in the development or progression of breast cancer. The transfection efficiency was confirmed using western blot and qRT-PCR analyses. As shown in Fig. 2A and B, the HCC1937 cells that had been transfected with the Cullin7 expression plasmid displayed significantly increased Cullin7 expression at both the mRNA and protein levels compared with the vector cell lines.

Fig. 2C and D shows that HCC1937 Cullin7 cells had significant increases in cell proliferation compared with vector-only controls. Cullin7-overexpressing cells generated more numbers of colonies and formed significantly larger colonies than vector-only controls.

The effect of Cullin7 on cell migration was first assessed by a wound healing assay. HCC1937 Cullin7 cells had significantly faster closure of the wound area compared with their respective control cells (Fig. 2E). This result was confirmed using Boyden's chamber assay; HCC1937 Cullin7 cells showed more than twofold cell migration through Transwell membranes than their control cells after 24 h of incubation (Fig. 2F). HCC1937 Cullin7 cells also exhibited a greater degree of invasion through Matrigel (Fig. 2F).

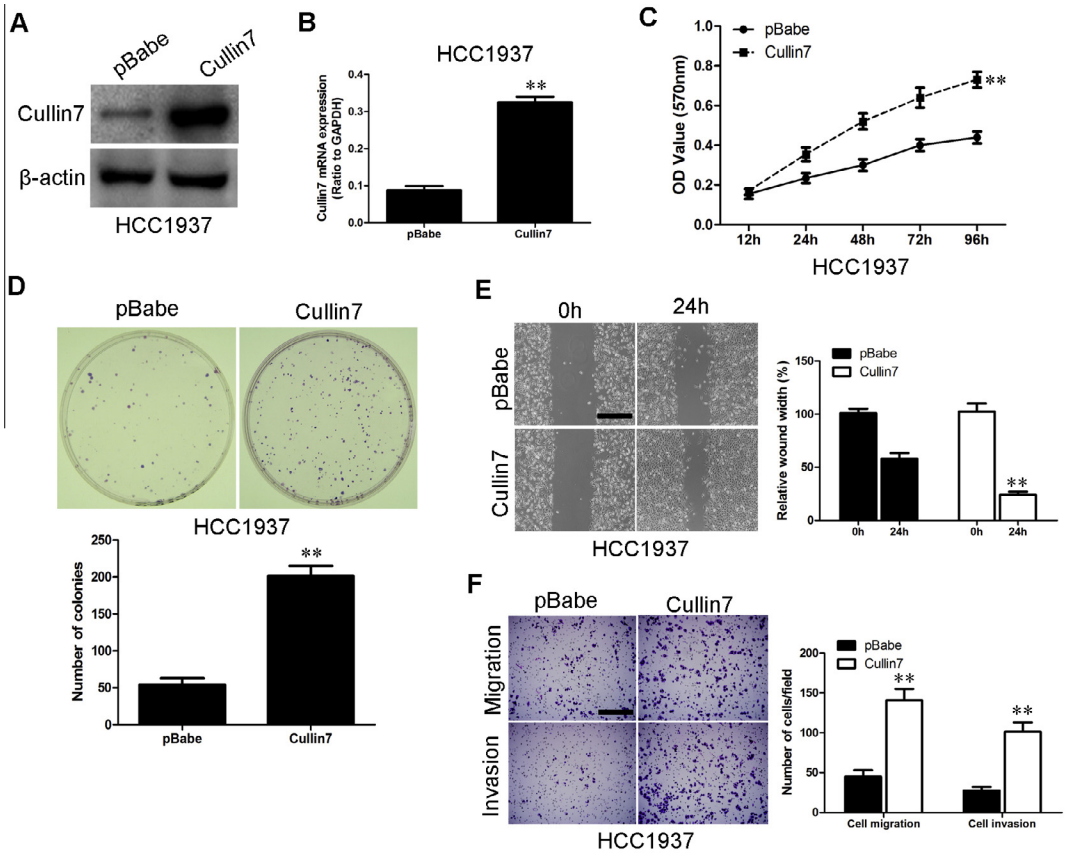

Fig. 2. Up-regulating Cullin7 promotes proliferation, migration, and invasion capacities of breast cancer cell in vitro. The transfection efficiency of Cullin7 overexpression was analyzed by measuring protein levels by western blot in HCC1937 cells (A). (B) The transfection efficiency of Cullin7 was analyzed by measuring transcript levels using qRT-PCR analyses in HCC1937 cells. (C) Cell proliferation after Cullin7 overexpression in HCC1937 cells was measured using MTT assays. (D) The results of colony formation assays that were conducted in Cullin7 overexpression in HCC1937 cells (upper panels), and the summary graphs are presented for the colony formation assay that was outlined (lower panels). (E) HCC1937 Cullin7 and its control vector cells were subjected to wound healing assay (left panels), the uncovered areas in the wound healing assays were quantified as a percentage of the original wound area (right panels). (F) HCC1937 Cullin7 and its control vector cells were subjected to Transwell migration (upper panels), and Matrigel invasion assays (lower panels). Quantifications of migrated cells through the membrane and invaded cells through Matrigel of each cell line are shown as proportions of their vector controls (right panels). Scale bars indicate 500 μm (E) and 200 μm (F). $^{**}P < 0.01$ is based on Student's *t*-test. All results are from three or four independent experiments. Error bars indicate standard deviation.

3.3. Down-regulating Cullin7 inhibits proliferation, migration, and invasion capacities of breast cancer cell in vitro

We used shRNA to generate a stable Cullin7 knockdown in the BT474 breast cancer cell line, which is the highest Cullin7 expression cell line in the 5 breast cancer cell lines (sFig. 1A and B). The transfection efficiency was confirmed using western blot and qRT-PCR analyses. As shown in Fig. 3A and B, the BT474 cells that had been transfected with the Cullin7 shRNA plasmid displayed significantly decreased Cullin7 expression at both the mRNA and protein levels compared with the control cells.

The effects of Cullin7 knockdown on cell growth was first assayed by MTT. As shown in Fig. 3C, Cullin7 knockdown significantly inhibited the growth of BT474 cells. Next, we performed a clonogenic assay to confirm the effects of Cullin7 on proliferation. We found that Cullin7 knockdown dramatically reduced the colony formation efficiency of BT547 cells. We next examined whether Cullin7 knockdown could inhibit the migratory and invasive capacities of breast cancer cells. The effect of Cullin7 knockdown on cell migration was first assessed by a wound healing assay. BT474 shCullin7 cells had significantly reduced closure of the wound area compared with their respective control cells (Fig. 3E). This result was confirmed using Boyden's chamber assay (Fig. 3F). BT474 shCullin7 cells also exhibited a lower degree of invasion through Matrigel (Fig. 3F).

3.4. p53 is a mediator for Cullin7-induced proliferation, migration, and invasion capacities of breast cancer cell

P53 inhibits tumorigenesis and metastasis in many carcinomas. To determine whether p53 was a downstream target of Cullin7 in breast cancer cells, p53 and its downstream molecule p21 and p27 expression in the cells with altered Cullin7 expression were evaluated by western blot. HCC1937 Cullin7cells exhibited greatly decreased p53 and its downstream molecule p21 and p27 expression (Fig. 4A), whereas the silencing of Cullin7 expression in BT474 cells dramatically increased p53 and its downstream molecule p21 and p27 expression at the protein levels (Fig. 4B). In addition, we also measured the expression of p53 mRNA in these cells by qRT-PCR, and we found that the mRNA level of p53 was not altered by Cullin7 up- or down- regulating (sFig. 2A and B).

To understand how Cullin7 exerts its effects on p53, 293T cells were co-transfected with FLAG-tagged p53 and HA-tagged Cullin7 constructs. Immunoprecipitation and subsequent immunoblot analyses revealed that HA-tagged Cullin7 co-immunoprecipitated with FLAG-tagged p53, confirming a physical interaction between the two proteins (Fig. 4C). The physical interaction between Cullin7 and p53 suggested that Cullin7 could target p53 for ubiquitination and subsequent degradation.

Indeed, overexpression of Cullin7 dramatically reduced endogenous p53 protein levels in a dose-dependent manner (Fig. 4D).

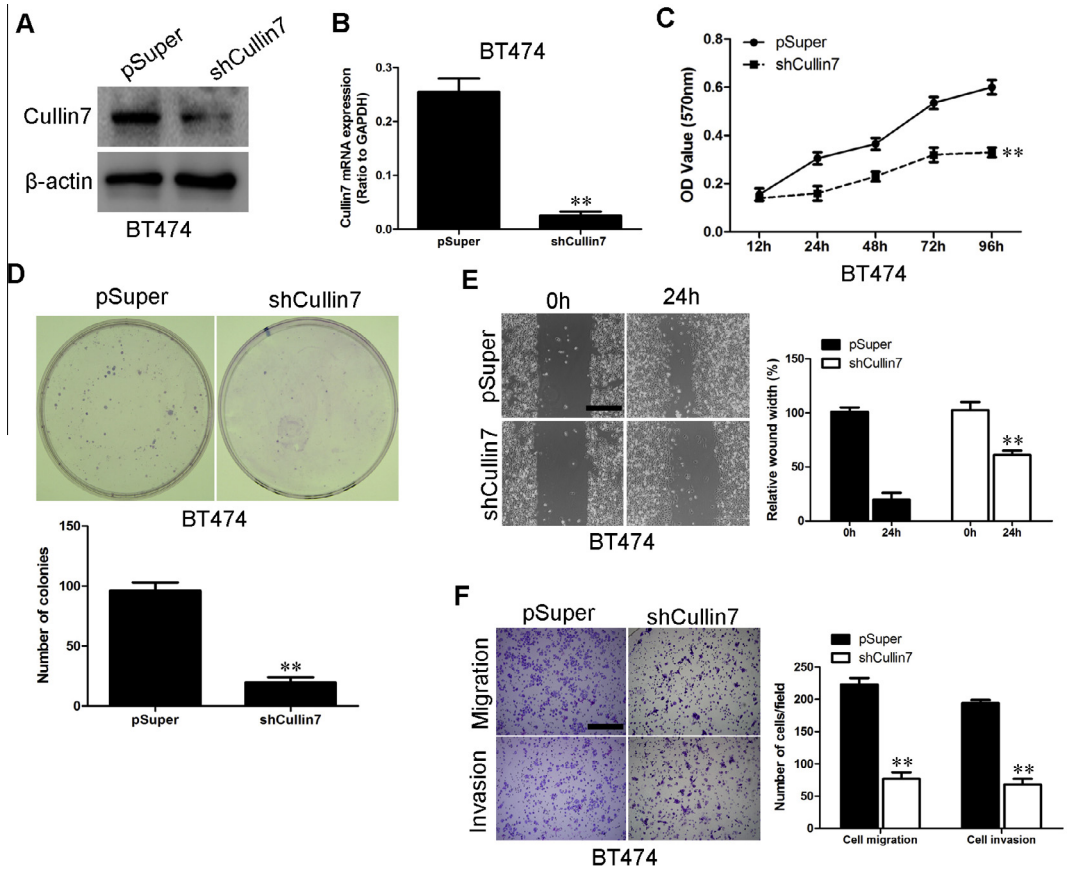

Fig. 3. Down-regulating Cullin7 inhibits proliferation, migration, and invasion capacities of breast cancer cell in vitro. (A) The transfection efficiency of Cullin7 knockdown was analyzed by measuring protein levels by western blot in BT474 cells. (B) The transfection efficiency of shCullin7 was analyzed by measuring transcript levels using qRT-PCR analyses in BT474 cells. (C) Cell proliferation after Cullin7 knockdown in BT474 cells was measured using MTT assays. (D) The results of colony formation assays that were conducted in Cullin7 knockdown in BT474 cells (upper panels), and the summary graphs are presented for the colony formation assay that was outlined (lower panels). (E) BT474 shCullin7 and its control vector cells were subjected to wound healing assay (left panels), the uncovered areas in the wound healing assays were quantified as a percentage of the original wound area (right panels). (F) BT474 shCullin7 and its control vector cells were subjected to Transwell migration (upper panels), and Matrigel invasion assays (lower panels). Quantifications of migrated cells through the membrane and invaded cells through Matrigel of each cell line are shown as proportions of their vector controls (right panels). Scale bars indicate 500 μm (E) and 200 μm (F). $^{**}P < 0.01$ is based on Student's *t*-test. All results are from three or four independent experiments. Error bars indicate standard deviation.

In addition, proteasome inhibition by addition of MG132 caused a significant increase in p53 levels (Fig. 4D), suggesting that Cullin7 promotes degradation of p53.

To test whether Cullin7-induced proliferation and metastatic capacity was mediated by down-regulating p53, siRNAs were used to silence *p53* gene expression by virally transfecting BT474-shCullin7 cells with *p53* siRNA (Fig. 4E). P53 knockdown in BT474 shCullin7 cells was accompanied with increased proliferation (Fig. 4F) and migratory and invasive capacities (Fig. 4G). These results show that p53 mediated Cullin7-induced migration, invasion, and proliferation capacities in breast cancer cells.

4. Discussion

Multiple molecular dysfunctions are associated with breast formation, growth, and metastasis, such as p53, p27, p21, VEGF, pRb, $p16^{INK4a}$, $p19^{ARF}$, and telomerase [13–17]. Despite the decline in breast cancer mortality, a number of breast cancer patients develop metastatic tumors even after surgical removal of the primary tumors [18]. Therefore, metastasis continues to be the main obstacle to the effective treatment of breast cancer, and there is an urgent need to identify novel molecular factors that lead to the invasiveness and metastasis of breast cancer.

Although a high incidence of Cullin7 expression in some cancers and a significant association with metastatic disease exist, limited information is available on its function in breast cancer. Previous studies showed that the Cullin7 was critical for vascular formation during development, and recent studies showed that Cullin7 was expressed in the endothelium of tumors in both humans and mice [3,6]. The present study is the first to define the functional roles of human Cullin7, specifically in breast cancer cells. Ectopic expression of Cullin7 in HCC1937 cells significant increase in cell proliferation, whereas knockdown of Cullin7 in BT474 cells resulted in a reduced growth rate. These results indicated that Cullin7 augmented breast cancer cell proliferation.

P53 has a major function in different types of cancer [19]. The majority of breast cancer lowers express p53 [20,21]. This study is the first to show that Cullin7 regulated p53 expression in protein and not in mRNA level in breast cancer cells, suggests that Cullin7 used an alternative mechanism to promote breast cancer cell aggressiveness. However, further research is needed to determine the effect of Cullin7 on the levels of other oncogene and anti-oncogene. Further evidence of the enhanced oncogenic potential of Cullin7 expressing cells was shown by their ability to undergo anchorage-dependent growth, a trait commonly used to determine the oncogenicity of cells in vitro. Cullin7-overexpressing cells generated more numbers of colonies and formed significantly larger colonies than the vector-only controls. By contrast, the silencing of Cullin7 expression in BT474 cells significantly reduced cell clonogenicity. P53 knockdown in BT474-shCullin7 cells was accompanied with increased of proliferation and clone formation. Colony

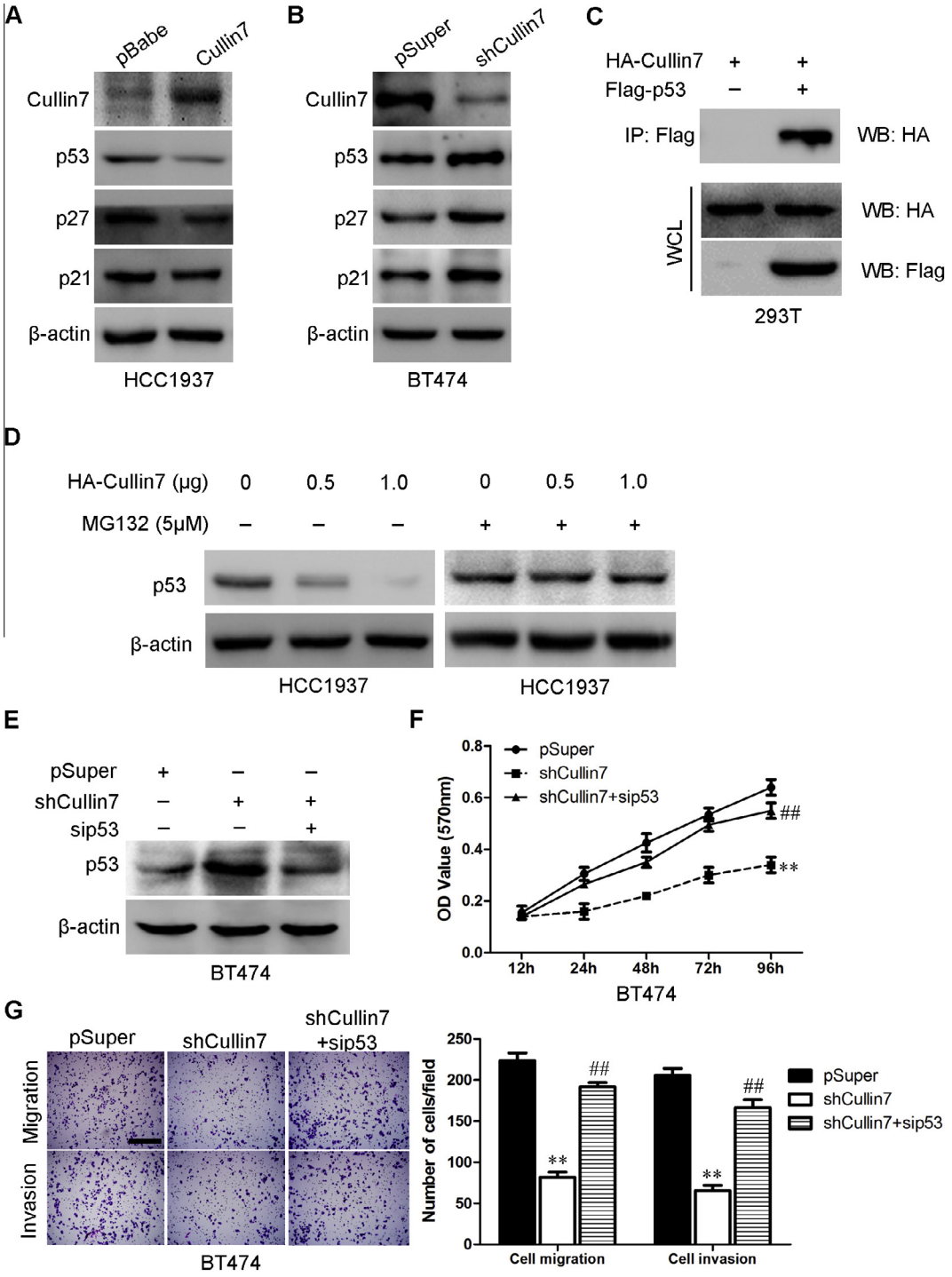

Fig. 4. p53 is a mediator for Cullin7-induced proliferation, migration, and invasion capacities of breast cancer cell. (A) Cullin7, p53, p21, and p27 expression in the HCC1937 cells with up-regulating Cullin7 expression was evaluated by western blot. (B) Cullin7, p53, p21, and p27 expression in the BT474 cells with down-regulating Cullin7 expression was evaluated by western blot. (C) HA-Cullin7 is specifically co-immunoprecipitated with Flag-p53 in 293T cell. In co-immunoprecipitated experiments, 5% of extracts used were shown as loading controls (WCL). (D) Proteasome inhibition by MG132 suppresses reduction of p53 by Cullin7 ectopic overexpression in HCC1937 cells. (E) The protein level of p53 in sip53 cells was verified by western blot. (F) BT474-pSuper, BT474-pSuper-shCullin7, and BT474-pSuper-shCullin7 + sip53 cells in vitro proliferation were examined by MTT. (G) BT474-pSuper, BT474-pSuper-shCullin7, and BT474-pSuper-shCullin7 + sip53 cells were subjected to Transwell migration (upper panels), and Matrigel invasion assays (lower panels). Quantifications of migrated cells through the membrane and invaded cells through Matrigel of each cell line are shown as proportions of their vector controls (right panels). Scale bars indicate 200 μm (G). Compared to pSuper group $^{**}P < 0.01$ and Compared to shcullin7 group $^{\#\#}P < 0.01$ are based on Student's *t*-test. All results are from three or four independent experiments. Error bars indicate standard deviation.

formation analyses and the functional indicators of oncogenesis showed that Cullin7 promoted oncogenic phenotypes of breast cancer cells by regulating p53 expression.

Motility and invasion are also major events in cancer metastasis and associated with poor prognosis in patients with cancer [22]. The metastasis process is a complex phenomenon regulated by

many components that facilitate the detachment of tumor cells from primary tumors to secondary sites. EMT promotes dissemination of a single carcinoma cell from the sites of primary tumors to distant organs and has a major function in breast cancer progression [5]. Previous studies showed that Cullin7 is a transcription factor and that it could induce EMT [12]. This study demonstrated that Cullin7 promoted the migratory and invasive potential of breast cancer cells. Ectopic expression of Cullin7 in breast cancer cells increased migratory and invasive behaviors in vitro. Decreased p53 expression by siRNAs reversed shCullin7-induced decreasing motility and invasion in BT474 shCullin7 cells. This observation is in agreement with recent findings that showing the involvement of Cullin7 in EMT in other cancer cells. Our results indicate that Cullin7 may potentiate the metastatic behavior of breast cancer cells by inhibiting p53 expression.

In conclusion, this study is the first to show that Cullin7 promotes breast cancer cell proliferation and invasion properties through down-regulating p53. The results of this study are useful in targeting novel proteins downstream of Cullin7 to disrupt signaling pathways involved in proliferation, motility, and invasion in breast cancer.

Conflict of interest

All authors certify that his manuscript has not been published in whole or in part nor is being considered for publication elsewhere. The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.134.

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