

RESEARCH COMMUNICATION

ER81-shRNA Inhibits Growth of Triple-negative Human Breast Cancer Cell Line MDA-MB-231 In Vivo and in Vitro

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Abstract

The lack of effective treatment targets for triple-negative breast cancers make them unfitted for endocrine or HER2 targeted therapy, and their prognosis is poor. Transcription factor ER81, a downstream gene of the HER2, is highly expressed in breast cancer lines, breast atypical hyperplasia and primary breast cancers including triple-negative examples. However, whether and how ER81 affects breast cancer carcinogenesis have remained elusive. We here assessed influence on a triple-negative cell line. ER81-shRNA was employed to silence ER81 expression in the MDA-MB-231 cell line, and MTT, colony-forming assays, and flow cytometry were used to detect cell proliferation, colony-forming capability, cell cycle distribution, and cell apoptosis in vitro. MDA-MB-231 cells stably transfected with ER81-shRNA were inoculated into nude mice, and growth inhibition of the cells was observed in vivo. We found that ER81 mRNA and protein expression in MDA-MB-231 cells was noticeably reduced by ER81-shRNA, and that cell proliferation and clonality were decreased significantly. ER81-shRNA further increased cell apoptosis and the residence time in G₀/G₁ phase, while delaying tumor-formation and growth rate in nude mice. It is concluded that ER81 may play an important role in the progression of breast cancer and may be a potentially valuable target for therapy, especially for triple negative breast cancer.

Keywords: ER81 - shRNA - breast cancer - triple-negative - gene therapy

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Introduction

Previous studies have shown that ETS-related protein 81 (ER81), a downstream gene of Human epidermal growth factor receptor 2 (HER2), may form a fusion protein with other proteins after chromosome translocation, leading to tumor formation, such as the fusion with the EWS gene and subsequent induction of Ewing's sarcoma (Jeon et al., 1995; Janknecht, 2005). Some research data from several groups have demonstrated that ER81 overexpressed in breast cancer cell lines, breast cancer, prostatic carcinoma (Monte et al., 1995; Cai et al., 2007) teratocarcinoma (Monte et al., 1995) megakaryocytic leukemia (Monte et al., 1995) melanoma (Monte et al., 1995; Jane-Valbuena et al., 2010) and gastrointestinal stromal tumor (Chi et al., 2010). Transduction of the ER81 gene can induce prostatic intraepithelial neoplasms and spermatocystic hyperplasia in transgenic mice, finally leading to prostatic carcinoma and adenocarcinoma of the seminal vesicles (Tomlins et al., 2007; Shin et al., 2009). Data showed ER81 expressed weakly in normal breast ductal epithelium and hyperplastic epithelium without atypia, but high expression were detected immunohistochemically in atypical hyperplasia of mammary glands, ductal carcinoma in situ, and infiltrating breast cancer tissues (Wang et al., 2011). Together with previous results, we assumed ER81 expression was related to the incidence and progression of

breast cancer. but the mechanism underlying its functions is still unclear. To further understand the functions of ER81 in the progression of breast cancer, we utilized ER81-shRNA to silence ER81 expression in the human breast cancer cell line MDA-MB-231 and observed the growth of MDA-MB-231 cells after ER81 knockout in vitro and the effects on in vivo tumor formation in nude mice. This study aimed to investigate the function of ER81 in the progression of breast cancer and to search for a new target for breast cancer treatment.

Materials and Methods

Cell line and cell culture

The MDA-MB-231 cell line (American Type Culture Collection number: HTB-26) was established from human metastatic mammary carcinoma cells showing high expression levels of ER81 (Baert et al., 1997) and no expression of HER2, ER and PR (Ha et al., 2009; Subik et al., 2010) and it can differentiate into poorly differentiated adenocarcinoma (grade III) in nude mice and antilymphocyte serum-treated BALB/c mice. The cell line was purchased from the cell line bank at Kunming Institute of Zoology, Chinese Academy of Sciences. The cells were cultured using the RPMI 1640 medium (Hyclone, SH30809.01B) containing 10% FCS fetal calf serum (Hyclone, SV30087.02) at 37 °C and 5% CO₂.

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Construction of ER81-shRNA eukaryotic expression vector (short-hairpin RNA (shRNA) plasmid constructs)

Three double-stranded shRNA fragments containing nine base “loop” regions, a terminator sequence, and restriction enzyme sites (HindIII and BamHI) specifically targeting ER81 mRNA (NCBI access number NM_004956.3) were designed and synthesized, and they were cloned into the HindIII and BamHI restriction enzyme site position in the eukaryotic vector (Wuhan GeneSil Biotechnology Co. Ltd, Wuhan, China) carrying green fluorescent protein and the neomycin resistance gene. The 65-68 nt oligonucleotide encoding ER81 specific shRNA, as follows: ER81-shRNA-1: 5'-GATCCGCTG GTCGAGGCATGGAATTCTCTATGGACAAATTCC ATGCCTCGACCAGTTTTTTGTCGACA-3', ER81-shRNA-2: 5'-GATCCACGTTGGGGCATTTCAGAAA TTCAAGACGTTTCTGAATGCCCAACGTTTTT TTGTCGACA-3', ER81-shRNA-3: 5'-GATCCGCCG TTCCTCCGCTATTATTCAAGACGTAATAGCGG AGTGAACGGCTTTTTTGTGTCGACA-3'. Non-targeting sequence was used for the control siRNA, As follow, Scrambled-shRNA: 5'-GATCCGACTTCATAAGGCG CATGCTTCAAGACGGCATGCGCCTTATGAAGT CTTTTTGTGTCGACA-3'. The shRNA fragment and vector pGenesh11 were digested with HindIII and BamHI and ligated. Subsequently, the following eukaryotic expression vectors carrying ER81-shRNA or Scrambled-shRNA were constructed: pGenesh11-ER81-shRNA-1, pGenesh11-ER81-shRNA-2, pGenesh11-ER81-shRNA-3, and pGenesh11-Scrambled-shRNA.

Establishment of MDA-MB-231 cell line stably transfected with ER81-shRNA

MDA-MB-231 cells were separately transfected with pGenesh11-ER81-shRNA-1, pGenesh11-ER81-shRNA-2, pGenesh11-ER81-shRNA-3, pGenesh11-Scrambled-shRNA, or empty plasmid (pGenesh11), and stably transfected cell lines were established. Plasmids were extracted using the PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen, K2100-04), and 3×10^4 cells/ml of MDA-MB-231 cells were plated in six-well plates. About 24 h later, after the fusion degree reached 90~95%, pGenesh11-ER81-shRNA-1, pGenesh11-ER81-shRNA-2, pGenesh11-ER81-shRNA-3, pGenesh11-Scrambled-shRNA, and empty vector (pGenesh11 as the empty vector control) were separately mixed with Lipofectamine™ 2000 (Invitrogen, 11668-019) at a ratio of 1:3 (plasmid : Lipofectamine). The mixture was incubated for 10 min at room temperature and added to the cells. At 6 h after transfection, the medium was replaced with complete culture medium. The cells were analyzed by fluorescence microscopy 24 h after transfection, to calculate the transfection efficiency. After 48 h, the medium was replaced with complete culture medium containing 400 µg/ml antibiotic G418 (Sigma-Aldrich, A1720), to screen positively transfected cells. The cells were dispersed and resuspended, and then individual colonies were selected and expanded. The complete culture solution containing 300 µg/ml G418 was changed for culture maintenance until cell lines stably transfected with each vector were obtained for further analyses.

RNA isolation and Q-PCR detection for ER81 mRNA expression

RNA was isolated from the transfected cell lines using TRIzol reagent (Invitrogen, 15596-026). One microgram of total RNA from each sample was used to synthesize first-strand cDNA using the RevertAid™ H Minus first strand cDNA synthesis kit (Fermentas, K1632). Expression of ER81 mRNA was detected by Q-PCR using the following primers: forward: 5'-GCAAGTGCCTTACATGGTCCAC-3', reverse: 5'-AGCCTGATAGTCTGGTACAAACT-3'. GAPDH was used as the internal reference; the primers were: forward: 5'-AGCCTGATAGTCTGGTACAAACT-3', reverse: 5'-ATGGGGAAGGTGAAGGTCG-3'. The Q-PCR reaction mixture contained 7.5 µl of 2x iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, 170-8882), 0.33 M sense primer, 0.33 M antisense primer, 1 µg of cDNA and water to a final volume of 15 µl. The PCR conditions were 94 °C for 1 min, followed by 40 cycles of 95 °C for 30 s, 60.0 °C for 30 s, and 72 °C for 30 s, with a final 10 min incubation at 72 °C. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot detection of ER81 protein expression

Transfected MDA-MB-231 cells were washed with cold phosphate-buffered saline (PBS). Then the cell protein was extracted using CytoBuster™ Protein Extraction Reagent (Novagen, 71009-4), according to manufacturer's instruction. The supernatant was collected, and the protein concentration was quantified using BCA protein assay kit (PIERCE, 23227). Samples (30 mg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride membrane. Next, the blots were stained with Ponceau S, blocked with 5% nonfat milk powder for 2 h after rinsing, and then probed with the appropriate dilution of ER81 polyclonal primary antibody (Abcam, ab81086) at 4 °C for 16-18 h. The membranes were rinsed, incubated with the appropriate horseradish peroxidase-conjugated goat anti-mouse (goat anti-mouse IgG-HRP) secondary antibody (Santa Cruz Biotechnology, sc2302) at room temperature for 1 h, and rinsed again (Yu et al., 2012). They were visualized by SuperSignal® West Pico Trial Kit (Pierce, 34079). The blots were exposed to film in the dark, and the film was then developed, fixed and examined.

MTT measures the cell viability

The cells in different transfection groups were collected, prepared as single-cell suspensions in complete culture medium, and inoculated into 96-well plates at a density of 2×10^3 cells per well. Six duplicates were set up for each phase in each group, and the cells were observed per 24h in 7 days. Empty vector control wells were set up simultaneously (the wells contained only the culture solution but no cells). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich, M5655) was added daily at the same phase to different groups, and the cells were incubated at 37 °C for 4-6 h. Dimethylsulfoxide (DMSO) (Sigma) was added to fully dissolve the crystal, and the absorbance (optical density)

was determined at the wavelength of 490 nm using a microplate reader (Chen et al., 2011).

Colony-forming assay

The cells in different transfection groups were collected at the exponential phase of growth and then digested and blown into single-cell suspensions. The suspensions were diluted and then inoculated into six-well plates at densities of 500 cells, with three duplicate wells set up for each cell density. The six-well plates were incubated at 37 °C in 5% CO₂ for 2 weeks (Cui et al., 2012). After macroscopically visible clones appeared, the culture was terminated; then, the cells were rinsed, fixed, and subjected to Giemsa stain (Sigma-Aldrich, G9641). The clones were counted, and the cloning efficiency was calculated according to the following formula: cloning efficiency (%) = number of clones/number of inoculations × 100%.

Flow cytometry detection for the effects of ER81-shRNA on the cell cycle

The cell cycle phase distribution was evaluated using flow cytometry according to the instructions of the CycleTest™ Plus DNA reagent kit (Becton Dickinson, 340242120). The sub-G₀/G₁, S, and G₂/M fractions of 3 × 10⁴ cells were determined by flow cytometry using FACS Vantage (Becton Dickinson). The results were analyzed using FlowJo software, where Proliferation Index = (S+G₂/M)/(G₀/G₁+S+G₂/M) × 100%.

Flow cytometry detection for effects of ER81-shRNA on cell apoptosis

To quantitate the level of apoptosis by flow cytometry, the Chromatin Condensation assay was performed using the Chromatin Condensation/Dead Cell Apoptosis Kit with Hoechst 33342 and PI (Invitrogen, V13244), which assay for apoptosis based upon fluorescence detection of the compacted state of the chromatin in apoptotic cells. blue-fluorescent Hoechst 33342 dye which stains the condensed chromatin of apoptotic cells more brightly than the chromatin of normal cells, and the red-fluorescent propidium iodide dye which is permeant only to dead cells. The cells in different transfection groups were collected at the exponential phase of growth, wash in cold PBS and adjust the cell density to 1 × 10⁶ cells/mL in PBS. For each assay, Add 1 μL of the Hoechst 33342 stock solution and 1 μL of the PI stock solution to each 1 mL of cell suspension, incubate the cells on ice for 20–30 minutes and then analyze the stained cells by flow cytometry. The population should separate into three groups: live cells will show only a low level of fluorescence, apoptotic cells will show a higher level of blue fluorescence, and dead cells will show both blue and red fluorescence. The rate of apoptosis was calculated using the average value of three experiments, apoptotic rate (%) = apoptotic cells/total cells × 100%. Confirm the flow cytometry results by viewing the cells under a fluorescence microscope.

Effects of ER81-shRNA on transplantation tumors in nude mice

Pathogen-free female 4-week-old BALB/c-nu/nu (nude) mice (Beijing Weitong Lihua Experimental Animal

Technology Co., Ltd., Beijing, China) were weighed, coded and divided randomly into experimental groups of three. Cells stably transfected with either a shRNA construct or empty plasmid were collected at the exponential growth phase and dispersed as single cells in PBS. A total of 10⁶ living cells in 0.2 ml of PBS were injected at each position in nude mice (Clay et al., 1999). Cells stably transfected with ER81-shRNA-1, ER81-shRNA-2 and ER81-shRNA-3 were inoculated into the subcutaneous fat pad inside the right anterior limb, and cells stably transfected with empty plasmid were inoculated into the subcutaneous fat pad inside the left anterior limb as a control. Tumor growth was observed every day, and the maximum and minimum widths of the tumors were measured daily after the tumors were visible, using vernier calipers. Tumor volume was calculated as: tumor volume V (mm³) = 0.5 × maximum width × minimum width², and the growth curves of the tumors were plotted. All nude mice were killed in week 11, and the transplantation tumor specimens were fixed in 4% neutral formalin, embedded in paraffin, and sectioned. Mouse anti-human ER81 polyclonal antibody was used as the primary antibody (Abcam, ab81086) for immunohistochemical staining to detect expression of ER81 protein, and the in situ TUNEL method was used to detect cell apoptosis in the transplantation tumor tissues.

Statistical analysis

All the data were analyzed using SPSS 11.5 software. Measurement data are presented as means ± standard deviation. The t-test was used to compare mean values between two groups, and single-factor analysis of variance (Student-Newman-Keuls) was used to compare mean values among multiple groups. The criterion for statistical significance was α = 0.05. Graphical procedures were performed using Microsoft Excel 2003.

Results

Effects of ER81-shRNA on the expression level of ER81 in cultured MDA-MB-231 cells

The MDA-MB-231 cell lines stably transfected with ER81-shRNA-1, ER81-shRNA-2, ER81-shRNA-3, Scrambled-shRNA, or empty plasmid were established by liposomal transfection, G418 screening, and monocloning of cells (Figure 1A). Q-PCR and RT-PCR analysis revealed that ER81-shRNA effectively inhibited the expression level of ER81 mRNA in the MDA-MB-231 cells (Figure 1B and Figure 1D). The expression level of ER81 mRNA in the Scrambled-shRNA group did not significantly differ from that in the empty vector group. The knockdown efficiencies in the ER81-shRNA-1 and ER81-shRNA-3 groups were 74.8% and 68.6%, respectively. The knockdown efficiency of ER81 mRNA in the ER81-shRNA-2 group was 39.3%. Western blot analysis showed decreased expression of ER81 protein in all three ER81-shRNA transfection groups (Figure 1C).

Effects of ER81-shRNA on growth, proliferation, cell cycle distribution, and cell apoptosis in cultured MDA-MB-231 cells

Cell proliferation was measured over 7 days, using

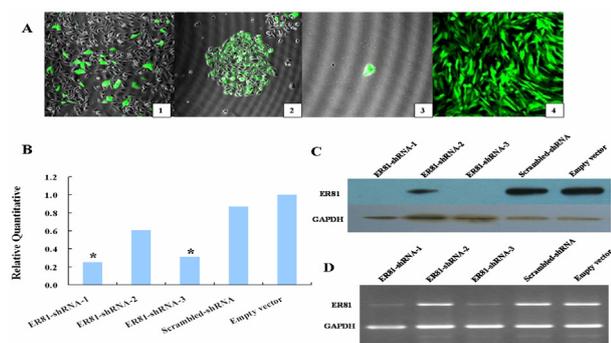


Figure 1. Establishment of MDA-MB-231 Cell Lines Stably Transfected with ER81-shRNA and Efficiency of ER81 Knockdown. A. Confocal microscopic observations of shRNA-transfected cells: 1. Green fluorescence was observed in MDA-MB-231 cells 24 h after transfection with ER81-shRNA. 2. Colonies of transfected cells formed after 22 days in G418 culture solution. 3. Single colonies were selected to establish a stably transfected cell line. 4. The MDA-MB-231 cell line stably transfected with ER81-shRNA was maintained in medium containing G418. B. Q-PCR of ER81 mRNA shows that the ER81 mRNA expression level was significantly lower in the three ER81-shRNA-transfected cell lines than in cells transfected with Scrambled-shRNA or empty vector (* P<0.05). C. Western blots revealed that the ER81 protein level was significantly lower in cells transfected with ER81-shRNA, particularly ER81-shRNA-1 and ER81-shRNA-3, than in the cells transfected with Scrambled-shRNA or empty vector. The expression level of GAPDH did not change. D. RT-PCR of ER81 mRNA shows that the ER81 mRNA expression level was significantly lower in the three ER81-shRNA-transfected cell lines than in cells transfected with Scrambled-shRNA or empty vector

the MTT assay. Cell proliferation index was significantly decreased in the ER81-shRNA-1 and ER81-shRNA-3 groups in 3 days after inoculation, compared with proliferation in the empty vector group (P < 0.01). Cell proliferation was significantly decreased in the ER81-shRNA-2 group compared with the empty vector group (P < 0.05) at 5 days after inoculation. Cell proliferation did not differ significantly between the Scrambled-shRNA and empty vector control groups (P > 0.05), indicating that ER81-shRNA-1, ER81-shRNA-2, and ER81-shRNA-3 all inhibited the growth of MDA-MB-231 cells. The inhibitory effects of ER81-shRNA-1 and ER81-shRNA-3 were clearly noticeable, whereas no inhibition was detected in the Scrambled-shRNA and empty vector groups (Figure 2A).

For the colony-forming assay, cells from each transfection group were inoculated in six-well plates and cultured for 2 weeks at a density of 500 cells/well. The numbers of colonies formed in the ER81-shRNA-1, ER81-shRNA-2 and ER81-shRNA-3 groups were 32.0 ± 6.25, 83.0 ± 8.19 and 24.33 ± 2.08, respectively, which are all significantly lower than the number in the empty vector group (140.00 ± 3.00) and Scrambled-shRNA (134.30 ± 7.77) (P < 0.01). The colony-forming rate did not differ significantly between the Scrambled-shRNA and empty vector groups (P > 0.05) (Figure 2B).

Cell apoptosis was measured assay using the TUNEL method and flow cytometry. The apoptotic rates in the ER81-shRNA-1, ER81-shRNA-2, ER81-shRNA-3, Scrambled-shRNA and empty vector groups were 20.67

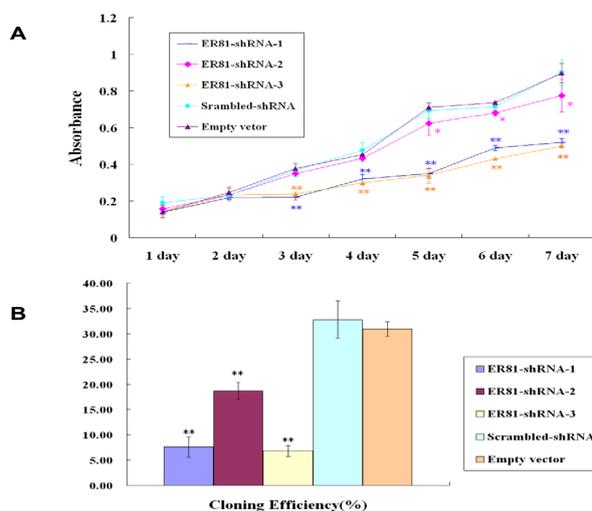


Figure 2. A. MTT assay of cell proliferation. The absorbance was significantly lower in the ER81-shRNA-1 and ER81-shRNA-3 groups, indicating decreased proliferation, compared with the Scrambled-shRNA and empty vector control groups (**P<0.01); the absorbance in the ER81-shRNA-2 group also showed a decrease (* P<0.05). The absorbance did not differ significantly between the Scrambled-shRNA and empty vector control groups. B. The colony-forming capability was significantly lower in the ER81-shRNA-1, ER81-shRNA-2 and ER81-shRNA-3 groups than in the Scrambled-shRNA and empty vector control groups (** P<0.01). The colony forming rate did not differ significantly between the Scrambled-shRNA and empty vector control groups

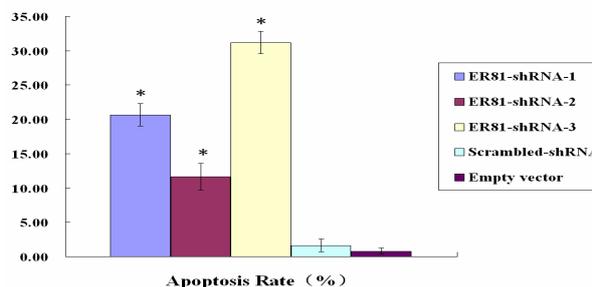


Figure 3. Flow Cytometric Analysis of Cell Apoptosis. The cell apoptotic rates in the ER81-shRNA interference groups were significantly higher than that in the control group. The rate of apoptosis was even higher in the ER81-shRNA-1, ER81-shRNA-2 and ER81-shRNA-3 groups (*P<0.05, in comparison with control). No significant difference in the apoptotic rate was detected between the Scrambled-shRNA and empty vector control groups

± 1.642%, 11.65 ± 1.734%, 31.18 ± 1.643%, 1.62 ± 0.949% and 0.79 ± 0.456%, respectively. The apoptotic rate was significantly higher in the ER81-shRNA-1, ER81-shRNA-2 and ER81-shRNA-3 groups than in the empty vector group (P < 0.05), but no significant difference between the Scrambled-shRNA and empty vector groups (P > 0.05) (Figure 3).

Cell cycle distributions in the transfection groups were determined by flow cytometry (Figure 4A). The percentage of cells in G₀/G₁ was higher and the percentages of cells in the S and G₂/M phases were lower in the MDA-MB-231 cell lines stably transfected with pGenesh11-ER81-shRNA-1, pGenesh11-ER81-shRNA-2 and pGenesh11-ER81-shRNA-3 compared with the cell line transfected with empty vector (Figure 4B). The

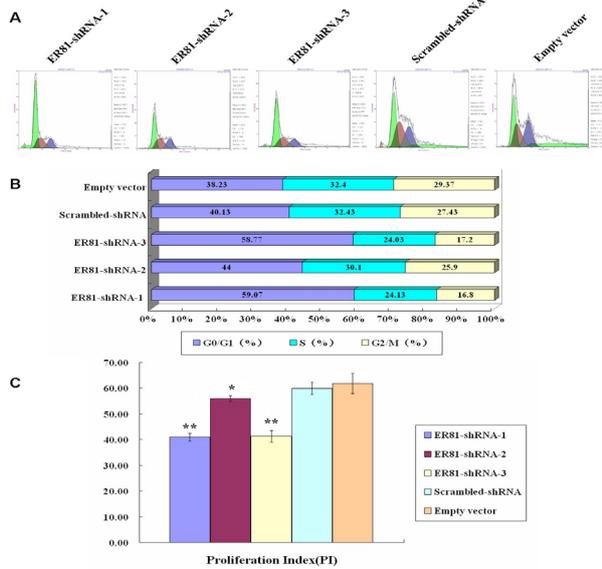


Figure 4. A. Flow cytometric analysis of the cell cycle distribution of the stably transfected cells. B. In the ER81-shRNA-1 and the ER81-shRNA-3 groups, 58.77% and 59.07% of the cells, respectively, were in G₀/G₁ phase, and this was significantly different from the distribution in the Scrambled-shRNA and empty vector groups. The ER81-shRNA-2 group also tended to show a prolonged G₀/G₁ phase. C. The proliferation index was significantly lower in the ER81-shRNA-1 and ER81-shRNA-3 groups compared with the Scrambled-shRNA and empty vector groups (**P<0.01); and was also decreased in the ER81-shRNA-2 group (*P<0.05)

proliferation index of the recombinantly transformed cells was significantly lower than that of the control cells (Figure 4C). The cell cycle distribution and proliferation index in the Scrambled-shRNA group showed no obvious changes (P > 0.05).

Effects of ER81-shRNA on the growth of transplantation tumors in MDA-MB-231 nude mice

After the nude mice were inoculated with the stably transfected MDA-MB-231 cells, tumors began to form in week 5 in the empty vector group and at week 10 in the ER81-shRNA groups. The tumor-formation capability in the ER81-shRNA-3 group was significantly lower than that in the other interference groups and the control group (Figure 5A), and the time of tumor formation in the ER81-shRNA-1, ER81-shRNA-2 and ER81-shRNA-3 groups was delayed compared with that in the empty vector group (Figure 5B). Additionally, the velocity of tumor formation decreased (P < 0.01), and the tumor size was significantly smaller, compared with the empty vector group (P < 0.01). Immunohistochemistry revealed significantly lower ER81 expression in tumors from ER81-shRNA-transfected cells than in tumors from empty vector-transfected cells (P < 0.01) (Figure 5C). In situ TUNEL detection demonstrated that the apoptotic rate was significantly higher in tumors from ER81-shRNA-transfected cells than in tumors from empty vector-transfected cells, which was consistent with the results of the in vitro experiments.

Discussion

Breast cancer accounting for 23% of all cancer cases

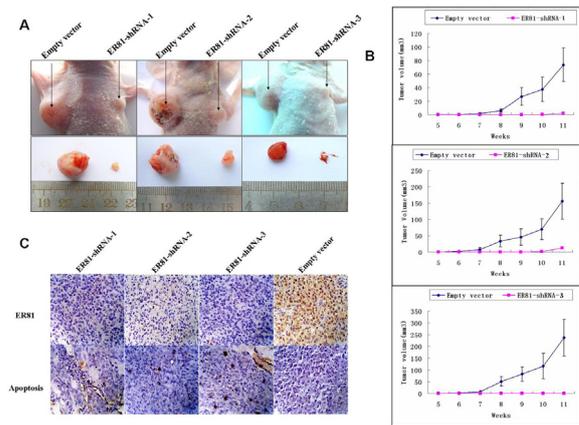


Figure 5. A. Nude mice were injected under the left forelimb with empty vector-transfected MDA-MB-231 cells and the under the right forelimb with MDA-MB-231 cells stably transfected with ER81-shRNA-1, ER81-shRNA-2 or ER81-shRNA-3. Tumor size was significantly larger in the empty vector group than in the ER81-shRNA groups. B. Tumor formation and growth were observed daily, and a tumor growth curve was plotted. Tumors began to form at week 5 in the empty vector-injected side and significantly later, at week 10, in the ER81-shRNA-injected side. Despite individual variation, tumor size on average was significantly smaller in the ER81-shRNA groups than in the empty vector group. Each bar represents the means±SD (n = 3). C. Immunohistochemical staining revealed significantly decreased ER81 protein expression levels in the ER81-shRNA-1, ER81-shRNA-2 and ER81-shRNA-3 groups. TUNEL assay detected cell apoptosis in the ER81-shRNA group, but not in the empty vector group

and 14% of all cancer deaths has become the major disease leading to the death of women in developing countries, and has the highest diagnostic frequency and mortality of any cancer in women, and its morbidity and mortality in both developing and developed countries rank first among malignant tumors in women (Jemal et al., 2011). So, studies regarding effective ways for preventing and treating breast cancer should focused on target therapy based on the molecular mechanism of the pathogenesis of breast cancers.

Transcription factors are a group of protein molecules that can bind to specific regions in the promoters of downstream genes, thus affecting growth, differentiation, and apoptosis of cells. The ER81 (ETS-related protein 81) (Brown and McKnight, 1992; Janknecht et al., 1993; Jeon, Davis, Braun et al., 1995; Monte et al., 1995) gene, also called ETV1 (ETS Variant 1), is a member of the PEA3 (Monte et al., 1995) subfamily of the Ets transcription factor family. ER81 executes its functions from upstream signals as a transcription factor, and it is the final executor of the upstream signaling pathway. Thus, ER81 may play an important role as a signaling molecule in the normal growth of cells and in individual development.

The human ER81/ETV1 gene locating on chromosome 7 is a downstream gene of HER2. The activation of ER81 depends on a great extent on both phosphorylation (Papoutsopoulou and Janknecht, 2000; Bosc et al., 2001) and acetylation (Goel and Janknecht, 2003), and activated ER81 can regulate the transcription of downstream genes by binding to their promoter regions via the DNA binding domain, a common region of the ETS family.

ER81 is expressed in neural tubes, mammary gland, myogenous cells, and cartilage-producing cells, as well as in mesoblasts and epiblasts of the heart, kidney, lung, and other organs during the embryonic stage, suggesting that ER81 is important in the growth and development of humans.

As a transcription factor, ER81 can regulate several genes associated with the incidence and progression of tumors, including the cell cycle-related genes *smad7* (Dowdy et al., 2003) and human telomerase reverse transcriptase (hTERT) (Fuchs et al., 2004; Goueli and Janknecht, 2004; Janknecht, 2004; Vageli et al., 2009). High expression levels of *Smad7* can cause avoidance of anti-growth signals in cells and subsequent apoptosis, which is related to short-term recurrence of tumors after treatment. Heparanase (Lu et al., 2003), which is related to vascularization and cell metastasis, is regulated by ER81. Heparanase, a unique enzyme that degrades heparan sulfate proteoglycan, can damage the extracellular matrix and basal membrane, participates in tumor vascularization, and is closely related to the invasion and metastasis of tumors. ER81 also regulates transcription of the matrix metalloproteinases MMP-1 (Bosc et al., 2001; Fuchs et al., 2003), MMP-2, and MMP-7 (Crawford et al., 2001), which degrade major basal membrane proteins such as type IV collagen and are closely associated to the infiltration and metastasis of tumors, VEGF (Fuchs et al., 2004), collagenase type I and collagenase type IV (Higashino et al., 1993).

More importantly, ER81 is regulated by the HER2/Neu-Ras-Raf-MAPK-ER81 signaling pathway (Janknecht, 1996; Bosc et al., 2001). As is well known, mutated or overexpressed Ras, Raf, and HER2/Neu are among the most prominent human oncoproteins. ER81 transcriptional activity is dramatically enhanced upon Her2/Neu overexpression (Bosc et al., 2001; Goel and Janknecht, 2003). ER81 can upregulate HER2 expression in breast cancer, indicating that HER2/Neu-activated ER81 may be part of a positive feedback loop that stimulates the expression of HER2/Neu (Bosc and Janknecht, 2002). Taken together, these findings suggest that ER81 may be an ideal downstream activator of the oncogenic potential of HER2/Neu in breast tumors. Clearly, ER81 may also be involved in tumor formation elicited by oncogenic Ras and Raf.

ER81 mRNA is increased in murine cell lines and tumors that overexpress HER2/Neu (Shepherd et al., 2001; Galang et al., 2004) and is expressed in human breast tumor specimens, including some human breast tumor cell lines such as MDA-MB-231, MDA-MB-436, MDA-MB-330, BT-549, and BT-20 (Baert et al., 1997; Bosc et al., 2001; Chung et al., 2002). In our preliminary investigations, a high expression level of ER81 was detected in 41.2% (7/17) of atypical hyperplastic tissues from mammary glands, 54.5% (12/22) of mammary gland ductal carcinoma in situ specimens, and 63.0% (51/81) of infiltrating breast cancer specimens, which was significantly different from the expression in normal mammary gland tissues (0%, 0/62) and usual hyperplasia (25.7%, 9/35) (Wang et al., 2011). Thus, ER81 may

contribute to breast tumorigenesis. ER81 overexpression may prime breast cells to become malignant upon overexpression of HER2/Neu. However, transgenic mice overexpressing ER81 in the breast do not develop mammary tumors (Netzer et al., 2002) suggesting that ER81 requires stimulation to become transcriptionally competent (Shin et al., 2008).

To further investigate the function of ER81 in the progression of breast cancer, we used shRNA to inhibit ER81 expression in human breast cancer MDA-MB-231 cells. ER81 mRNA and protein expression levels were significantly inhibited in MDA-MB-231 cells stably transfected with ER81-shRNA. Knockdown of ER81 significantly decreased the cell growth rate, proliferation index, and colony-forming capability, increased the percentage of cells in G₀/G₁ phase and the cell apoptosis rate in vitro. The tumor-forming ability of MDA-MB-231 cells in nude was decreased markedly, with a 90% of tumor inhibition rate. Our results further indicate that ER81 plays an important role in the progression of breast cancer and may be a potential new and valuable target gene for breast cancer gene therapy.

Breast cancer is increasingly regarded as a heterogeneous disease which can be classified into distinct molecular subtypes with prognostic significance. Triple-negative breast cancer is larger percentage than other three subtypes (Lv et al., 2011). In recent years, some anti-tumor drugs aimed at molecular targets in breast cancer, for example, an anti-estrogen drug for breast cancer patients with high expression levels of estrogen and progesterone receptors or the anti-HER2 monoclonal antibody Herceptin for HER2-amplified breast cancer, (Holbro et al., 2003) have shown relatively satisfactory efficacy in clinical trials. Nevertheless, proper target therapeutic methods are still unavailable for triple-negative breast cancer that is negative expression of HER2, estrogen receptor (ER) and progesterone (PR). It is interesting that MDA-MB-231, a triple-negative breast cancer line, showed high sensitivity for the treatment of ER81 knockdown. The mechanism is still not clear, may be there is another gene regulated ER81 expression on the upstream besides HER2. As we know, this is the first description about ER81 target therapy for triple negative breast cancer which represents a new way of target therapy for this special type of breast cancers.

ER81-shRNA significantly inhibited the expression of ER81 in the human breast cancer MDA-MB-231 cell line and inhibited the growth of MDA-MB-231 cells in vivo and in vitro, indicating that ER81 plays an important role in the progression of breast cancer and may be a valuable new target for breast cancer therapy, especially for triple negative breast cancer therapy.

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