

LHX6 inhibits breast cancer cell proliferation and invasion via repression of the Wnt/ β -catenin signaling pathway

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Abstract. LIM homeobox domain 6 (LHX6), a member of the LHX family of proteins, has been implicated in cancer development. However, the involvement of LHX6 in the development of breast cancer remains unclear. In the present study, the epigenetic regulation, biological function and associated molecular mechanisms of LHX6 in breast cancer were analyzed. The expression levels of LHX6 were demonstrated to be markedly decreased in breast cancer tissues and cell lines. In addition, it was found that increased LHX6 expression in breast cancer cell lines inhibited cell proliferation and invasion. Furthermore, increased LHX6 expression significantly decreased the expression of β -catenin in MDA-MB-231 breast cancer cells, and small interfering RNA- β -catenin enhanced LHX6-induced inhibition of cell proliferation and invasion in MDA-MB-231 breast cancer cells. These results indicate that LHX6 inhibits breast cancer cell growth and invasion through suppression of the Wnt/ β -catenin signaling pathway. Thus, the present study provides a novel insight into the underlying mechanism of tumorigenesis in breast cancer, indicating the therapeutic potential of LHX6 in the treatment of breast cancer.

Introduction

Breast cancer is a common breast malignancy and a major cause of cancer-associated mortality in females worldwide (1). The percentage of female breast cancer incidences, out of all female cancers, is 27% in the developed countries (2) and the most prevalent type of malignant tumor in females, in China, is breast cancer (3). Despite earlier detection and development of novel treatment protocols, a number of patients continue to

have a poor prognosis, exhibit early metastasis and are likely to succumb to the disease within five years of diagnosis (4). To date, the mechanisms of breast cancer oncogenesis remain unclear. The identification of novel genes that are functionally involved in breast cancer development and progression may facilitate with the elucidation of potential diagnostic and therapeutic targets.

The human LIM homeobox domain (LHX) gene regulates developmental processes at multiple levels, including tissue patterning, cell fate specification and growth (5). Recently, increasing evidence has revealed that various LHX genes have been implicated in tumorigenesis. LHX4 may act as a tumor suppressor by downregulation of α -fetoprotein expression in hepatocarcinogenesis (6). The transgenic expression of LHX2 promotes vessel maturation, primary tumor growth, tumor cell intravasation and metastasis during breast carcinogenesis (7). Epigenetic alteration of the LHX9 gene contributes to glioma cell invasiveness and migration (8).

LHX6 is a member of the LHX family of proteins, and encodes an LIM homeodomain transcription factor involved in embryogenesis (9). LHX6 is important in the development of the central nervous system, including the specification and migration of interneuron subtypes, such as parvalbumin- and somatostatin-positive interneurons (10,11). Furthermore, LHX6 hypermethylation has been shown to act as a sensitive methylation marker in head and neck carcinomas (12). In a previous study, LHX6 suppressed lung cell viability, colony formation and migration, induced apoptosis and G₁/S arrest, and inhibited their tumorigenicity in nude mice (13). However, the role of LHX6 in breast cancer remains unclear. In the current study, the epigenetic regulation, biological function and molecular mechanism of LHX6 in breast cancer was investigated.

Materials and methods

Breast cancer tissue samples. All cases examined were of breast cancer patients who underwent surgical resection at the Department of Breast and Thyroid Surgery, Huaihe Hospital of Henan University (Kaifeng, China) during the period from 2008 to 2013. The patients with breast cancer included 23 females, aged 43-58 years, with a median age of 51 years. Informed consent for research use was obtained from all of

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the subjects providing samples. The tumors were classified and graded according to the criteria of the World Health Organization (14). Following surgical resection, samples were collected and maintained in ice-cold Tris-buffered saline [10 mM Tris (pH 7.2) and 0.9% NaCl] and immediately processed for future analysis. Healthy tissue adjacent to the tumor was obtained from the same breast and served as a control.

Breast cancer cell culture. The breast cancer cell lines, MCF7 and MDA-MB-231 were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Sigma-Aldrich, St. Louis, MO, USA).

Construction of the LHX6 expression vector and cell transfection. cDNA corresponding to the full-length human LHX6 gene was verified by sequencing and subcloned into the mammalian expression vector, pIRES2-EGFP (Invitrogen Life Technologies, Carlsbad, CA, USA). The MCF7 and MDA-MB-231 cells were transfected using X-tremeGene HP DNA transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Clones with stable transfection of LHX6 or an empty vector were established with G418 (Invitrogen Life Technologies) selection for 2-3 weeks. Gene and protein expression of LHX6 was evaluated using reverse-transcription quantitative polymerase chain reaction and western blot analysis.

Small interfering (si)RNA transfection. siRNA- β -catenin and siRNA-mock were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). For transfection, 3×10^4 cells were seeded in each cell of 24-well microplates, grown for 24 h to reach 30-50% confluence, and subsequently incubated with a mixture of siRNA and Lipofectamine 2000 reagent (Invitrogen Life Technologies) in 100 μ l serum-free Opti-MEM (Sigma-Aldrich), according to the manufacturer's instructions. The transfection efficiency was examined by RT-qPCR and western blotting.

RT-qPCR. Total RNA, from breast cancer tissues and cells, was prepared with TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions. Total RNA (500 ng) was used for cDNA synthesis with Transcriptase Reverse Transcriptase (Invitrogen Life Technologies), and 1 μ l cDNA was used per PCR reaction. The PCR was performed using the Bio-Rad iQ5 quantitative PCR system (Takara Biotechnology Co., Ltd., Dalian, China). The specific primers for LHX6 were as follows: Sense, 5'-GCAGAACAGCTGCTACATCAAGAA-3' and antisense, 5'-CAGTCGCTGGCGTAGATCTGTTC-3'. All reactions were run in triplicate and the results were normalized to an internal control, β -actin, with the following primer sequences: Sense 5'-ATTGGCAATGAGCGGTTC-3' and antisense, 5'-GGATGCCACAGGACTCCAT-3'. The PCR cycling program was 95°C for 3 min, then 28 cycles of 95°C for 20 sec, 60°C for 20 sec and 72°C for 15 sec, and a final extension at 72°C for 5 min. The expression levels of the relative genes were calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis. Total protein was extracted from breast cancer cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer's instructions. Approximately 20 μ g lysate was resolved on 10% SDS denaturing gels (Sigma-Aldrich). Following SDS gel electrophoresis, the proteins were transferred to polyvinylidene fluoride filters (EMD Millipore, Boston, MA, USA) and immunoblotted with mouse anti-LHX6 (1:1,000; sc-271433; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or mouse anti- β -catenin (1:1,500; sc-53484; Santa Cruz Biotechnology, Inc.). After washing with Tris-buffered saline with Tween-20 buffer, the membranes were incubated for 1 h at 25°C with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000; sc-55227; Santa Cruz Biotechnology Inc.). Enhanced chemiluminescence reagent was used for detection and the fluorescence was scanned using a Typhoon scanner (9400, GE Healthcare Life Sciences, Piscataway, NJ, USA). All experiments were performed in triplicate.

Cell proliferation assay. To determine the cell growth of transfected clones, 2,000 cells of each clone were plated in triplicate in 96-well plates and cell viability was assessed by 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. After 24, 48, 72 or 96 h, MTT reagent [diluted from a 4-mg/ml solution in phosphate-buffered saline (PBS) Sigma-Aldrich] was added to all the wells at a final concentration of 0.8 mg/ml and the cells were further incubated for 4 h at 37°C. The reaction was terminated by adding 200 μ l/well dimethyl sulfoxide (Sigma-Aldrich) and absorbance was read at 570 nm in an ELISA plate reader (DNM-9602; Nanjing Perlove Medical Equipment Co., Ltd., Nanjing, China).

Cell invasion assay. Invasion assays were conducted using modified Boyden chambers with filter inserts for 24-well dishes containing 8- μ m pores (EMD Millipore). Filters coated in 40 μ g Matrigel (Gibco Life Technologies, Rockville, MD, USA) were used for the invasion assays. Cells treated with the LHX6 expression vector in 100 μ l RPMI 1640 (Invitrogen Life Technologies) were plated into the upper chamber. The lower chamber was filled with 1 ml RPMI. After a 24-h incubation at 37°C, the cells were fixed with methanol. Non-invaded cells were carefully removed from the upper surface of the filter with a cotton swab. Invaded cells in the lower chamber of the filter were stained with crystal violet (Sigma-Aldrich) and counted under a microscope (BX53; Olympus Corporation, Tokyo, Japan). Each clone was plated in triplicate for each experiment and each experiment was repeated a minimum of three times.

In vivo tumorigenicity. MDA-MB-231 cells (1×10^6 cells in 0.2 ml PBS) stably transfected with pIRES2-EGFP-LHX6 or the empty vector were injected subcutaneously into the dorsal flank of 4-week-old female BALB/c nude mice (n=10; Experimental Animal Center of Henan University, Henan, China). All mice were housed at an ambient temperature of $24 \pm 1^\circ\text{C}$ and were provided with free access to food and water. The tumor volume was measured weekly using calipers and calculated using the following formula: Tumor volume = $ab^2/2$ (where a is the long diameter and b is the short diameter of

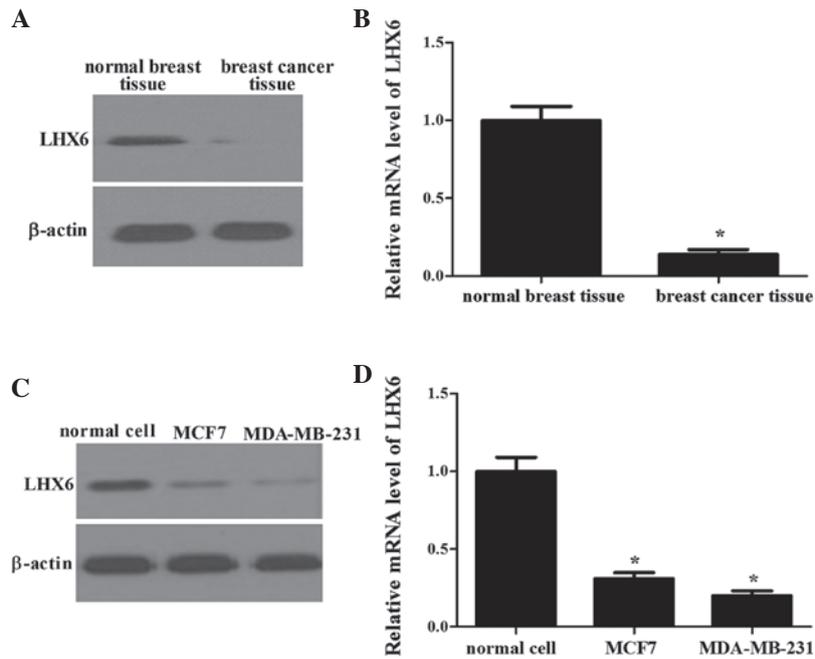


Figure 1. LHX6 expression in breast cancer tissues and cell lines, MCF7 and MDA-MB-231. (A) Representative results of LHX6 protein expression in breast cancer tissues. (B) Representative results of LHX6 mRNA expression in breast cancer tissues. LHX6 expression was significantly decreased in the breast cancer tissues compared with LHX6 expression in the corresponding normal breast tissues, as detected by RT-qPCR. (C) Representative protein results of LHX6 in the breast cancer cell lines. (D) LHX6 mRNA expression in the breast cancer cell lines, as determined by RT-qPCR. *P<0.05 compared with the normal cell control group. LHX6, LIM homeobox domain 6. RT-qPCR, reverse-transcription quantitative polymerase chain reaction.

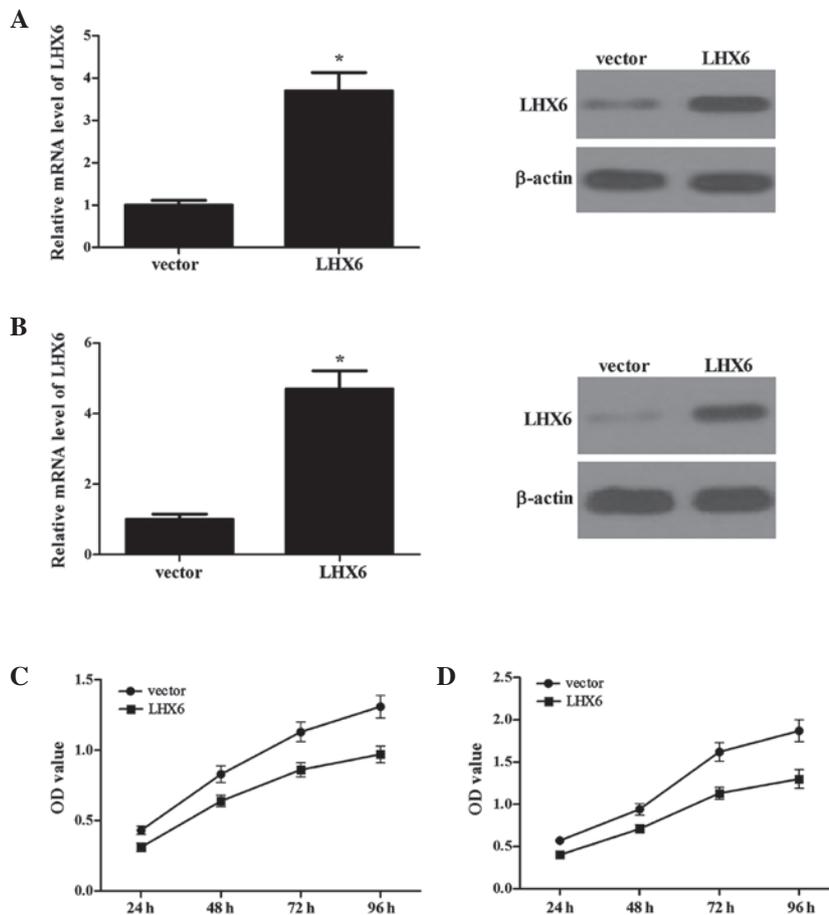


Figure 2. LHX6 inhibits cell proliferation. Expression of LHX6 mRNA and protein in transfected breast cancer cell lines, (A) MCF7 and (B) MDA-MB-231 was analyzed using reverse-transcription quantitative polymerase chain reaction and western blotting. LHX6 markedly inhibited the proliferation of (C) MCF7 and (D) MDA-MB-231 cells, when compared with that of the control group. Data are presented as the mean \pm standard deviation of three independent experiments. *P<0.05 compared with the control group (vector). LHX6, LIM homeobox domain 6; OD, optical density.

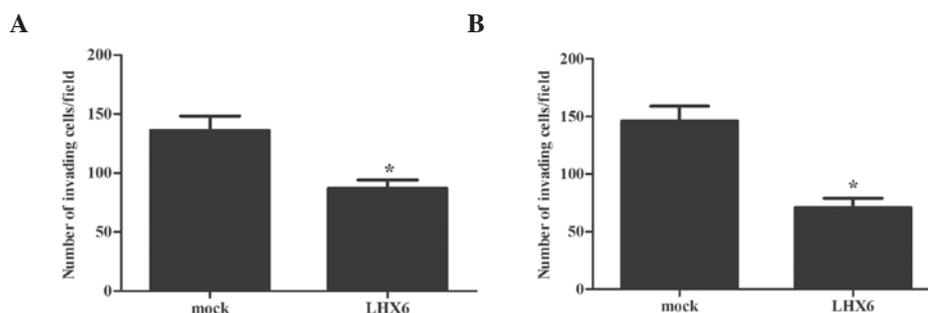


Figure 3. LHX6 inhibits cell invasion. Cell invasion was determined using a Boyden chamber assay. The number of invaded cells in LHX6-transfected (A) MCF7 and (B) MDA-MB-231 cells was significantly reduced compared with that of the control cells (mock). Data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$ compared with the control group (mock). LHX6, LIM homeobox domain 6.

the tumor). Mice were sacrificed using pentobarbitol sodium (40 mg/kg; Sigma-Aldrich) on completion of the follow-up and tumor tissue samples were collected. Total weight, tumor weight, and tumor volume were recorded. All animal studies were approved by the Institutional Animal and Committee of Huaihe Hospital of Henan University.

Statistical analysis. Statistical analyses were performed with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The results are expressed as the mean \pm standard deviation. The statistical significance was determined using Mann-Whitney U-test for the variables of the two sample groups and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

LHX6 expression is downregulated in breast cancer tissues and cell lines. Western blot analysis and RT-qPCR were performed to detect LHX6 expression in primary breast cancer tissues and in their corresponding adjacent healthy tissues. As shown in Fig. 1A and B, LHX6 expression was downregulated in breast cancer tissues when compared with that in the adjacent healthy tissues. LHX6 expression was then examined in MCF7 and MDA-MB-231 cells. As compared with the normal cell line, LHX6 expression was observed to be significantly reduced in MCF7 (Fig. 1C) and MDA-MB-231 cells (Fig. 1D). These results indicate that LHX6 expression is downregulated in breast cancer.

LHX6 inhibits breast cancer cell proliferation. To evaluate the role of LHX6 in breast cancer, an LHX6 expression vector was established and transfected in the breast cancer cell lines, and whether LHX6 participates in cell proliferation was evaluated. The transfection efficiency was determined by RT-qPCR and western blot analysis of the MCF7 and MDA-MB-231 cells. As shown in Fig. 2A and B, the mRNA expression of LHX6 was significantly increased in the MCF7 and MDA-MB-231 cells ($P < 0.05$). Consistent with the results of RT-qPCR, western blot analysis demonstrated that protein expression of LHX6 was also markedly increased in the breast cancer cells. Furthermore, the cell proliferation assay demonstrated that ectopic expression of LHX6 significantly inhibited the proliferation of MCF7 and MDA-MB-231 cells, when compared with that of the control groups (Fig. 2C and D). These results

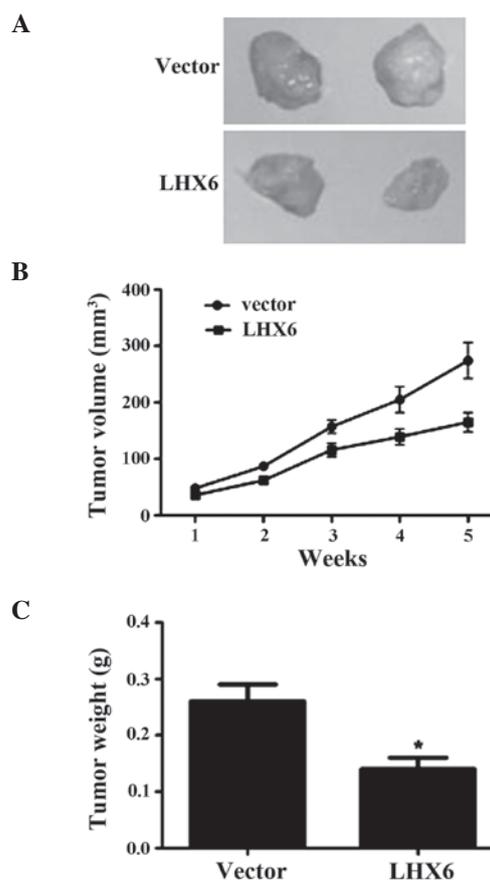


Figure 4. LHX6 inhibits tumor formation in BALB/c nude mice. (A) Representative images of tumors dissected from each group. (B) The tumor growth curve of LHX6-expressing MDA-MB-231 cells was compared with that for the vector only cells. (C) Tumors from the LHX6 and vector only (control) groups were weighed and the mean tumor weight is presented. * $P < 0.05$ compared with the vector group. LHX6, LIM homeobox domain 6. Data are presented as the mean \pm standard deviation of three independent experiments.

indicate that the expression of LHX6 inhibits breast cancer cell proliferation.

LHX6 inhibits breast cancer cell invasion. The impact of LHX6 on cell invasion was also investigated in the current study. As shown in Fig. 3A, enforced expression of LHX6 resulted in a 1.5-fold reduction in the number of MCF7 cells migrating across the filter. A similar inhibitory effect was

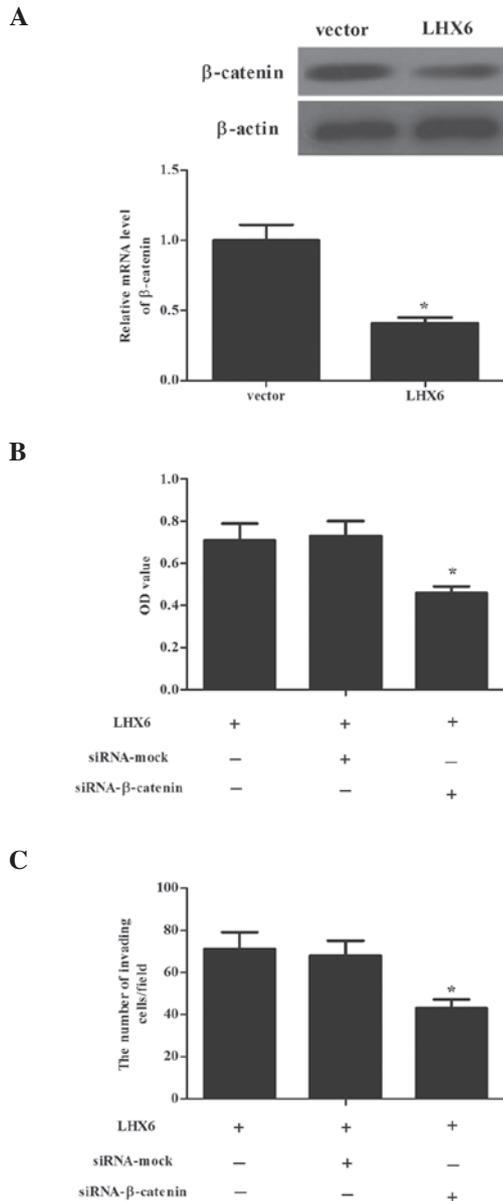


Figure 5. LHX6 inhibits breast cancer cell proliferation and invasion by suppression of the Wnt/ β -catenin signaling pathway. (A) The protein and mRNA expression level of β -catenin following transfection of LHX6 in MDA-MB-231 cells. (B and C) siRNA- β -catenin potentiates the LHX6-induced inhibition of cell proliferation and invasion in MDA-MB-231 breast cancer cells. All experiments were repeated a minimum of three times. * $P < 0.05$ compared with siRNA-mock-transfected cells. LHX6, LIM homeobox domain 6; siRNA, small interfering RNA; OD, optical density. Data are presented as the mean \pm standard deviation.

observed on MDA-MB-231 cells (Fig. 3B). These results suggest that LHX6 inhibits breast cancer cell invasion.

LHX6 inhibits breast cancer growth in vivo. Next the effect of LHX6 *in vivo* was investigated. As shown in Fig. 4A and B, mice injected with LHX6-expressing MDA-MB-231 cells exhibited tumors of significantly reduced volume, when compared with that of the empty vector group ($P < 0.05$). Furthermore, the mean tumor weight was significantly decreased in nude mice that were injected with the LHX6-expressing MDA-MB-231 cells, when compared with that of mice injected with the control vector-transfected cells (Fig. 4C; $P < 0.05$).

SiRNA- β -catenin potentiates LHX6-induced inhibition of cell proliferation and invasion in MDA-MB-231 cells. Aberrant activation of Wnt/ β -catenin signaling is significant in the pathogenesis of breast cancer. To investigate the potential effects of LHX6 on the Wnt/ β -catenin signaling pathway, the expression of Wnt/ β -catenin signaling molecules was examined by RT-qPCR and western blot analysis following transfection of LHX6 in MDA-MB-231 cells. As shown in Fig. 5A, the mRNA and protein levels of β -catenin were significantly reduced in LHX6-transfected MDA-MB-231 cells ($P < 0.05$). The role of β -catenin in LHX6-transfected MDA-MB-231 cells was subsequently analyzed. Upon treatment with siRNA- β -catenin, the inhibitory effect of LHX6 on breast cancer cell proliferation and invasion was identified to be significantly enhanced in LHX6-transfected MDA-MB-231 cells (Fig. 5B and C; $P < 0.05$). These results reveal that LHX6 inhibits breast cancer cell proliferation and invasion through suppression of the Wnt/ β -catenin signaling pathway.

Discussion

Epigenetic silencing and genetic deletion of tumor suppressors contribute to the development and progression of breast cancer. In the present study, LHX6 expression was demonstrated to be downregulated in breast cancer tissues and cell lines, and the enforced expression of LHX6 appeared to inhibit cell proliferation and invasion. Furthermore, increased LHX6 expression reduced the expression level of β -catenin, and siRNA- β -catenin appeared to enhance the LHX6-induced inhibition of cell proliferation and invasion in breast cancer cells.

Epigenetic alterations of tumor-suppressor genes, including promoter methylation and histone modification, are critical in the initiation and progression of multiple types of cancer, including breast cancer. Various studies have shown that LHX proteins are involved in cytoskeletal organization, organ development and oncogenesis (15,16). In the present study, increased expression of LHX6 was found to inhibit breast cancer cell growth and invasion. Various LHX genes have also been implicated in tumorigenesis. Increased expression of LHX6 inhibits cervical cancer cell proliferation and suppresses the tumorigenic phenotype (16). Choi *et al* (17) demonstrated that inactivated LHX8 reduces transcription of the proapoptotic genes, bcl-2-like protein, caspase-2 and caspase-3 in mouse oocytes. Vladimirova *et al* (8) showed that the LHX9 gene is frequently silenced in pediatric malignant astrocytomas by hypermethylation, and that this epigenetic alteration is involved in glioma cell invasiveness and migration. These results indicate that LHX6 functions as a tumor suppressor in breast cancer progression.

Aberrant activation of Wnt signaling, which is key in the regulation of cell growth, development and differentiation, has been implicated in many cancer types, including colorectal, prostate, liver and breast cancer (18,19). During canonical Wnt signaling, binding of Wnt ligands to Frizzled/low-density lipoprotein receptor-related protein 5/6 receptor complexes causes stabilization of β -catenin. Stabilized β -catenin may translocate to the nucleus and modulate the expression of specific target genes, which control cell proliferation and invasion (20,21). The most common alternation of the Wnt signaling pathway

includes mutation of β -catenin, adenomatous polyposis coli and T-cell factor/lymphoid enhancer factor (22-24). Therefore, inhibition of Wnt signaling pathway-associated molecules may potentially be an effective approach to the treatment of various types of human cancer. For example, DACT1 (a homologue of a Dapper, located at chromosomal region 14q23.1) was first identified as a Dishevelled-associated antagonist of Wnt/ β -catenin, and it reduced the expression of active β -catenin and its downstream target gene, c-Myc in breast cancer cells, thus inhibiting cell proliferation of breast cancer by inducing apoptosis, as well as tumor cell migration (25). Recently, one report demonstrated that LHX6 directly interacts with pituitary homeobox 2 (PITX2) to inhibit PITX2 transcriptional activity and activation of multiple promoters (26). PITX2 directly interacts with β -catenin to synergistically regulate lymphoid enhancer-binding factor 1 expression, which regulates the activity of various growth control genes (27). In the present study, the expression level of β -catenin was identified to be significantly reduced in LHX6-transfected breast cancer cells. Additionally, siRNA- β -catenin enhanced LHX6-induced inhibition of cell proliferation and invasion. These results demonstrate that LHX6 inhibits breast cancer cell proliferation and invasion via suppression of the Wnt/ β -catenin signaling pathway.

In conclusion, LHX6 was demonstrated to be significant in human breast cancer pathogenesis. It is proposed that LHX6 expression inhibits tumor progression by suppressing cell proliferation and invasion. Therefore, LHX6 may serve as a potential therapeutic target for breast cancer.

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