

## Original Article

# Downregulation of Dickkopf-1 is responsible for high proliferation of breast cancer cells via losing control of Wnt/ $\beta$ -catenin signaling

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**Aim:** To investigate the role of DKK-1/Wnt/ $\beta$ -catenin signaling in high proliferation of LM-MCF-7 breast cancer cells, a sub-clone of MCF-7 cell line.

**Methods:** Two cell lines (MCF-7 and LM-MCF-7) with different proliferation abilities were used. LM-MCF-7 cells were transiently transfected with the pcDNA3-DKK-1 plasmid encoding the DKK-1 gene (or MCF-7 cells were transfected siRNA targeting DKK-1 mRNA). Flow cytometry analysis and 5-bromo-2'-deoxyuridine (BrdU) incorporation assay were applied to detect the cell proliferation. The expression levels of  $\beta$ -catenin, phosphorylated  $\beta$ -catenin, c-Myc, cyclin D1 and Survivin were examined by Western blot analysis. The regulation of Survivin was investigated by Luciferase reporter gene assay.

**Results:** Western blot and RT-PCR analysis showed that the expression level of DKK-1 was downregulated in LM-MCF-7 relative to MCF-7 cells. Flow cytometry and BrdU incorporation assay showed DKK-1 could suppress growth of breast cancer cells. Overexpression of DKK-1 was able to accelerate phosphorylation-dependent degradation of  $\beta$ -catenin and downregulate the expression of  $\beta$ -catenin, c-Myc, cyclin D1 and Survivin. Luciferase reporter gene assay demonstrated that Survivin could be regulated by  $\beta$ -catenin/TCF4 pathway.

**Conclusion:** We conclude that the downregulation of DKK-1 is responsible for the high proliferation ability of LM-MCF-7 breast cancer cells via losing control of Wnt/ $\beta$ -catenin signaling pathway, in which c-Myc, cyclinD1 and Survivin serve as essential downstream effectors. Our finding provides a new insight into the mechanism of breast cancer cell proliferation.

**Keywords:** Dickkopf-1; Wnt/ $\beta$ -catenin; breast cancer; cell proliferation; MCF-7 cell line; survivin

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## Introduction

Breast cancer is a very common tumor worldwide, and its metastasis is a major cause of death. Our laboratory previously established a metastatic subclone from the MCF-7 breast cancer cell line, called LM-MCF-7, derived from a lung metastasis of a severe combined immunodeficient (SCID) mouse<sup>[1]</sup>. Both in vivo and in vitro experiments, our results showed that LM-MCF-7 had high malignant phenotype in cell proliferation. Results of gene chips assay, containing 21,329 kinds of human genes, demonstrated that 67 kinds of genes were markedly different between LM-MCF-7 cells and MCF-7 cells<sup>[2]</sup>, suggesting that the two cell lines were remarkably different in molecular events associated with malignance. Therefore, the

two cell lines, having the similar genetic background, are ideal parallel cell lines to investigate the mechanism of proliferation of breast cancer cells<sup>[1, 3, 4]</sup>. However, the endogenous signal pathways which are responsible for the high proliferation ability of LM-MCF-7 cells remain unclear.

The aggressive nature of malignant cancer cells is determined by complex signaling pathways that regulate key functions including cell proliferation, survival, migration, and invasion. The enhanced and independent cell proliferation is an essential step in tumor metastasis<sup>[5]</sup>. Recent studies show that the signal transduction pathways, such as NOTCH3-mediated signaling, ErbB-mediated cascade, serum and glucocorticoid-inducible kinase (SGK)-mediated signaling, G protein-coupled receptor GPR40-mediated signaling and ERK/MAPK signaling, are involved in the regulation of breast cancer cell proliferation<sup>[6–8]</sup>. It has been reported that Wnt signaling plays an important role in regulation of cell proliferation in several cancers, such as prostate cancer, lung cancer, myeloma and

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colon cancer<sup>[9-12]</sup>.

Dickkopf-1 (DKK-1) was first identified as a secreted inhibitor of the canonical Wnt signaling pathway<sup>[13]</sup>. DKK-1 mediates its inhibitory effects on Wnt signaling by binding to the Kremen receptor. Frizzled, the receptor for Wnt, and Kremen both use LRP5/6 as a co-receptor. As a result DKK-1 can sequester LRP5/6 away from Frizzled, thereby inhibiting Wnt signaling<sup>[14-16]</sup>. An increasing number of studies have indicated that the members of the DKK gene family are involved in a variety of carcinomas, such as colon cancer, colorectal cancer and melanoma<sup>[17-21]</sup>. DKK-1 mediated inhibition of TCF/Lef transcription can inhibit ovarian carcinoma cell proliferation<sup>[19]</sup>. Our previous study showed that DKK-1 secreted by mesenchymal stem cells could inhibit the growth of tumor cells via depressing Wnt signaling<sup>[22]</sup>. However, the role of DKK-1 in the growth of LM-MCF-7 breast cancer cells remains unclear.

In the present study, we investigated the mechanism of growth of LM-MCF-7 breast cancer cells involving Wnt/ $\beta$ -catenin signal pathway. Our finding shows that DKK-1 is downregulated in LM-MCF-7 cells relative to MCF-7 cells, which is responsible for the high proliferation ability of LM-MCF-7 breast cancer cells via losing control of Wnt/ $\beta$ -catenin signaling pathway.

## Materials and methods

### Cell culture

MCF-7 and LM-MCF-7 cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, and 100 U/mL penicillin/streptomycin in humidified 5% CO<sub>2</sub> at 37 °C.

### Cell growth curve analysis

MCF-7 cells and LM-MCF-7 cells in exponential growth phase were counted and seeded into flasks (1×10<sup>5</sup> cells/flask, 21 flasks per cell line). Medium was changed every 3 days as usual. Three flasks of cells were harvested daily by trypsinisation and counted using a hemocytometer. Draw the growth curve by using the average number. Tumor cell doubling time:  $TD = T \lg 2 / \lg(N/N_0)$  (TD: doubling time; T: time interval, N<sub>0</sub>: initial cell number, N: end-point cell number)<sup>[23]</sup>.

### RNA isolation and RT-PCR

Extraction of total RNA of the cells and reverse transcription were carried out according to a previously published protocol<sup>[24]</sup>. The specific primers for DKK-1 were: 5'-TAGCACCTTGGATGGGTATT-3' (forward) and 5'-ATCCTGAGGCACAGTCTGAT-3' (reverse). The amplification was done for 25 cycles (94 °C 30 s, 56 °C 30 s, 72 °C 30 s). cDNA synthesis was normalized by PCR with GAPDH primers; 5'-CCAAGGTCATCCATGACAAC-3' (forward), 5'-AGAGGCAGGGATGATGTTCT-3' (reverse).

### Plasmids and Transfections

pcDNA3-DKK-1 plasmid encoding DKK-1 was a gift from Dr Vincent J HEARING<sup>[21]</sup>. Plasmids pGL3-sur contain-

ing Survivin promoter gene and pcDNA-Tcf4 $\delta$  containing a dominant-negative Tcf-4 gene lacking the portion responsible for the protein binding to DNA were constructed previously in our lab<sup>[3]</sup>. Short interfering RNA (siRNA) duplex composed of sense and antisense strands were synthesized (Guangzhou RiboBio Co, Ltd, China). RNA oligonucleotides used for targeting DKK-1 were 5'-AAUGGUCUGGUAC-UUAUUCdGdC-3' (sense) and 3'-dCdGUUACCAGACCAUGAAUAAGG-5' (antisense). Control siRNA oligonucleotides were 5'-AAUGGUCUUAUUCdGdC-3' (sense) and 3'-dCdGUUACCAGUACCAGAAUAAGG-5' (antisense)<sup>[25]</sup>. One day before transfection, cells were collected, and seeded into 6-well plates at 1×10<sup>5</sup> cells per well (*n*=3, each group). LM-MCF-7 cells were transfected with 2  $\mu$ g plasmids such as pcDNA3 empty vector, pcDNA3-DKK-1 encoding DKK-1, or pcDNA-Tcf4 $\delta$  respectively (MCF-7 cells were transfected with 20 nmol/L DKK-1 siRNA or control siRNA), using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The transfection mixture was removed after 6 h. Transfection efficiency in the cells was monitored in separated wells which paralleled with experimental group by co-transfection of 0.2  $\mu$ g pEGFP-C2 plasmid, which expresses green fluorescence protein (GFP).

### Flow cytometry analysis

After 48 h of transfection, adherent cells were washed with cold PBS and fixed in 70% ethanol at 4 °C. Cells were pelleted and treated with RNase A (50  $\mu$ g/mL, Takara, Otsu, Japan) and stained with propidium iodide (50  $\mu$ g/mL, Sigma-Aldrich, St Louis, MO, USA). The cell cycle was determined by a FACSCalibur™ Flow Cytometer (Becton Dickinson, Bedford, Mass). The proliferation indexes (PIs) were calculated by using the formula<sup>[26]</sup>:

$$PI = (G_2/M+S) \div (G_0/G_1+S+G_2/M) \times 100\%$$

### BrdU incorporation assay

5-Bromo-2'-deoxyuridine (BrdU) incorporation assay was performed described as previously<sup>[27]</sup>. In brief, cells were seeded in 6-well culture plate and were grown overnight prior to transfection. After 48 h transfection, all groups (*n*=3 in every group) were incubated with fresh medium containing 10  $\mu$ mol/L BrdU (Sigma-Aldrich, St Louis, MO, USA) for 4 h prior to immunofluorescence staining with mouse anti-BrdU antibody. The cells were fixed for 15 min with 4% paraformaldehyde in phosphate buffered saline (PBS). After 1 h incubation with PBS containing 2 mol/L HCl to denature DNA, cover slips were washed 3 times with 0.5% bovine serum albumin (BSA) and 0.5% Tween 20 in PBS, and incubated overnight (4 °C) with a mouse anti-BrdU antibody (NeoMarkers, Fremont, CA, USA) at 1:300 dilution. Reactions were developed using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark) at 1:100 dilution for BrdU staining. The BrdU labeling index was assessed by point counting through a Nikon TE200 inverted microscope (Nikon, Tokyo, Japan) using a 40× objective lens. A total of 700–800 nuclei were counted in 6–8 representative fields. The label-

ing index was expressed as the number of positively-labeled nuclei/total number of nuclei. Propidium iodine (Sigma-Aldrich, St Louis, MO, USA) staining for nuclei in 50 µg/mL was used as the control to all cells in each group.

#### Western blot analysis

After 48 h of transfection, cells were washed three times with ice-cold PBS and extracted directly in the lysis buffer (20 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 10 mmol/L EDTA pH 8.0, 1% TritonX-100, 1% DOC, 1 mmol/L DTT, and protein inhibitor cocktails) for 30 min at room temperature. Then, the cell lysates were clarified by centrifugation at 13000 r/min for 20 min and the supernatants were collected for protein determination by the Bradford assay. Equal amounts of protein were separated using 10%–15% SDS-PAGE. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in PBS/0.1% Tween 20 containing 5% dry milk and probed with primary antibodies: rabbit anti-human DKK-1 polyclonal antibody (1:400 dilution; Sigma-Aldrich, St Louis, MO, USA), mouse anti-c-Myc antibody (1:500 dilution, Sigma-Aldrich), rabbit anti-β-catenin antibody (1:4000 dilution, Sigma-Aldrich), mouse anti-phospho-β-catenin antibody (1:1000 dilution, Sigma-Aldrich), mouse anti-cyclin D1 antibody (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA), mouse anti-survivin antibody (1:1000 dilution, Santa Cruz Biotech, Delaware Avenue, Santa Cruz, CA, USA). Protein bands were visualized by using the enhanced chemiluminescence (ECL, Amersham pharmacia biotech, Tokyo, Japan) system. For loading control, membranes were stripped and reprobed with mouse anti-human β-actin monoclonal antibody (1:20000 dilution, Sigma-Aldrich). All experiments were repeated 3 times. We further confirmed the results by applying Glyco Band-Scan software (PROZYME®, San Leandro, Calif, USA).

#### Luciferase reporter gene assay

The cells, such as MCF-7 and LM-MCF-7 were placed into 24-well plate respectively. The plasmids of 0.8 µg pGL3-sur (containing survivin promoter), 0.5 µg pcDNA3-Tcf4δ (or 0.5 µg pcDNA3-DKK-1) and 0.1 µg pRL-TK (containing renilla luciferase gene) were co-transfected into the LM-MCF-7 cells in triplicate by using lipofectAMINE 2000 according to the manufacture's recommendation. In MCF-7 cells, plasmids of 0.8 µg pGL3-sur, 20 nmol/L DKK-1 siRNA and 0.5 µg pcDNA3-Tcf4δ were co-transfected into the MCF-7 cells in triplicate. After 48 h transfection, the dual luciferase reporter gene assay was performed by Dual-Glo luciferase Assay kit (Promega, WI, USA). The intensity of luminescence was measured by a TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA). Data were normalized by Renilla luciferase luminescence intensity.

#### Statistical analysis

All data are presented as mean±standard error of the mean and were analyzed by ANOVA or Student's *t* test using Prism 4.0 (GraphPad Software, CA). A *P* value of <0.05 was con-

sidered statistically significant. All statistical tests were two-sided.

## Results

### DKK-1 is downregulated in LM-MCF-7 breast cancer cells

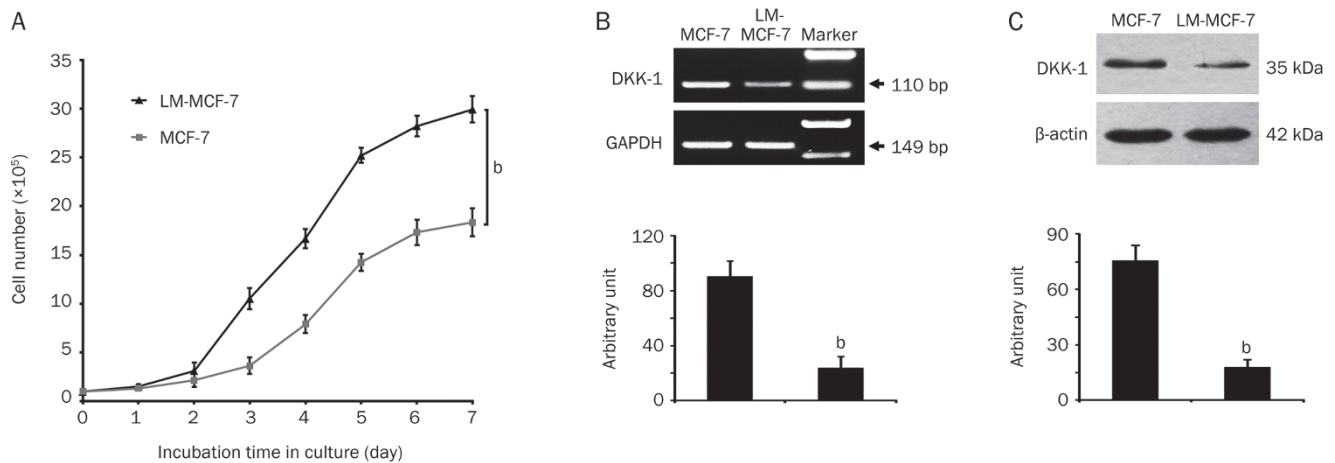
Malignant breast cancer cells require an independent proliferation signal pathway to adapt foreign microenvironment for survival. Recent studies show that DKK-1 is involved in regulation of a variety of tumor cells growth<sup>[17–21]</sup>. Our laboratory previously established a metastatic subclone from the MCF-7 breast cancer cell line, called LM-MCF-7, derived from a lung metastasis of a severe combined immunodeficient (SCID) mouse. Our previous results showed that LM-MCF-7 had high malignant phenotype in cell proliferation<sup>[1]</sup>. In this study, we showed that the population doubling time *in vitro* was estimated as 34.3 h and 40.0 h in the exponential phase of LM-MCF-7 cells and MCF-7 cells, respectively (Figure 1A). To identify the role of DKK-1 in LM-MCF-7 breast cancer cell proliferation, we investigated the expression of DKK-1 at the levels of mRNA and protein in MCF-7 and LM-MCF-7 cells. RT-PCR and Western blot analysis showed that the expression of DKK-1 at the levels of mRNA and protein was downregulated in LM-MCF-7 cells relative to MCF-7 cells (Figures 1B and 1C), suggesting that DKK-1 may be involved in the high proliferation of LM-MCF-7 breast cancer cells.

### DKK-1 suppresses the growth of LM-MCF-7 breast cancer cells

Accordingly, we examined the role of DKK-1 in proliferation of breast cancer cells by transfection. Transfection efficiency revealed that approximately 70%–80% of cells showed green fluorescence (Figure 2A). BrdU incorporation analysis showed that the downregulation of expression of DKK-1 by RNA interference led to the percentage of BrdU-positive MCF-7 cells increased significantly ( $P<0.05$ , *vs* control, Student's *t* test, Figure 2B). However, the percentage of BrdU-positive LM-MCF-7 cells reduced significantly when overexpressing DKK-1 in LM-MCF-7 cells by transfecting with pcDNA3-DKK-1 plasmids ( $P<0.05$ , *vs* control, Student's *t* test, Figure 2B). Flow cytometry analysis showed that the downregulation of DKK-1 by RNA interference led to the increase of cell proliferation index (PI) of MCF-7 cells from 30.87% to 41.44% ( $P<0.05$ , *vs* control, Student's *t* test). However, the PI of LM-MCF-7 declined from 50.61% to 32.99% after overexpressing DKK-1 by transfecting pcDNA3-DKK-1 plasmids ( $P<0.05$ , *vs* control, Student's *t* test, Figure 2C). Thus, our finding suggests that the DKK-1 is involved in proliferation of breast cancer cells.

### β-catenin, c-Myc, cyclin D1, and Survivin serve as downstream effectors of DKK-1

Our previous results showed that β-catenin, c-Myc, cyclin D1, and Survivin were upregulated in LM-MCF-7 cells by cDNA microarray<sup>[2]</sup>. Therefore, we further examined the protein expression levels of them in the cells. Western blot analysis showed that β-catenin, c-Myc, cyclin D1, and Survivin were highly expressed in LM-MCF-7 cells relative to MCF-7 cells, while the phosphorylation level of β-catenin was decreased



**Figure 1.** DKK-1 is downregulated in LM-MCF-7 breast cancer cells. (A) Cell growth curve of MCF-7 cells and LM-MCF-7 cells was measured, respectively (<sup>b</sup> $P < 0.05$ , Student's  $t$  test). (B) RT-PCR showed the mRNA expression level of DKK-1 in MCF-7 and LM-MCF-7 cells. Histogram shows the results by applying Glyco Band-Scan software (<sup>b</sup> $P < 0.05$ , Student's  $t$  test). (C) Western blot analysis showed the expression level of DKK-1 in MCF-7 and LM-MCF-7 cells. Histogram shows the results by applying Glyco Band-Scan software (<sup>b</sup> $P < 0.05$ , Student's  $t$  test).

in LM-MCF-7 cells (Figure 3A, 3B), suggesting that  $\beta$ -catenin, c-Myc, cyclin D1, and Survivin may serve as downstream effectors of DKK-1 in the cells. Then, we found that the downregulation of DKK-1 in MCF-7 cells by RNA interference could lead to the upregulation of  $\beta$ -catenin, c-Myc, cyclin D1, and Survivin, and decrease of phosphorylated  $\beta$ -catenin (Figure 3C, 3D). Contrarily, the overexpression of DKK-1 in LM-MCF-7 cells by transfection with pcDNA3-DKK-1 plasmid could result in the opposite results in MCF-7 cells (Figure 3E, 3F). Therefore, we conclude that  $\beta$ -catenin, c-Myc, cyclin D1, and Survivin were downstream effectors of DKK-1 in breast cancer cells.

#### Survivin is a downstream target gene of $\beta$ -catenin

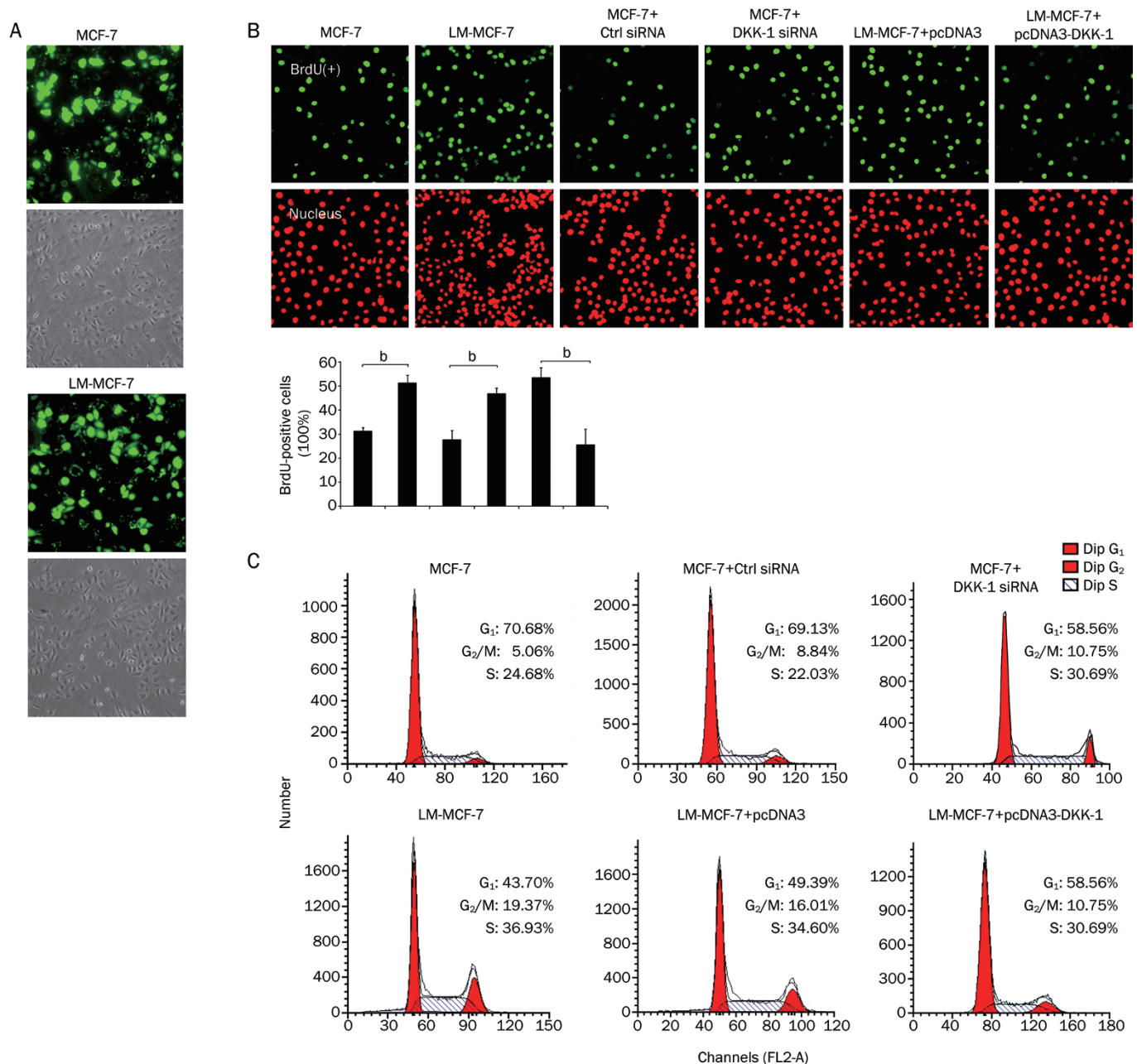
To examine whether Survivin is a downstream target gene of  $\beta$ -catenin, we examined the promoter activity of Survivin by luciferase reporter gene assay when co-transfecting with pcDNA-Tcf4 $\delta$  plasmids. Tcf4 $\delta$ , containing a dominant-negative Tcf-4 gene lacking the portion that responsible for the Tcf-4 protein binding to DNA, can block  $\beta$ -catenin/Tcf4 signal pathway<sup>[24]</sup>. Luciferase reporter gene assay showed that the Survivin promoter luciferase activity was higher in LM-MCF-7 cells than in MCF-7 cells ( $P < 0.05$ , Student's  $t$  test, Figure 4A). Transfection of LM-MCF-7 cells with pcDNA-DKK-1 plasmids or pcDNA-Tcf4 $\delta$  plasmids could downregulate the Survivin promoter luciferase activity of LM-MCF-7 cells ( $P < 0.05$ , Student's  $t$  test, *vs* Control, Figure 4A). Meanwhile, downregulation of DKK-1 by RNA interference could enhance Survivin promoter luciferase activity in MCF-7 cells. It failed to change Survivin promoter luciferase activity when DKK-1 RNAi and overexpression of Tcf4 $\delta$  were performed simultaneously in MCF-7 cells (Figure 4B). Moreover, Western blot assay showed that transfection with pcDNA-Tcf4 $\delta$  plasmids in LM-MCF-7 cells could efficiently downregulated the expression of Survivin (Figure 4C, 4D), suggesting that Survivin is a down-

stream target gene of  $\beta$ -catenin.

To further confirm the effect of Wnt/ $\beta$ -catenin on the proliferation of LM-MCF-7 breast cancer cells, we transfected LM-MCF-7 cells with pcDNA-Tcf4 $\delta$  plasmid for blocking  $\beta$ -catenin/Tcf4 signal pathway. Flow cytometry analysis showed that the overexpression of Tcf4 $\delta$  could potentially inhibit the proliferation of LM-MCF-7 cells, whose proliferation index (PI) of LM-MCF-7 cells declined from 54.57% to 30.54% (Figure 4E,  $P < 0.05$ , *vs* control, Student's  $t$  test), suggesting that the inhibition of Wnt/ $\beta$ -catenin mediated by DKK-1 is responsible for the high proliferation of breast cancer cells.

#### Discussion

Breast cancer is the most common malignant disease in women. Our laboratory previously established a metastatic subclone from the MCF-7 breast cancer cell line<sup>[1]</sup>. We have reported that a positive feedback cascade of Gi/o proteins-PLC-PKC-pERK1/2 mediated by cyclooxygenase (COX) and lipoxygenase (LOX) contributes to the high proliferation of LM-MCF-7 breast cancer cells<sup>[4]</sup>. Additionally, a cross-talk between myosin light chain kinase (MLCK) and phosphorylated ERK1/2 also leads to the high proliferation of LM-MCF-7 breast cancer cells<sup>[3]</sup>. In the present study, we investigated the mechanism of high proliferation of LM-MCF-7 cells involving Wnt/ $\beta$ -catenin signaling. The malignant cancer cell growth needs independent signaling pathways which were generated due to the expression levels of certain effectors changes. According to our previous gene chips assay<sup>[2]</sup>, we supposed that an endogenous signaling pathway, such as Wnt/ $\beta$ -catenin, may be responsible for the high proliferation of LM-MCF-7 breast cancer cells. We first examined the expression level of DKK-1 in MCF-7/LM-MCF-7 breast cancer cells. Our data demonstrated that the expression level of DKK-1 was downregulated in LM-MCF-7 cells relative to MCF-7 cells (Figure 1), suggesting that DKK-1 may be involved in the pro-

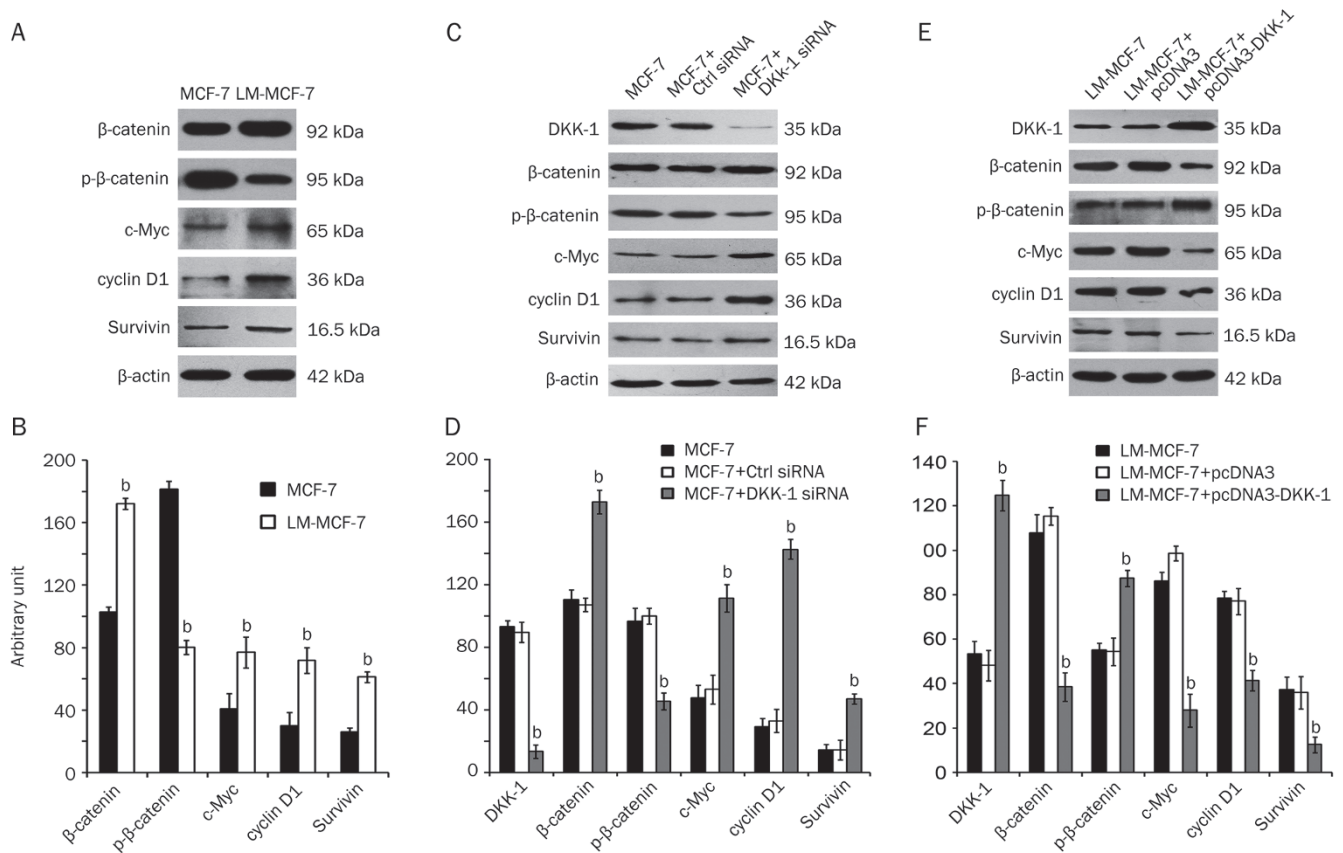


**Figure 2.** DKK-1 suppresses the growth of LM-MCF-7 breast cancer cells. (A) In transfection efficiency, co-transfection was performed in cells, such as pcDNA3-DKK-1 plasmid and pEGFP-C2 plasmid in LM-MCF-7 cells, and DKK-1 siRNA and pEGFP-C2 plasmid in MCF-7 cells.  $\times 100$ . (B) Measurement of cell proliferation by BrdU incorporation assay. Positive PI staining is in red in the nucleus, showing the numbers of cells as the control. Green fluorescence shows the number of BrdU-positive cells.  $\times 100$ . Histogram shows the positive rates of BrdU-positive cells ( $^bP < 0.05$ , vs control Student's *t* test). (C) Examination of cell cycle by flow cytometry analysis in MCF-7 cells and LM-MCF-7 cells.

liferation of LM-MCF-7 cells. Then, we identified the function of DKK-1 in the growth of LM-MCF-7 cells. Flow cytometry analysis and BrdU incorporation assay showed that elevating expression of DKK-1 could suppress proliferation of breast cancer cells (Figure 2). Our results are consistent with that the expression of DKK family of proteins (DKK-1, -2, and -3) is reduced in most of melanoma cell lines and most of tumor samples<sup>[20]</sup>. The overexpression of DKK-1 could result in the

suppression of tumor growth of human prostate and lung cancers<sup>[28, 29]</sup>. Previous study also shows ectopic expression of a dominant negative p53 gene in the p53 wild type MCF-7 breast cancer cell line results in a dramatic decrease in levels of DKK-1 mRNA expression<sup>[30]</sup>. All the data suggest that DKK-1 is a very important factor in tumor cell proliferation.

Next, we try to identify the downstream effectors of DKK-1 in promotion of proliferation of breast cancer cells.

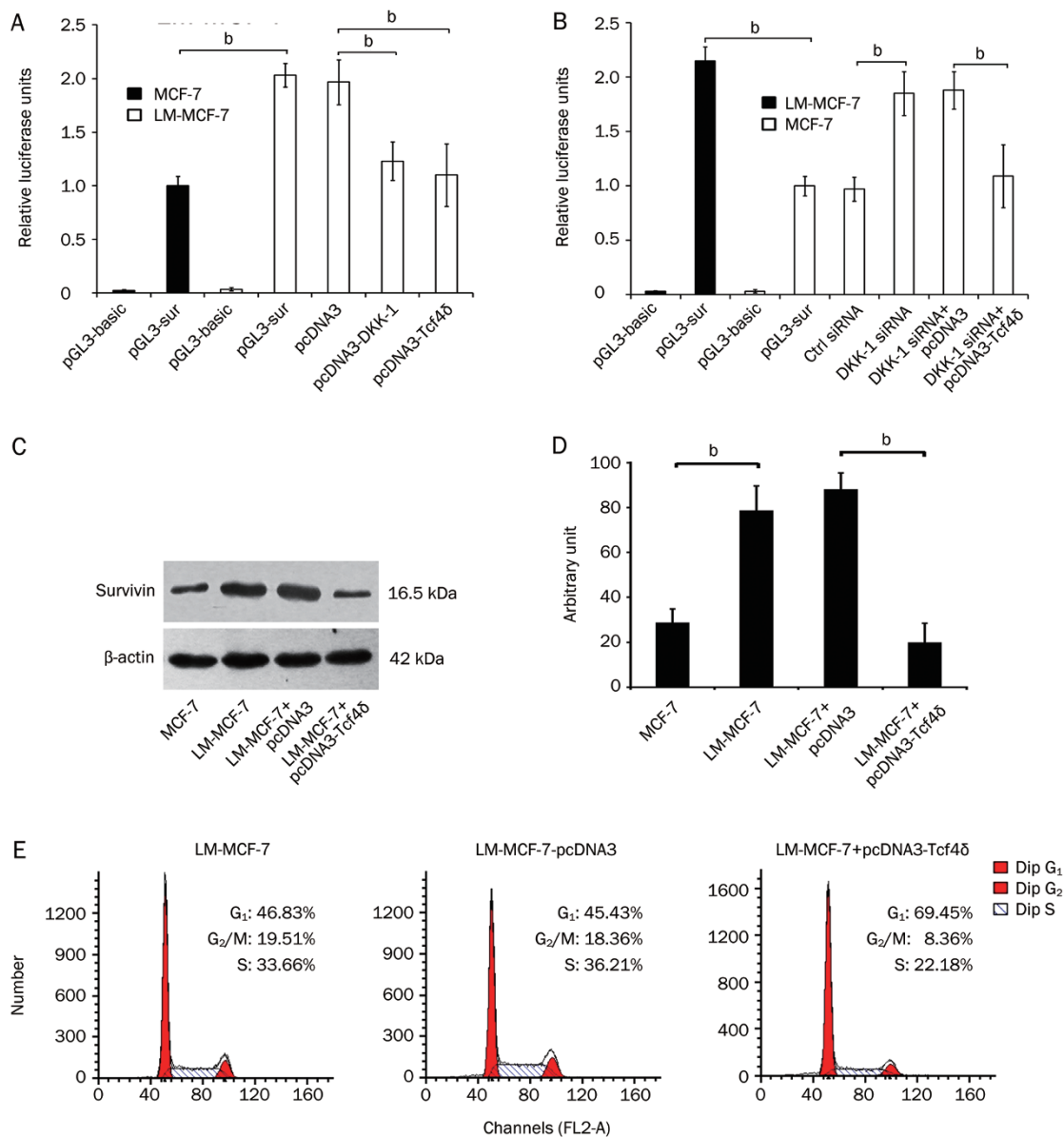


**Figure 3.** β-Catenin, c-Myc, cyclin D1 and Survivin serve as downstream effectors of DKK-1. (A) Western blot analysis showed the expression level of β-catenin, phosphorylated β-catenin, c-Myc, cyclin D1, and Survivin in MCF-7 and LM-MCF-7 cells. The data are representative of three independent experiments. (B) Histogram shows the results by applying Glyco Band-Scan software ( $^bP < 0.05$ , Student's *t* test). (C) Western blot analysis showed the expression level of the proteins when DKK-1 was downregulated by RNA interference in MCF-7 cells. (D) Histogram shows the results by applying Glyco Band-Scan software ( $^bP < 0.05$ , vs control siRNA, Student's *t* test). (E) Western blot analysis showed the expression level of the proteins when DKK-1 was upregulated by transfection of LM-MCF-7 cells with pcDNA3-DKK-1 plasmid. (F) Histogram shows the results by applying Glyco Band-Scan software ( $^bP < 0.05$ , vs pcDNA3, Student's *t* test). The data are representative of three independent experiments.

A hallmark of canonical Wnt pathway activation which can enhance proliferation ability of several tumor cell lines shows an increase in β-catenin-dependent transcription, primarily as a result of β-catenin protein stabilization<sup>[9, 10, 31, 32]</sup>. Thus, we assessed the effects of DKK-1 on the phosphorylation of β-catenin which led to β-catenin degradation. Western blot analysis demonstrated that the expression of DKK-1 could result in declined expression of β-catenin and increased phosphorylation of β-catenin protein (Figure 3), suggesting that the overexpression of DKK-1 in breast cancer cells results in phosphorylation-dependant degradation of β-catenin. This result is consistent with previous study that DKK-1 expression resulted in increased phosphorylation and accelerated half-life of β-catenin protein in T47D carcinoma cells<sup>[33]</sup>. Next, we examined the expression of β-catenin-dependent transcription effectors<sup>[12, 34]</sup>, such as c-Myc and cyclin D1. c-Myc is a nuclear phosphoprotein that functions as a transcription factor stimulating cell-cycle progression. It responds directly to mitogenic signals and plays a critical role in cell-cycle progression, especially during transition from G<sub>1</sub> to the S phase<sup>[35]</sup>. cyclin D1

has a rate-limiting role in G<sub>1</sub>-S phase transition. Both transgenic mouse models and clinical studies indicate a pivotal role for cyclin D1 in normal and malignant cell growth, particularly in breast cancer<sup>[36]</sup>. In our experiments, Western blot analysis showed that DKK-1 was able to downregulate the expression of c-Myc and cyclin D1 (Figure 3), suggesting that c-Myc and cyclin D1 are downstream effectors of DKK-1.

We previously showed that Survivin mRNA was upregulated in LM-MCF-7 cells relative to MCF-7 cells by cDNA microarray<sup>[2]</sup>. Then, we confirmed that at protein level by Western blot analysis (Figure 3A, 3B). Next, we found that DKK-1 could negatively regulate the expression of Survivin (Figure 3D-3F). Luciferase reporter gene assay and Western blot analysis showed that Survivin was a β-catenin/Tcf4 pathway-dependent transcription gene via transfecting breast cancer cells pcDNA-Tcf4δ plasmids which contains a dominant-negative Tcf-4 gene lacking the portion that responsible for the protein binding to DNA (Figure 4). Our results are consistent with previous reports that blockade of Wnt signaling decreased the Survivin protein expression level in melanoma



**Figure 4.** Survivin is a downstream target gene of  $\beta$ -catenin. Effect of DKK-1 (or Tcf4 $\delta$ ) on Survivin promoter was examined by luciferase reporter gene assay. Presented results are relative luciferase activities. (A) Activity of the Survivin promoter could be decreased by the overexpression of DKK-1 (or Tcf4 $\delta$ ) in LM-MCF-7 cells ( $^bP < 0.05$  vs pcDNA3, Student's *t* test). (B) Activity of the Survivin promoter could be increased by DKK-1 RNA interference in MCF-7 cells ( $^bP < 0.05$  vs control siRNA, Student's *t* test). However, the overexpression of Tcf4 $\delta$  could abolish the increase ( $^bP < 0.05$  vs DKK-1 siRNA+pcDNA3, Student's *t* test). (C) Western blot analysis showed the expression of Survivin could be inhibited by overexpressing Tcf4 $\delta$  in LM-MCF-7 cells. (D) Histogram shows the results by applying Glyco Band-Scan software ( $^bP < 0.05$  vs pcDNA3, Student's *t* test). The data are representative of three independent experiments. (E) Cell cycle of LM-MCF-7 cells and LM-MCF-7 cells transfected with pcDNA-Tcf4 $\delta$  (or pcDNA3 empty vector) was examined by flow cytometry analysis.

cell lines and colon cancer cells<sup>[37, 38]</sup>. Survivin is an anti-apoptotic protein which is overexpressed in most human cancers<sup>[39]</sup>. Therefore, we conclude that DKK-1 can also regulate breast cancer cell proliferation by suppressing Survivin expression via  $\beta$ -catenin.

Our finding provided evidence of correlation between expression levels of DKK-1 and changes of Wnt/ $\beta$ -catenin signal pathway targets (Figure 3 and 4). Moreover, the Wnt signal pathway targets, such as cyclin D1, c-Myc, and Sur-

vivin, were all verified as  $\beta$ -catenin/Tcf4 pathway-dependent transcription genes (Figure 4A–4D)<sup>[12, 34]</sup>. The flow cytometry analysis showed that the blockage of  $\beta$ -catenin/Tcf4 pathway by transfection with pcDNA-Tcf4 $\delta$  plasmids could potentially inhibit the proliferation of LM-MCF-7 cells (Figure 4E). Therefore, we conclude that the downregulation of DKK-1 is responsible for the high proliferation of breast cancer LM-MCF-7 cells via losing control of Wnt/ $\beta$ -catenin signaling. In addition, it has been reported that DKK-1 is able to inhibit tumor growth

through noncanonical CamKII pathway in human breast carcinoma cells<sup>[33]</sup>. DKK-1 mediated activation of the JNK signaling pathway was responsible for inhibition of cell growth in  $\beta$ -catenin-deficient human mesothelioma cells<sup>[40]</sup>. Thus, it suggests that DKK-1 plays an important role in tumor cell malignance through not only Wnt/ $\beta$ -catenin signal pathway but also other signal pathways.

Taken together, our data demonstrate that the downregulation of DKK-1 is responsible for the high proliferation ability of LM-MCF-7 breast cancer cells via losing control of  $\beta$ -catenin/Tcf transcription cascade, in which c-Myc, cyclinD1, and Survivin serve as essential downstream effectors of  $\beta$ -catenin. Here, our finding provides a new insight into the mechanism of high proliferation of LM-MCF-7 breast cancer cell. Overexpression of DKK-1 may be used in the gene therapy to inhibit the development of breast cancer cells.

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### Author contribution

Xiao-lei ZHOU, Xiao-dong ZHANG, Li-Hong YE designed research; Xiao-lei ZHOU, Xiao-ran QIN performed research; Xiao-dong ZHANG, Li-Hong YE contributed new reagents or analytic tools; Xiao-lei ZHOU, Xiao-dong ZHANG, Li-Hong YE analyzed data; Xiao-lei ZHOU, Xiao-dong ZHANG, Li-Hong YE wrote the paper.

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