Int J Clin Exp Pathol 2015;8(9):10420-10427
www.ijcep.com /ISSN:1936-2625/IJCEP0012507

Original Article

Knockdown of eIF3D inhibits breast cancer cell proliferation and invasion through suppressing the Wnt/β-catenin signaling pathway

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Received July 7, 2015; Accepted August 21, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: eIF3D (eukaryotic translation initiation factor 3subunit D) is one member of the eIF3 family and plays a critical role in translation initiation. Previous studies showed that it was involved in the development and progression of several tumors. However, the role of eIF3D in breast cancer and the underlying mechanism is still unclear. Therefore, this study set out to investigate the role of eIF3D in breast cancer. Our results demonstrated that eIF3D is up-regulated in breast cancer cells. Knockdown of eIF3D inhibited breast cancer cell proliferation and invasion. In addition, knockdown of eIF3D inhibited the expression of β-catenin, cyclin D1 and c-Myc in breast cancer cells. Taken together, our findings show that siRNA-eIF3D inhibits breast cancer cell proliferation and invasion through suppressing the Wnt/β-catenin signaling pathway. Therefore, eIF3D may be a good molecular target for the prevention and treatment of breast cancer.

Keywords: eIF3D (eukaryotic translation initiation factor 3 subunit D), breast cancer, proliferation, invasion

Introduction

Breast cancer is the leading malignancy in women worldwide and the incidence rates have been increasing annually [1]. Although various treatments for breast cancer, such as chemotherapy, radiation and hormone therapy, have been used and have been improved recently, there is currently no effective therapy to control the recurrence and metastasis of breast cancer [2]. Therefore, understanding the molecular mechanisms underlying the breast cancer may reveal new therapeutic targets, and can improve the management of patients with breast cancer.

Numerous tumor suppressor genes and oncogenes have been identified in breast cancer and further studies of these gene alterations and functions will assist in revealing the molecular mechanisms of breast cancer initiation and progression [3]. Eukaryotic translation initiation factor 3 (eIF3) is a highly complex multiprotein assembly with multiple functions in translation [4]. It consists of10-13 subunits, and misregulation of eIF3 subunit expression correlates with cancer development and progression [5]. eIF3D (eukaryotic translation initiation factor 3 subunit D) is one member of the eIF3 family and plays a critical role in translation initiation [6]. Recently, some studies have found that eIF3D was involved in the development and progression of several tumors [7-9]. Gao et al. demonstrated that eIF3D knockdown significantly inhibited non-small cell lung cancer (NSCLC) cell proliferation and colony formation, and blocked the cell cycle [10].

However, the role of eIF3D in breast cancer and the underlying mechanism is still unclear. Therefore, this study set out to investigate the role of eIF3D in breast cancer. Our findings showed that eIF3D gene silencing by small interfering RNA (siRNA) can inhibit proliferation and invasion of breast cancer cells, suggesting the potential of targeting eIF3D to improve the therapeutic outcome of breast cancer.

Materials and methods

Cell culture and siRNA transfection

Human breast cancer cell lines (MDA-MB-231 and MCF-7) and human mammary epithelial

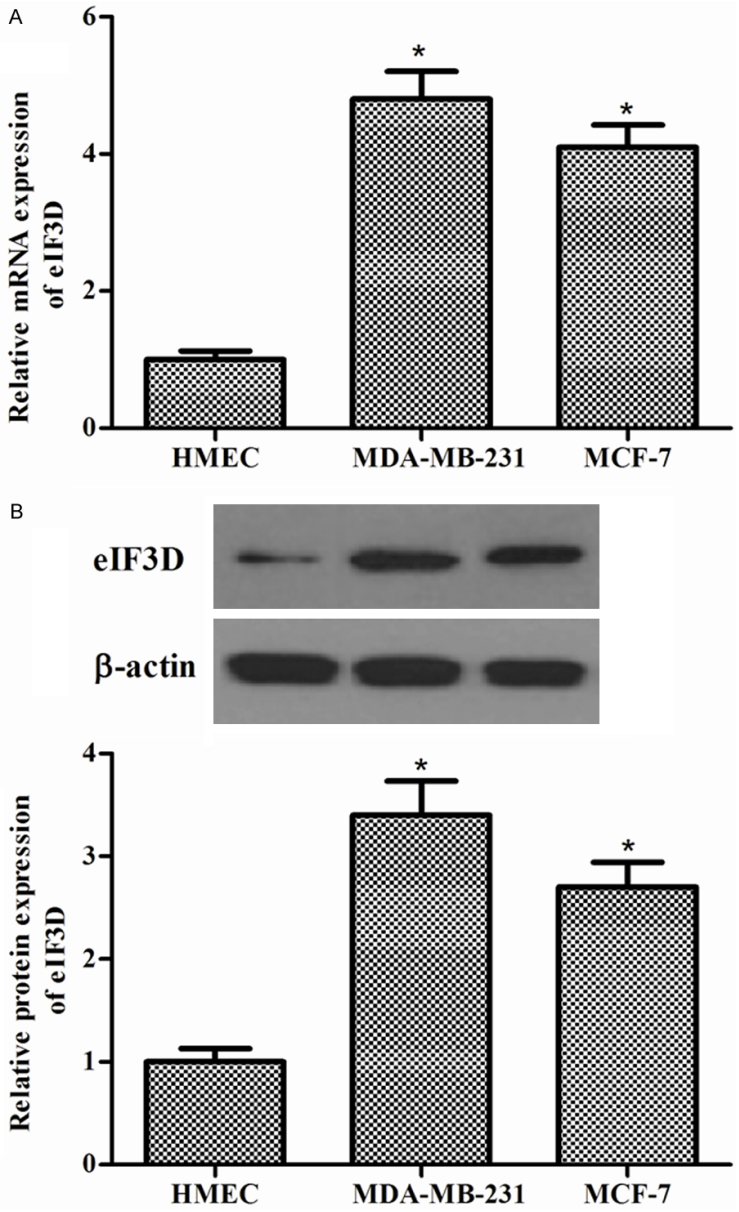

Figure 1. Expression of eIF3D in breast cancer cells. (A) RT-qPCR analysis of EIF3D mRNA expression in breast cancer cell lines. (B) Western blot analysis of EIF3D protein expression in breast cancer cell lines. The expression levels of eIF3D mRNA (A) and protein (B) in breast cancer cells were obviously increased than those in the breast epithelial cell. Date was mean ± SD of three independent experiments. *P < 0.05 vs. control group.

cell (HMEC) line were purchased from The American Type Culture Collection (ATCC, Manassas, VA). The cells maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, 100 units/mL penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA) in a humidified 5% CO_2 atmosphere at 37°C.

For transfection, cells were transfected with siRNA targeting eIF3D (siRNA-eIF3D) and non-targeting siRNA (siRNA-scr) (GenePharma, Shanghai, China) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA extraction and quantitative real-time RT-PCR analysis

Total RNA was extracted from breast cancer cells using Trizol reagent (Abcam, Cambridge, UK) according to the manufacturer's instructions. cDNA was synthesized from the extracted RNA (5 μg) using the EasyScript First-Strand cDNA Synthesis SuperMix kit (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed using the following primers: eIF3D, 5'-CTGGAGGAGGGCAAATACCT-3' (sense) and 5'-CTCGGTGGAAGGACAAACTC-3' (antisense); and β-actin, 5'-TTAGTTGCGTTACACCCTTTC-3' (sense) and 5'-ACCTTCACCGTTCCAGTTT3' (antisense). The steps used for RT-qPCR were as follows: 94°C for 2 min for initial denaturation; 94°C for 20 sec, 59°C for 15 sec, and 72°C for 15 sec; 2 sec for plate reading for 35 cycles; and a melt curve from 65 to 95°C. Relative quantification of eIF3D mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot

Cells were homogenized and lysed with RIPA lysis buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% TritonX-100, 1 mM EDTA, 10 mM β-glycerophosphate, 2 mM sodium vanadate and protease inhibitor). Protein concentration was assayed using a micro BCA protein kit (Pierce, Rockford, IL, USA). The equal amount of protein samples was separated by 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Millipore, Boston,

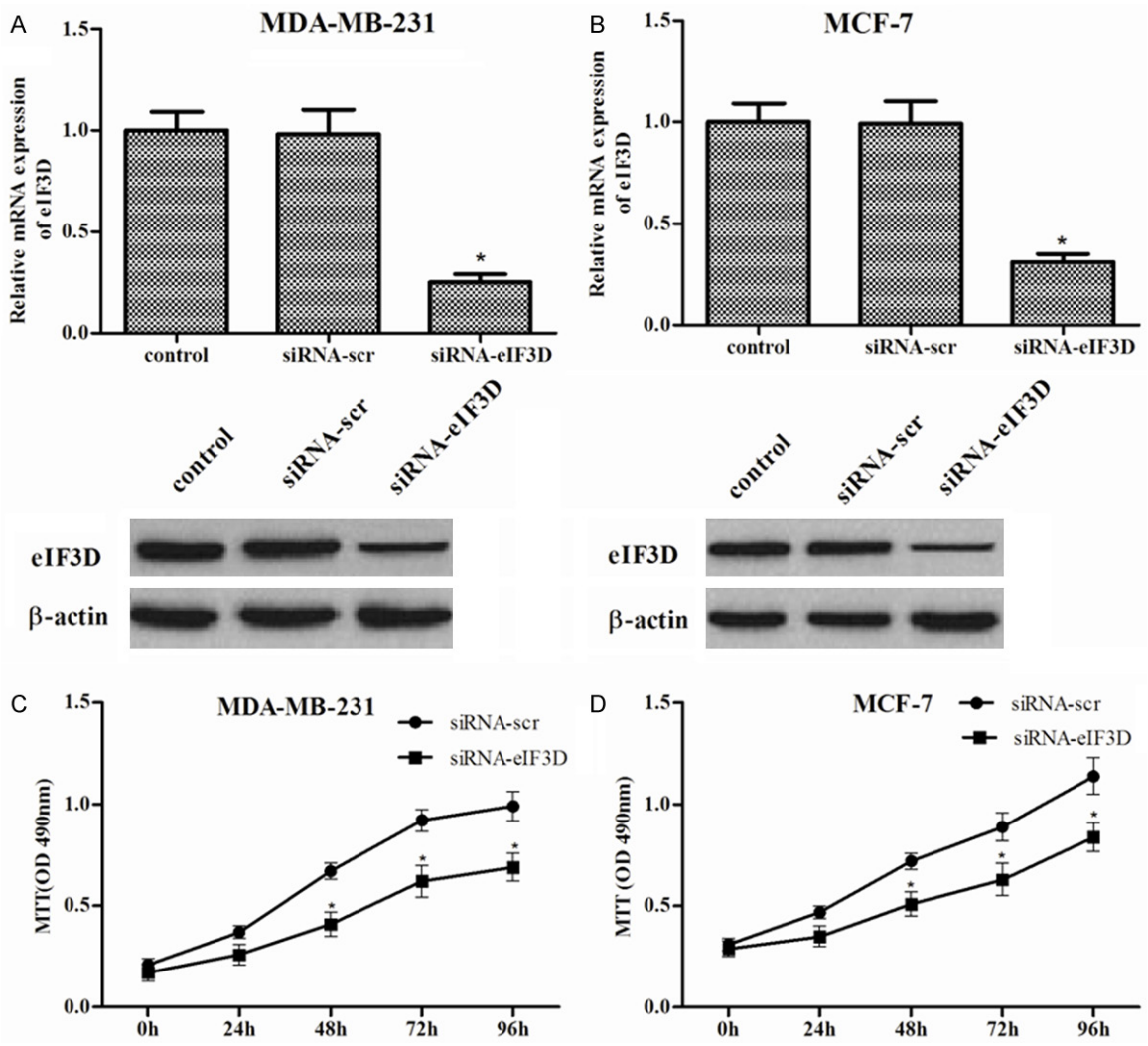

Figure 2. Effect of eIF3D on breast cancer cell proliferation. MDA-MB-231 (A) and MCF-7 (B) cells were transiently transfected with siRNA-eIF3D or siRNA-scr for 48 h. RT-qPCR and Western blot assays were performed to detect the expression of eIF3D after siRNA transfection. (C) and (D) *in vitro* cell growth was examined by cell proliferation assay at the indicated time. Date was mean ± SD of three independent experiments. $^{*}P < 0.05$ vs. siRNA-scr group.

MA, USA). Blots were blocked with 5% fat-free milk, followed by incubation with eIF3D, β-catenin, cyclin D1, c-Myc and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Then the blots were washed and incubated with horse radish peroxidase-conjugated secondary antibody. Protein bands were evaluated by enhanced chemiluminescence (Thermo Fisher Scientific, RockFord, IL, USA).

Cell proliferation assay

Cell proliferation was evaluated by 3-(4.5-methylthiozol-2yl)-2.5-diphenyltetrazolium bromide (MTT) assay. Cells (1×10^4 cells/well) transfected with siRNA-scr or siRNA-eIF3D were seeded into 96-well plates and cultured for 24, 48, 72, and 96 h. At the indicated times, MTT (10 μl) was added into each well, and cells were cultured for an additional 4 h. Following incubation, the culture medium in each well was replaced with 200 μl of DMSO, and the plates were agitated to dissolve the dark blue crystals. Absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay plate reader (Roche Diagnostics GmbH, Penzberg, Germany).

Cell cycle assay

Cell cycle progression was evaluated by flow cytometry. Cells (1×10^4 cells/well) transfected with siRNA-scr or siRNA-eIF3D were cultured in complete medium for 24 h. Cells were harvest-

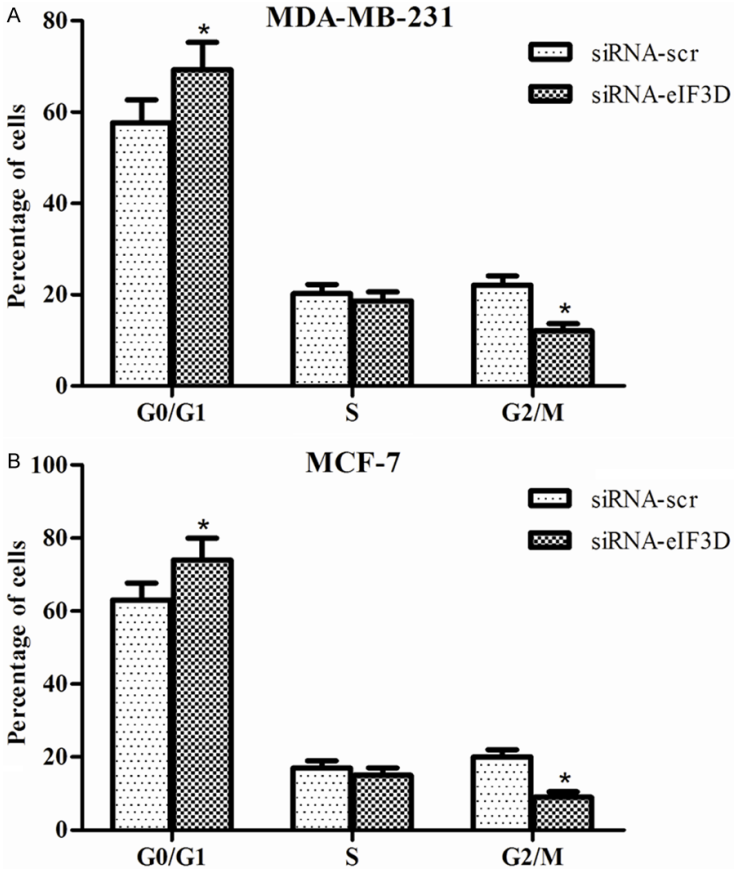

Figure 3. Effect of eIF3D on cell-cycle distribution. Cell cycle progression was evaluated by flow cytometry after 24 h of siRNA-eIF3D transfection. Percentages of cell cycle distribution among different groups are presented as a histogram graph in MDA-MB-231 (A) and MCF-7 (B) cells. Date was mean ± SD of three independent experiments. $^{*}P < 0.05$ vs. siRNA-scr group.

ed and fixed in 70% ethanol and stored at -20°C overnight. Followed by washed twice with phosphate buffered saline (PBS), cells were harvested by trypsinization, centrifuged, and suspended with 1 ml cold PBS and then fixed in methanol for 30 min on ice. Fixed cells were washed with PBS twice, then incubated in RNAse solution (100 μg/ml) for 30 min at 37°C. Subsequently, the cells were incubated in propidium iodide (PI) solution (100 μg/mlin PBS) at room temperature for 30 min. The cell cycle was detected by flow cytometry.

Cell invasion assay

The cell invasion assay was performed using a Boydenchamber coated with matrigel, according to the manufacturer's protocol. Cells transfected with siRNA-scror siRNA-eIF3D were serum-starved, and 1×10^5 cells/ml were suspended in serum-free media; the cell suspension was added on to the top of the chamber and the lower chamber was filled with 500 μl DMEM media and incubated at 37°C in 5% CO_2. After 24 h, the cells that did not invade through the pores were wiped out with cotton wool. Then cells located on the lower surface of the chamber were stained with 0.1% crystal violet (Sigma) and counted using a microscope (Olympus, Tokyo, Japan). These experiments were repeated three times.

Statistical analysis

All results are reported as means ± SD. Statistical analysis involved using the Student's t test for comparison of 2 groups or 1-way ANOVA for multiple comparisons. $P < 0.05$ was considered to be significant.

Results

Expression of eIF3D in breast cancer cells

To investigate the potential role of eIF3D in the tumor igenesis of breast cancer, we detected the expression of eIF3D in breast cancer cell lines. As indicated in **Figure 1A** and **1B**, the expression levels of eIF3D mRNA and protein in breast cancer cells were obviously increased than those in the breast epithelial cell. These results suggest that eIF3D is up-regulated in breast cancer.

Effect of eIF3Don breast cancer cell proliferation

To study the function of eIF3D in breast cancer, MDA-MB-231 and MCF-7 cells were transduced with siRNA-eIF3D. As indicated in **Figure 2A** and **2B**, levels of eIF3D mRNA were obviously decreased following transfection of siRNA-eIF3D in the MDA-MB-231 and MCF-7 cells, respectively, as compared with the siRNA-scramble and control groups. In addition, Western blot analysis demonstrated that the protein level of eIF3Dwas also significantly decreased. These results suggest that the expression of eIF3D was significantly downregulated

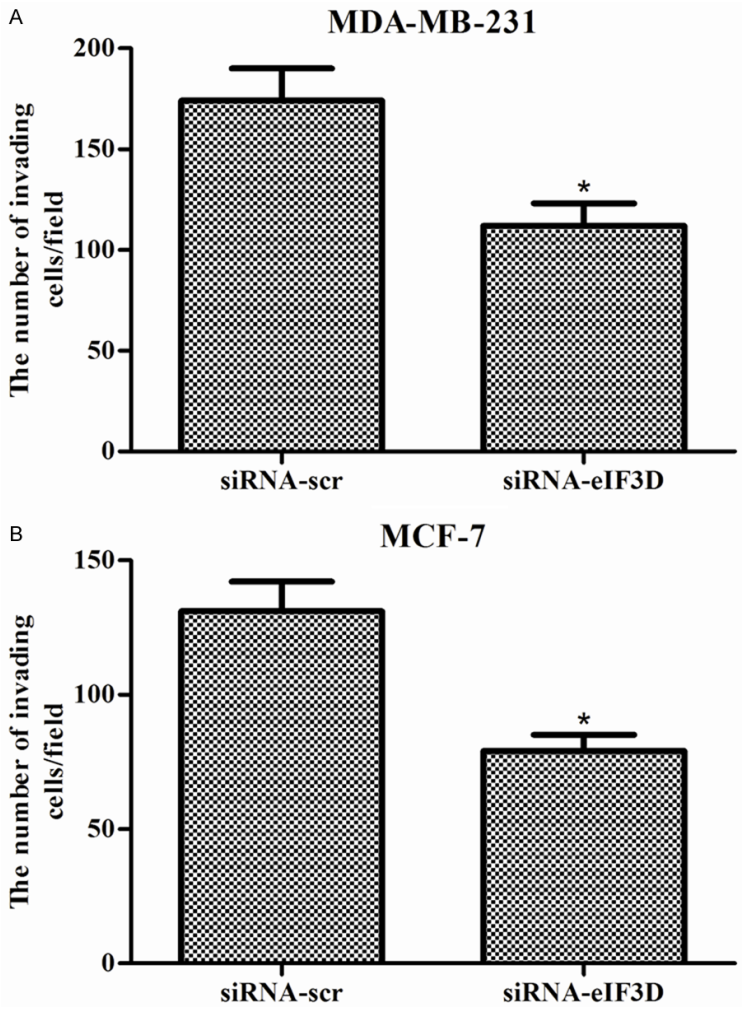

Figure 4. Effect of eIF3Don breast cancer cell invasion. Matrigel invasion assay showing that knockdown of eIF3D decreased cell invasion in MDA-MB-231 (A) and MCF-7 (B) cells compared to control cells. Date was mean ± SD of three independent experiments. *P < 0.05 vs. siRNA-scr group.

after transfection of siRNA-eIF3D (**Figure 2A** and **2B**). Then, we evaluated the effect of eIF3D on breast cancer cell using a MTT assay. For MDA-MB-231 cells, the growth curve of siRNA-eIF3D-transfected cells started to drop from the second and fourth day, as compared with the siRNA-scr group. Similarity, for MCF-7 cells, cell proliferation was also decreased by siRNA-eIF3D. The data indicated that siRNA-eIF3D inhibited the proliferation of both types of cells in a time-dependent manner (**Figure 2C** and **2D**).

Effect of eIF3D on cell-cycle distribution

To elucidate whether siRNA-eIF3D had any impact on the cell-cycle progression of breast cancer cells, MDA-MB-231 and MCF-7 cells were subjected to a flow cytometry assay 24 h after transfection of siRNA-eIF3D. As indicated in **Figure 3**, MDA-MB-231 and MCF-7 cells transfected with siRNA-eIF3D exhibited a greater portion of cells in the G0/G1 phase, respectively, as compared with the siRNA-scr group. The results showed that siRNA-eIF3D could significantly induce G0/G1 phase arrest in human breast cancer cells.

Effect of eIF3Don breast cancer cell invasion

We next evaluated the effect of eIF3D on breast cancer cell invasion. As indicated in **Figure 4A**, the number of invaded MDA-MB-231 cells was significantly reduced after transfection with siRNA-eIF3D when compared with the siRNA-scr group. Similarly, siRNA-eIF3D also inhibited the invasion of MCF-7 cells (**Figure 4B**).

Knockdown of eIF3D inhibits the activation of Wnt/β-catenin signaling pathway

To further illuminate the molecular mechanisms by which eIF3D affects breast cancer cell growth and invasion, we investigated the effects of eIF3D on the downstream target genes of Wnt pathway, β-catenin, cyclin D1 and c-Myc. As indicated in **Figure 5**, the expression levels of β-catenin, cyclin D1 and c-Myc were significantly decreased in siRNA-eIF3D-treated cells, respectively. The data indicated that knockdown of eIF3D inhibited breast cancer cell growth and invasion via blockade of β-catenin, cyclin D1 and c-Myc activations.

Discussion

In this study, we demonstrated that eIF3D is up-regulated in breast cancer cells. Knockdown of eIF3D inhibited breast cancer cell proliferation and invasion. In addition, knockdown of eIF3D inhibited the expression of β-catenin, cyclin D1 and c-Myc in breast cancer cells.

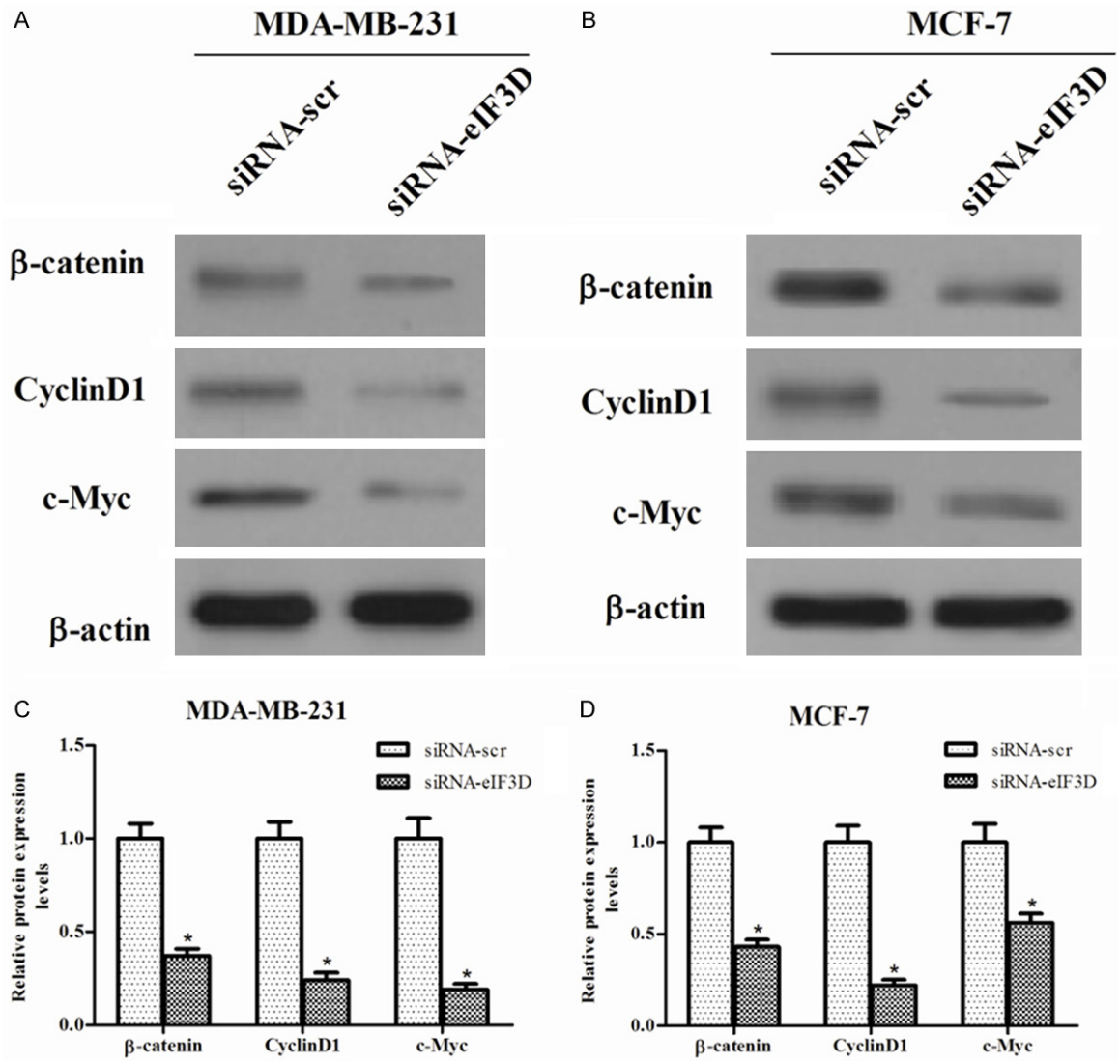

Figure 5. Knockdown of eIF3D inhibits the activation of Wnt/β-catenin signaling pathway. Western blot to detect the protein levels of β-catenin, cyclin D1 and c-Myc in MDA-MB-231 (A) and MCF-7 (B) cells with the indicated antibodies. The intensity of β-catenin, cyclin D1 and c-Myc bands in MDA-MB-231 (C) and MCF-7 (D) cells was normalized to the intensity of their respective β-actin bands and quantified against each other. Date was mean ± SD of three independent experiments. *P < 0.05 vs. siRNA-scr group.

Translation control, involving alterations in translation factor levels and activities, plays a critical role in the process of carcinogenesis [11]. The genes of the eIF3 family have been found to be deregulated in a diverse range of solid tumors [5, 12-14]. It has been reported that the expression of eIF3e was elevated in breast cancer [15]. In accordance with the role of eIF3e in breast cancer, in this study, we found that eIF3D is up-regulated in breast cancer cells, which suggests that eIF3D may serve as an oncogene in the development and progression of breast cancer.

It has been reported that knockdown of eIF3D inhibits cell proliferation through G2/M phase arrest in non-small cell lung cancer [10]. Another study showed that knockdown of eIF3D inhibits cell proliferation through G2/Mphase arrest in melanoma [8]. Consistent with previous findings, in this study, we found that siRNA-eIF3Dalso inhibited breast cancer cell proliferation through G2/Mphase arrest. All of these data suggest that eIF3D plays an important role in breast cancer cell growth.

Previous studies have showed that increased level of EMT is associated with progression and

metastasis of cancers [16-18]. In breast cancer, the EMT process is related to increased invasiveness and metastatic potential [19]. In this study, we observed that knockdown of eIF3D inhibited the invasiveness of breast cancer cells, which was associated with significantly reduced levels of N-cadherin and vimentin, but increased levels of E-cadherin in breast cancer cells. Our findings suggest that eIF3D may be a potential therapeutic target for the prevention of breast cancer metastasis.

Misregulation of the canonical Wnt/β-catenin pathway and aberrant activation of Wnt signaling target genes contribute to breast cancer progression [20-22]. A primary consequence of Wnt signaling activation is the stabilization of β-catenin in the cytoplasm, resulting in an increased translocation of β-catenin to the nucleus. Then, nuclear β-catenin forms a complex with the TCF/LEF transcription factor leading to activation of Wnt target gene expression [23, 24]. Xu et al. reported that β-catenin small hairpin RNA (shRNA) breast cancer cells formed markedly smaller tumors than control cells, and β-catenin-knockdown cells were also significantly impaired in their ability to migrate in wound-filling assays and form anchorage-independent colonies in soft agar [25]. Cyclin D1 is a well-known Wnt target gene. It was related to aggressive breast cancer for increasing the ability of proliferation, migration, and invasion for human breast cancer cells [26]. Recently, Qi et al. showed that eIF3i promotes colon oncogenesis by regulating COX-2 protein synthesis and β-catenin activation [27]. In this study, we found that siRNA-eIF3D inhibited the expression levels of β-catenin, cyclin D1 and c-Myc in MDA-MB-231 and MCF-7 cells. These results suggest that siRNA-eIF3D inhibits cell proliferation and invasion through suppressing the Wnt/β-catenin signaling pathway.

In conclusion, our findings show that siRNA-eIF3D inhibits cell proliferation and invasion through suppressing the Wnt/β-catenin signaling pathway. Therefore, eIF3D may be a good molecular target for the prevention and treatment of breast cancer.

Disclosure of conflict of interest

None.

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