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BCL6 induces EMT by promoting the ZEB1-mediated transcription repression of E-cadherin in breast cancer cells

Jin-Mei Yu [1], Wei Sun [1], Fang Hua, Jing Xie, Heng Lin, Dan-Dan Zhou, Zhuo-Wei Hu *

Molecular Immunology and Pharmacology Group, The State Key Laboratory of Natural Product Structure and Function, The Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, 1 Xian Nong Tan Street, Beijing 100050, China

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ABSTRACT

B-cell CLL/lymphoma 6 (BCL6), a transcriptional repressor, is involved in the development and progression of breast cancers with uncertain mechanism. The purpose of this study is to investigate the potential effect and mechanism of BCL6 in the regulation of epithelial–mesenchymal transition (EMT), a critical cellular process for controlling the development and progression of breast cancers. We found that BCL6 promoted invasion, migration and growth by stimulating EMT in breast cancer cells. BCL6 induced EMT by enhancing the expression of transcriptional repressor ZEB1 which bound to the E-cadherin promoter and repressing the E-cadherin transcription. Deletion of ZEB1 protected against the pro-EMT roles of BCL6 by restoring the expression of E-cadherin in these cells. Moreover, inhibition of BCL6 with BCL6 inhibitor 79-6 suppressed these functions of BCL6 in breast cancer cells. These findings indicate that BCL6 promotes EMT via enhancing the ZEB1-mediated transcriptional repression of E-cadherin in breast cancer cells. Targeting BCL6 has therapeutic potential against the development and progression of breast cancer.

Introduction

BCL6 is a transcriptional repressor and plays an oncogenic role in human B-cell lymphomas [1,2]. Recently, a number of studies suggest that BCL6 may be involved in the pathogenesis of breast cancer. For instance, it has been shown that BCL-6 inhibits mammary epithelial cell differentiation [3]. The expression of BCL6 positively associates with the tumor-promoting function of CCND1 and HIF1α in invasive breast cancer [4]. Moreover, the expression level of BCL6 positively associates with poor survival of breast cancer patients [5]. Therefore, BCL6 has been found to have a potential acting as a therapeutic target for breast cancer [6]. However, the mechanism of BCL6 regulating the development and progression of breast cancer remains to be clarified.

BCL6 is a member of the BTB/POZ zinc finger family. By binding to specific DNA sequences, BCL6 controls the transcriptional expression of target genes involved in the pathogenesis of B-cell lymphomas or breast cancers [6,7]. Once it binds to the target DNA sequences, BCL6 recruits corepressor SMRT, NCOR or BCOR through its BTB domain and regulates cellular proliferation and survival [8]. The BCL6 inhibitors RI-BPI and 79-6 both protect against the recruitment of SMRT, NCOR or BCOR by binding to the BTB domain of BCL6 [9,10]. Small molecular BCL6 inhibitor 79-6 is a cell-permeable oxindole compound that selectively inhibits the transcriptional repression activity of BCL6 but does not affect BCL6 protein levels. 79-6 blocks corepressor BBD (BCL6 binding domain)-mediated BCL6 interaction via binding to a pocket in the lateral groove of the BCL6 BTB domain, resulting in upregulation of BCL6 target genes [9]. BCL6 can also interact with MTA3 through its RD2 domain and represses the terminal differentiation [11]. Interestingly, BCL6 recruits CtBP through its DBD domain and represses its own expression [12].

Epithelial–mesenchymal transition (EMT) is a cell process by which epithelial cells converse into mesenchymal cells and obtain the capacity of cell motility. EMT plays an important role in maintaining the invasion and metastasis of malignancies [13,14]. Aggressive breast cancers exhibit phenotypic characteristics of EMT [15]. Epithelial cells undergoing EMT lose the expression of epithelial marker (E-cadherin and ZO-1) and acquire the expression of mesenchymal markers (N-cadherin, α-SMA and vimentin) and EMT biomarker β-catenin [16,17]. There are a variety of transcriptional repressors that repress the E-cadherin transcription. These transcription repressors, including Snail, Twist, Slug, ZEB1, ZEB2 and CtBP2, commonly function as oncogenes in a variety of cancers [18–23]. Generally, these transcriptional repressors bind to the E-box elements and repress the E-cadherin transcription. However, whether and how BCL6 regulates EMT response in breast cancer remains unknown.

In this study, we hypothesized that BCL6 plays a role in the regulation of EMT process in breast cancer cells. We found that BCL6 promotes cell invasion, migration and growth by inducing EMT response via enhancing the ZEB1-mediated transcription repression

* Corresponding author. Tel.: +86 10 8316 5034; fax: +86 10 8316 5034.
E-mail address: huzhuowei@imm.ac.cn (Z.-W. Hu).
[1] These authors contributed equally to this work.

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of E-cadherin in breast cancer cells. The inhibition of BCL6 with a small molecule BCL6 inhibitor 79-6 reverses these functions of BCL6 in breast cancer cells. Our studies suggest that BCL6 acts as a potential therapeutic target against breast cancer progression.

Materials and methods

Materials

The breast cancer tissue microarrays were purchased from Alenabio (Shanxi, China). The control-shRNA and BCL6-shRNA (h) Lentiviral Particles, ChIP-grade antibodies against BCL6 and ZEB1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The vector and BCL6-myc plasmid were purchased from Sino Biological (Beijing, China).The antibodies against E-cadherin, ZO-1, ZEB1, vimentin, α-SMA, N-cadherin and β-catenin were obtained from Cell Signaling Technology (Boston, MA, USA). The BCL6 Inhibitor 79-6, EZ-Zyme Chromatin Prep Kit and Chromatin Immunoprecipitation Kit were obtained from Merck Millipore (Billerica, MA, USA). Other materials were purchased from commercial resources.

Cells lines and transfection

All breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained according to the manufacturer's instructions. To generate MDA231 cell populations stably expressing BCL6 shRNA, MDA231 cells were infected with the control-shRNA or BCL6-shRNA (h) Lentiviral Particles according to the manufacturer's instructions. The vector or BCL6-myc plasmid was transfected into MCF-7 cells and selected as previously described [24].

To knock down BCL6 expression, BCL6-siRNA or control siRNA (Ribobio, Guangzhou, China) was transfected into breast cancer cells with Lipofectamine® RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. BCL6 protein and mRNA levels were analyzed by Western blotting or Quantitative Real-time PCR (qRT-PCR) respectively.

Tissue microarray and immunohistochemistry

The breast cancer tissue microarray contains 89 cases of ductal or lobular breast carcinoma, and 19 cases of normal breast tissue specimens. The tissue microarray was detected by immunohistochemical analysis as previously described [24].

Western blotting and protein stability assay

Cellular protein was extracted with RIPA lysis buffer containing protease inhibitor and analyzed using BCA protein assay kit. SDS-PAGE and Western blotting were performed as described before [25].

Cycloheximide (CHX) is originally isolated from *Streptomyces griseus* and has been shown to block the elongation phase of eukaryotic translation through binding to the ribosome and inhibiting eEF2-mediated translocation. CHX has been widely used as a protein synthesis inhibitor to assess the stability of the existing proteins [26,27]. To assess protein stability, cells were treated with CHX (20 mmol/L) for the indicated times. Cell extracts were prepared and the expression levels of indicated proteins were detected with immunoblotting as described before [28].

Immunofluorescence microscopy

Breast cancer cells were plated on sterile coverlips and transfected with the expression plasmids or siRNAs as indicated for 48 hours. The expression of E-cadherin was stained and imaged as previously described [24].

Cell invasion assays

Cell invasion assays were performed using Millicell (8.0 μm, Millipore, Billerica, MA, USA). Millicells were precoated with 10 μg/mL fibronectin and Matrigel (1:8; BD Bioscience, Bedford, MA, USA), and then dried. Millicells were inserted into a 24-well plate containing culture medium with 10% FBS. Cells were starved overnight and then seeded on the upper chamber (1×10^5 cells per well in 0.4% FBS culture medium). After 24 hours, cells migrated were stained and counted as described before [24].

3D culture on Matrigel

The 24-well plate was coated with Matrigel (BD Bioscience, Bedford, MA, USA) and dried, then hydrated with the medium for 1 hour at 37 °C. The MCF-7 cells (5000 cells) or MDA231 cells (3000 cells) were cultured on Matrigel with indicated treatment. After 7 days incubation, colony formation was measured.

ChIP

ChIP assay was performed as described before [29,30]. In brief, cells were fixed in 1% formaldehyde for 10 min with shaking. Cells were lysed by repetitive freeze-thawing. Immunoprecipitation was then performed overnight at 4 °C with anti-RNA Polymerase, normal Mouse IgG, and ZEB1 or BCL6 antibody. The protein G agarose was added to collect the immunocomplexes. The beads were resuspended in elution buffer and incubated overnight at 65 °C, then DNA was extracted. DNA was purified using Spin Columns. Equal amounts of product and input were analyzed by qRT-PCR. Data are expressed as fold binding over background (using both input and negative control). The primers used to amplify specific regions of the indicated genes are shown in Table 1.

Table 1
Primer sequences used for qRT-PCR.

Gene name	Primer sequence
E-cadherin-F(ChIP)	AATTAGCCTGGCGTGGTGGTGTG
E-cadherin-R(ChIP)	GGGGTCTCACTCTTTCACCCAAGC
ZEB1-F(ChIP)	TATTCGAAGGAGGTGGGAAGCAGG
ZEB1-R(ChIP)	CGTGCAGGACCTTAAGGCAAGAAG
GAPDH-F	GACCTGCCGTCTAGAAAAACCTGC
GAPDH-R	TCGCTGTTGAAGTCAGAGGAGACC
E-cadherin-F	TCAGCCAAGATCCTGAGCTCCCT
E-cadherin-R	AGGTCAGCAGCTTGAACCACCAG
BCL6-F	GTCCTGCAGCAGTAAGAATGCCTG
BCL6-R	GGCTGTTGAGCACGATGAACTTGT
ZEB1-F	CCAGACAGTGTTACCAGGGAGGAG
ZEB1-R	TGCCCTTCCTTTCCTGTGTCATCC

Quantitative polymerase chain reaction

Total cellular RNA was extracted using the trizol reagent (TransGen Biotech, Beijing, China) according to manufacturer's instructions. The KAPA SYBR FAST qRT-PCR kit was used to detect mRNA levels of BCL6, GAPDH, E-cadherin and ZEB1 according to the manufacturer's instructions. The primer sequences that were used for qRT-PCR are shown in Table 1.

Statistical analysis

Data are shown as mean ± standard error of the mean (SD). Student's t-test was used for two-group comparisons. Comparisons between three or more groups were analyzed by one-way ANOVA followed by Duncan's test in SPSS 17.0 (SPSS Inc.). $P < 0.05$ was considered statistically significant.

Results

BCL6 positively correlates with the malignant property of breast cancers

To determine the pro-tumor role of BCL6 in breast cancers, we analyzed the breast cancer tissue microarrays with the correspondent clinical records. We found that the expression of BCL6 was much higher in the invasive breast cancer tissues as compared to the normal breast tissue specimens (Fig. 1A). Also, higher expression of BCL6 was found in the invasive breast cancer cell lines such as MDA231 cells in comparison to the noninvasive breast cancer cell lines such as MCF-7 cells (Fig. 1B). Interestingly, we found that the invasive ability and colony-forming ability were enhanced in the MCF-7-BCL6-myc cells in comparison to the MCF-7-vector cells while these abilities were reduced in the MDA231-BCL6-shRNA cells as compared with the MDA231-control-shRNA cells (Fig. 1C and E). Moreover, BCL6 inhibitor 79-6 suppressed these activities in both of MCF-7 and MDA231 cells (Fig. 1D and F). Indeed, the smooth border of the MCF-7-vector cells was changed to spike-like border in the MCF-7-BCL6-myc cells (Fig. 1D). However, depletion of BCL6 in MDA231 cells caused the cells from the spike-like border of cells to the smooth border in comparison to the wide type MDA231 cells (Fig. 1D). The spike-like borders of MCF-7 and MDA231 cells were changed to the smooth borders after the treatment of cells with BCL6 inhibitor 79-6 (Fig. 1F). These data suggest that the expressing levels of BCL6 positively determine the malignant property of breast cancers.