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Expression and Functions of the Repressor Element 1 (RE-1)-Silencing Transcription Factor (REST) in Breast Cancer

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ABSTRACT

The repressor element 1 (RE-1)-silencing transcription factor (REST), also known as the neuron-restrictive silencer factor (NRSF) or repressor binding to the X2 box (XBR), REST/NRSF/XBR, is originally discovered as a transcriptional repressor of a large number of primarily terminal neuronal differentiation genes in non-neuronal cells and neural stem cells (NSCs). Recently, the tumor-suppressor function of REST is finally proved. However, the expression profile and function of REST in breast cancer are not very clear. In this study, the expression of REST was detected in breast cancer tissue by immunohistochemistry. The results showed that REST expression was significantly lower in breast cancer samples compared to normal and benign breast samples ($P < 0.05$). Furthermore, the shRNA approach was used to investigate the function of REST in human breast cancer cells. Knocking down REST expression by shRNA in the human breast cancer MCF-7 cells resulted in an increase in cell proliferation, suppression in apoptosis, and reduced sensitivity to anticancer drug with a concurrent significantly up-regulated expression of Bcl-2. These data implied a significant role of REST in breast cancer. The reduced expression of REST might contribute to the breast cancer pathogenesis. J. Cell. Biochem. 110: 968–974, 2010.

KEY WORDS: REST; BREAST CANCER; PATHOGENESIS; CHEMOSENSITIVITY

Breast cancer is the most common cancer of women and the second leading cause of female cancer mortality in the western world [Jemal et al., 2008]. Nearly 200,000 women are diagnosed, and about 40,000 women died due to breast cancer every year in the United States [Ahmedin Jemal et al., 2009]. During early stages, breast cancer can be effectively treated with surgery and chemotherapy. And like most solid tumors, metastatic disease rather than the primary tumor itself is responsible for death [Welch et al., 2000; Yang et al., 2004]. The mortality rate of breast cancer could be lowered by identification of novel molecular markers of early breast cancer and understanding the molecular mechanisms of the early events of breast cancer.

The repressor element 1 (RE-1)-silencing transcription factor (REST), also known as the neuron-restrictive silencer factor (NRSF) or repressor binding to the X2 box (XBR), REST/NRSF/XBR, is originally discovered as a transcriptional repressor of a large number of primarily terminal neuronal differentiation genes in non-neuronal cells and neural stem cells (NSCs) [Chong et al., 1995; Schoenherr and Anderson, 1995]. The human zinc finger transcription factor REST gene encoding a 1,096-aminoacid protein is expressed at high levels in the non-neural tissues. It was suggested that REST is involved in the regulation of neural cell fate determination, and also in the repression of neuron-specific genes in differentiated non-neuronal cells. Most of the known biological processes regulated by REST involve the neuronal target genes [Ballas and Mandel, 2005; Ballas et al., 2005]. However, it likely controls other non-neuronal genes that may impact cell motility, angiogenesis, apoptosis, cell replication, and protein synthesis

Abbreviations used: REST, the repressor element 1 (RE-1)-silencing transcription factor; 5-Fu, 5-fluorouracil; Syn, synaptophysin.

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[Shimojo and Hersh, 2004; Coulson, 2005]. The tumor-suppressor function of REST is finally proved by an innovative study from Stephen Elledge's group [Westbrook et al., 2005]. While using an RNAi-based screen for tumor-suppressor genes in human mammary epithelial cells, they discover REST as one such gene. Blockade of REST function in the epithelial cells is found to cause a transformation phenotype, such as anchorage-independent growth. These observations suggest that REST plays important roles in breast cancer. In further support of a role in tumorigenesis, REST expression was recently shown to be absent in a subset of SCLC cells [Neumann et al., 2004]. And an excellent work from Magnus von Knebel Doeberitz's Laboratory showed that reconstitution of REST expression induced apoptosis in SCLC cells, suggesting that inhibition of REST activity is a crucial step in the carcinogenesis of subgroup of SCLC [Moss et al., 2009]. Also, REST gene was mutated in several primary colorectal cancer samples and established cell lines [Westbrook et al., 2005]. All of these indicated the possible role of REST in the development of the cancer.

In the present study, we showed aberrant expression of REST in breast cancer samples by immunohistochemical analysis. Additionally, we elucidated the functions of REST in breast cancer cell. RNA interference approach was used to knockdown REST expression in the human breast MCF-7 cell. Then the biologic consequences of knocking down REST expression in regard to cell proliferation, apoptosis, and cytotoxic response to 5-fluorouracil (5-Fu) were determined. These results showed the direct association of REST with breast cancer and that downregulation expression of REST influenced cellular characteristics.

MATERIALS AND METHODS

TISSUE SPECIMENS

Sixty-eight archived breast cancer samples and 32 non-neoplastic breast tissues were procured from Xiang Ya Hospital. The cancer patients had received neither chemotherapy nor radiation therapy before tumor resection. The utilization of archived cancer samples and non-neoplastic breast tissues used in this study was approved by our university Ethical Committee.

CELL CULTURE

MCF-7, MDA-MB-231, MDA-MB-435s MDA-MB-453, and MDA-MB-468 human breast cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. All cells were cultured at a constant temperature of 37°C in a 5% CO_2 humidified atmosphere.

IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

The slides were stained with anti-human REST antibody (ab21635, Abcam, Inc., Cambridge, MA) by heat-induced epitope retrieval immunohistochemistry technique using a Histostain-plus kit (Jiamay Biotech Co., Ltd) according to the manufacturer's instructions. The immunohistochemical reaction color was developed by treating the tissue sections with 3,3′-diaminobenzidine (DAB) substrate (DAB substrate kit). All slides were analyzed using an Olympus BX 41 Microscope (Olympus Corporation). The intensity of immunoreactivity of the REST was scored by a pathologist (Guoqing Pan).

Cells were grown on sterilized coverslips for 20 h and processed for immunofluorescence. Cells were fixed and blocked by 10% goat serum containing 0.05% Tween-20 for 30 min. Cells were incubated with the anti-REST antibody for 60 min, 3 × 5 min washed in PBS, and then incubated with FITC-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min, 3 × 5 min washed in PBS.

CONSTRUCTION OF SHRNA EXPRESSION VECTORS TARGETING REST (SHRNA–REST) AND CONTROL VECTOR: TRANSFECTION, SELECTION, AND DEVELOPMENT OF STABLE TRANSFECTANTS

Two homologous single-stranded DNA (ssDNA) molecules were chemically synthesized according to Ferrer et al. [Barrachina et al., 2007]. Annealing of the ssDNA molecules was cloned into the pGenesil vector (MCF-7/shREST). For controls, scrambled non-targeting sequences were used in pGenesil (MCF-7/Mock).

Cells were incubated overnight with a combination of shRNA–REST (4 μg) or control sequences as described above using Lipofectamine™ 2000 (Invitrogen Life Technologies, Frederick, MD) as the transfection reagent in accordance to instruction provided by the manufacturer. Stable transfectants were selected with G418 (800 μg/ml) for the shRNA–REST and control transfectants. Drug-resistant colonies were isolated using cloning rings, expanded, and screened by RT-PCR and Western blots to determine the level of REST expression.

SEMI-QUANTITATIVE REVERSE TRANSCRIPTION-PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen Life Technologies). The reverse transcription reaction was performed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences). The newly synthesized cDNA was amplified by PCR (REST primer: left 5′-gagcgagtatcactggagga-3′, right 5′-atggcttct-cacctgaatga-3′; GAPDH primer: left 5′-gtcagtggtggacctgacct-3′, right 5′-tgaggaggggagattcagtg-3′).

WESTERN BLOTTING

Cellular proteins were fractionated by SDS–PAGE and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane, blocked, and immunostained with primary antibodies. After three washes in TBST, the membranes were incubated with goat anti-rabbit/mouse secondary antibody, followed by enhanced chemiluminescence (KPL, Gaithersburg). α-Tubulin expression (using anti-human α-tubulin antibody (Santa Cruz Biotechnology))was used as internal controls for equal protein loading.

CELL PROLIFERATION ASSAY

Cells were seeded in 96-well plates at a density of 2,000 cells/well and cultured for 6, 24, 48, 72, and 96 h, respectively. Also, 20 μl of MTT (Sigma) stock solution (5 mg/ml) was added to 200 μl of medium in each well, and plates were incubated for an additional 4 h at 37°C, and subsequently, 150 μl of dimethyl sulfoxide (DMSO) was added to each well. The plate was shaken on a rotary platform for 10 min, and then the absorbance at wavelength 490 nm was measured by the use of a microplate reader.

SOFT AGAR GROWTH ASSAY

Anchorage-independent growth as a characteristic of in vitro tumorigenicity was assessed by soft agar clonogenic assay. Cells were detached and plated in 0.3% agarose with a 0.7% agarose underlay (4×10^3 cells per well in six-well plates). Colonies larger than 100 μm in diameter were counted after 20 days.

APOPTOSIS ASSAY

Detection of apoptosis by annexin V staining. The annexin V-fluorescein isothiocyanate (FITC)-labeled Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) was used to detect and quantify apoptosis by flow cytometry, according to the manufacturer's instructions.

STATISTICAL ANALYSIS

All experiments were performed at least three times, and the results were from representative experiments. The results were presented as means ± SD. Statistical analysis was performed using SPSS Version 10.0 for Windows. A paired *t*-test and χ^2 test were used to analyze the REST staining in breast cancer samples and functional assay data. *P*-values of <0.05 were considered significant.

RESULTS

IMMUNOSTAINING PROFILE OF REST IN NORMAL/BENIGN BREAST AND BREAST CANCER SAMPLES

The expression profile of REST in breast cancer was unknown. We examined the expression pattern of REST in normal/benign breast and breast cancer tissue samples by immunohistochemistry. REST expression was detectable in normal and benign breast samples, whereas breast cancer samples showed a significantly lower REST expression (Fig. 1). Low expression of REST in primary breast carcinoma was found in 35 (16 + 19) of 68 cases examined (51.5%). It was worth noting that REST protein was not detected in 30.9% (21/68) breast cancer samples (Table I).

REST EXPRESSION IN BREAST CANCER CELL LINES

In order to study the function of REST in breast cancer, we first examined the expression of REST in five different human breast carcinoma cell lines by RT-PCR and Western blot. As shown in Fig. 2A, the level of REST was varied in five different human breast carcinoma cell lines. It was strong in MCF-7 and MDA-MB-468 cell line, and relative low expression in MDA-MB-231 and MDA-MB-435s cells line. MCF-7 cell was selected for the further research.

GENERATION OF STABLE SHRNA-EXPRESSING BREAST CANCER CELL LINE

To further delineate the role of REST in cancer progression, MCF-7 cells were transfected with either REST-specific shRNA construct or a non-specific SCR shRNA construct (Mock). After G418 selection, RT-PCR, Western blot, and Immunofluorescence analysis confirmed that MCF-7/shREST clone showed lower level of REST compared to MCF-7/Mock and parental MCF-7 cells (Fig. 2B–D). We also found that the level of synaptophysin (Syn), the target gene of REST, was up-regulated when REST was shut down (Fig. 2C).

KNOCKDOWN OF REST SUPPRESSED THE APOPTOSIS OF BREAST CANCER CELL LINE MCF-7

Loss or reduced REST expression led to an increase in malignant cell behavior in terms of the propensity for anti-apoptosis (Fig. 3). The role of REST in the apoptosis of MCF-7 cells was assessed using Annexin V and PI staining followed by flow cytometry assay. As shown in Figure 3A, three groups of cells were induced apoptosis by treating with 2.5 mg/ml 5-Fu. The result indicated that the 5-Fu-induced apoptotic rate of MCF-7/shREST was obviously lower than that of MCF-7/Mock and parental MCF-7 cell. We then examined the effects of REST knockdown on caspase-7 activity. As shown in Figure 3B, REST depletion decreased the activity of caspase-7 when compared with parental cell and control cell. These data indicated that knockdown of REST expression attenuated the apoptosis in MCF-7 cells.

KNOCKDOWN OF REST ENHANCED THE PROLIFERATION OF MCF-7 CELLS

MTT assay was employed to examine the effect of reduced REST on MCF-7 cell proliferation. As shown in Figure 4A, MCF-7/shREST cells exhibited increase in proliferation, whereas the proliferation in MCF-7/Mock cells was unaffected when compared with the parental

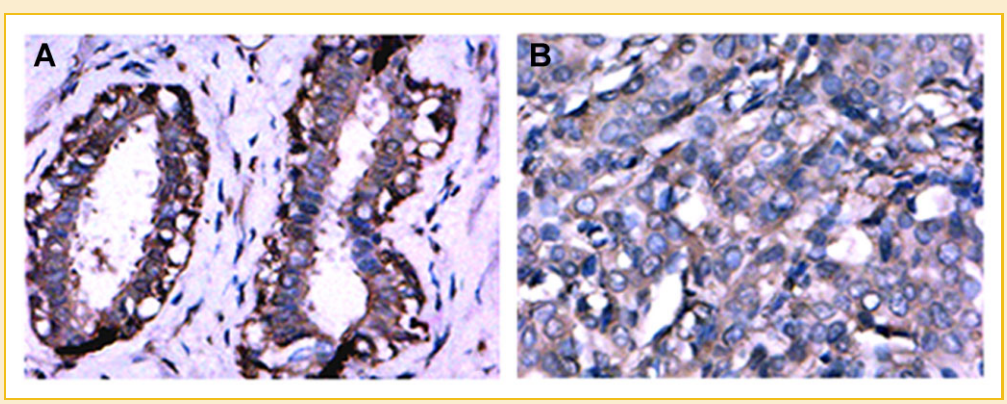

Fig. 1. Immunohistochemical staining of REST in human normal breast tissues and breast cancer. Representatives of staining. A: REST strong staining in normal breast tissue. B: REST weak staining in breast cancer. Original magnification, 200×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Expression of REST in Primary Breast Cancer

Tissue type	REST immunostaining					
	−	+	++	+++	% positive	*P*-value
Normal breast (10)	0	0	4	6	100	<0.05*
Benign tissues (n = 22)	0	3	7	12	100	
Breast cancer (68)	21	16	19	12	69.1	

Staining grades (semi-quantitative scale)—percentage of cells stained and degree of staining: negative (−), 0–5%; weakly positive (+), 6–25%; moderately positive (++), 26–50%; and strongly positive (+++), 51–100%.
*REST expression was significantly lower in breast cancer compared to normal, benign samples.

MCF-7 cells. And soft agar clonogenic assay showed the number of colony about MCF-7/shREST group was higher than MCF-7/Mock group (Fig. 4B). Furthermore, the expression of PI3K and *p*-Akt was detected by Western blot. As shown in Figure 4C, the expression of PI3K and *p*-Akt was increased in MCF-7/shREST cell compared with parental cell and MCF-7/Mock cell. These results indicated that the proliferation was enhanced when REST was shut down.

LOSS OF REST EXPRESSION RESULTED IN AN INCREASE IN BCL-2 EXPRESSION AND A REDUCTION IN CHEMOSENSITIVITY TO 5-FU

The MTT assay [Kawabata et al., 2001] was used to identify the chemosensitivity to 5-Fu of the two group cells. Cells were treated with 5-Fu at different concentrations for 48 h. MCF-7/shREST showed a slightly decreased chemosensitizing activity to 5-Fu compared to MCF-7/Mock (Fig. 5A). That means the sensitivity to 5-Fu was attenuated when REST expression was silenced. Because knocking down REST expression resulted in an increase in malignant cell behavior and the expression of Bcl-2 is associated with malignant progression and drug resistance [Alireza et al., 2008; Sekine et al., 2009], we hypothesized that knocking down REST expression would alter the expression of Bcl-2 and alter the cellular response to cytotoxic drug. Indeed, knocking down REST expression resulted in an increase in the expression of Bcl-2 (Fig. 5B) with a concurrent reduction in sensitivity to 5-Fu.

DISCUSSION

REST is expressed at high levels in the non-neural tissues. It is a zinc finger protein that binds to a conserved 23 bp motif known as RE-1 (also called NRSE) in a large number of genes encoding fundamental neuronal traits such as ion channels [Schoenherr and Anderson, 1995], synaptic vesicle proteins, and neurotransmitter receptors [Schoenherr et al., 1996]. REST not only maintains transcriptional silencing of a range of neuronal genes in differentiated non-neuronal cells but also has key roles during early lineage commitment in neurogenesis and in mediating transcriptional responses associated with neural plasticity. Furthermore, REST and its cofactors can alter chromatin structure and regulate transcription through histone deacetylation, chromatin remodeling, and methylation [Huang et al., 1999; Battaglioli et al., 2002; Lunyak et al., 2002]. Recently, REST was linked with specific cancers such as medulloblastoma, neuroblastoma, prostate cancer, and small cell lung cancer [Palm et al., 1999; Coulson et al., 2000; Lawinger et al.,

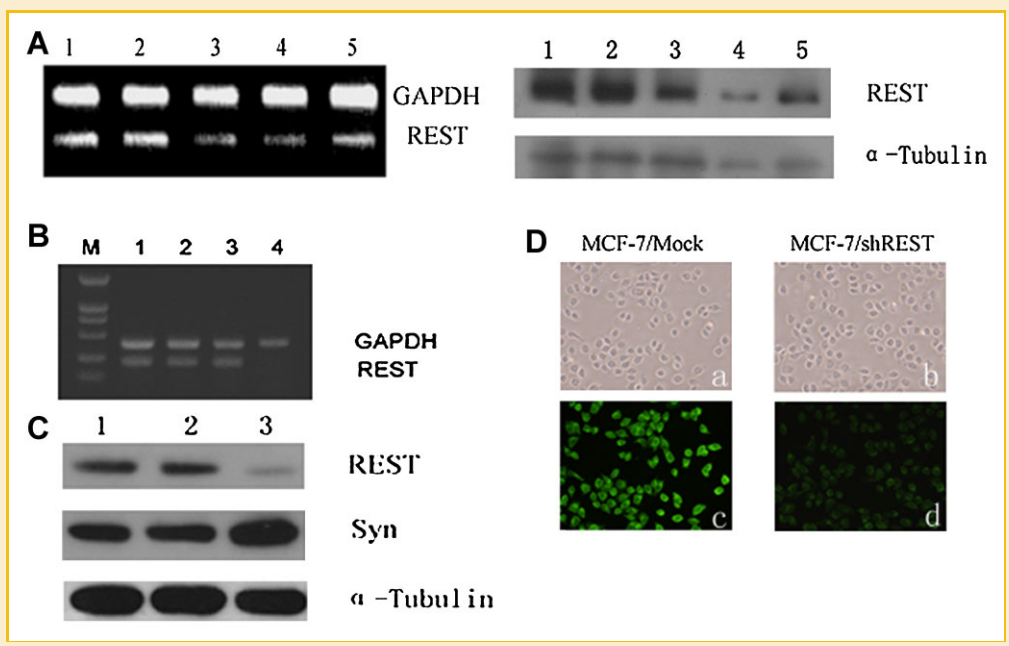

Fig. 2. Knocking down REST expression in MCF-7 cells. A: Representative result of RT-PCR and Western blot analysis for REST expression in breast cancer cell lines. The REST was differently expressed in five breast cancer cell lines examined. Lanes 1–5: MCF-7; MD-MBA-468; MD-MBA-453; MD-MBA-231; MD-MBA-435S. B: RT-PCR analysis. Lane 1: DL2000 marker; lanes 2–4: parental MCF-7 cells, vector plasmid-transfected cells, and control-transfected cells (Mock), respectively. Lane 5: shREST-transfected clone. GAPDH expression was used as internal controls. C: Western blot analysis. Lane 1: Parental MCF-7 cells; lanes 2: MCF-7/Mock cells; Lane 3: MCF-7/shREST cells. D: Immunofluorescence analysis. REST expression was obviously reduced in MCF-7/shREST cells (d) compared with MCF-7/Mock cells (c). Original magnification, 200×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

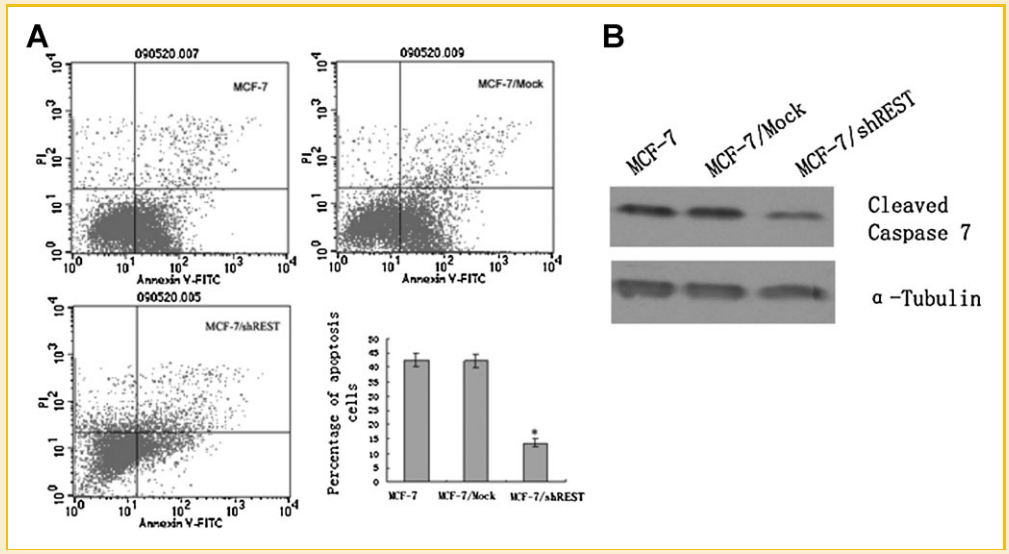

Fig. 3. Loss of REST expression suppressed apoptosis. A: Flow cytometry analysis. Representative images and statistical plots of flow cytometry analysis for apoptotic cells by Annexin V and PI staining. The percentage of apoptotic cells after treated with 5-Fu was significantly reduced in MCF-7/shREST cells compared to parental cells or MCF-7/Mock cells. $^{*}P < 0.01$ when compared with control cells. B: Downregulation of REST led to decreased activation of Caspase-7.

2000; Tawadros et al., 2005]. And now, a role in tumorigenesis seems more likely to be widely recognized. REST has emerged as one of the first tumor suppressors [Westbrook et al., 2005]. Nature has reported that REST is a key target in beta-TRCP-driven transformation [Westbrook et al., 2008]. More and more evidence have proofed the importance role of REST in pathogenesis of cancer. In this study, we investigated the overall expression profile of REST in breast cancer by immunohistochemistry. The result showed that REST expression in breast cancer was lower compared with normal breast tissue. It was worth noting that REST may take part in the development of breast cancer. However, how REST performs its functions in breast carcinogenesis remains to be determined. Also,

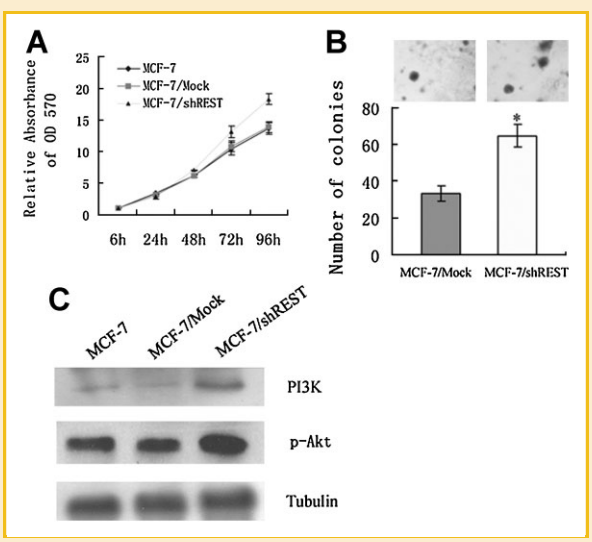

Fig. 4. Loss of REST expression promotes cellular proliferation. A: The growth curve revealed that downregulation of REST increased the proliferation rate of MCF-7 cells. B: Soft agar growth assay. The number of colonies was highly increased in MCF-7/shREST cells. $^{*}P < 0.05$ when compared with control cells. C: Downregulation of REST led to increased expression of PI3K and *p*-Akt.

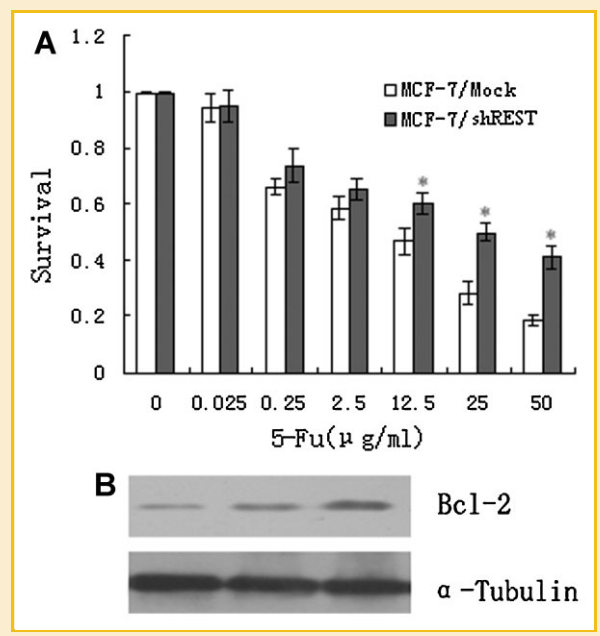

Fig. 5. Loss of REST expression results in a reduction in chemosensitivity to 5-Fu. A: Survival rate of MCF-7/shREST cells was higher than MCF-7/Mock cells. This indicated the sensitivity of MCF-7/shREST cells to 5-Fu was reduced compared to MCF-7/Mock cells. $^{*}P < 0.05$. B: Immunoblot assay. The expression of Bcl-2 in parental cells, MCF-7/Mock, and MCF-7/shREST cells were detected. The expression of Bcl-2 was increased in MCF-7/shREST cells, whereas the expression of REST reduced. α-Tubulin was used as the loading control.

the mechanism of the downregulation of REST in breast cancer will be investigated in the future.

To further investigate the function of REST in breast cancer, the expression of REST was detected in five breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-435s MDA-MB-453, and MDA-MB-468). The level of REST was relatively higher in MCF-7 cells and MDA-MB-468 cell lines, and it was relatively low in MDA-MB-231 and MDA-MB-435s cell lines. It was well known that MCF-7 cells and MDA-MB-468 cell were less malignant than MDA-MB-231 and MDA-MB-435s cell. It may indicate the role of REST in determining the biological behavior of cancer cells. REST-specific shRNA construct was transfected to MCF-7 cells to establish stable cell line. We found that shRNA-mediated REST knockdown significantly enhanced the proliferation and suppressed the apoptosis of MCF-7 cell. Furthermore, loss of REST expression resulted in a reduction in chemosensitivity to 5-Fu via an increase in Bcl-2 expression. These findings also shed light on the mechanistic basis of the multidrug resistance of breast cancer. However, future studies are necessary to be performed to determine whether REST regulates Bcl-2 expression either by direct interaction with its promoter or by an indirect pathway.

To our knowledge, Syn was used as the marker for neuroendocrine differentiation (NED). Breast cancer also displays some neuroendocrine features. However, the mechanism of it still remains to be discovered. In this line, our research confirmed that Syn was the target gene of REST. The result showed that downregulation of REST was associated with increased level of Syn (Fig. 2C). So the finding suggested that aberrant expression of REST may be involved in the NED of breast cancer. This would help us to understand the mechanism of NED and investigate its role in the development of tumor.

Recently, Reddy et al. [2009] have reported the role of REST in breast cancer. Some of our results were consistent with the results from their studies. We all revealed the possible function of REST in the development of breast cancer cell. They concentrated on the central role for REST in the oncogenic function of TAC1 and suggest a tumor-suppressor role for REST in breast cancer. We revealed that the tumor-suppressor role for REST has also including other mechanism, such as promote apoptosis through activation of caspase or inhibition of Bcl-2.

In conclusion, this work demonstrates the direct association of REST expression with breast cancer. It also sheds some light on the pathogenesis of breast carcinoma, and probably represents a new therapeutic target for breast carcinoma treatment.

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