PRECLINICAL STUDY

Tumor-specific RNAi targeting eIF4E suppresses tumor growth, induces apoptosis and enhances cisplatin cytotoxicity in human breast carcinoma cells

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Abstract *Background* Eukaryotic initiation factor eIF4E, an important regulator of translation, plays a crucial role in the malignant transformation, progression and chemoresistance of many human solid tumors. The overexpression of this gene has been found in a variety of human malignancies including breast carcinoma. In the present study, we attempted to explore the possibility of eIF4E as a therapeutic target for the treatment of human breast carcinoma using breast carcinoma cell line (MCF-7). *Materials and methods* The survivin promoter-driven eIF4E-shRNA vector was constructed on the basis of pSUPER.retro vector. Then, we established stably transfected MCF-7 and MCF210 (normal human mammary epithelial cell) cells expressing eIF4E-shRNAs or control-shRNAs. Firstly, the changes of eIF4E expression were detected by RT-PCR and Western blot assays. Next, the optimal shRNA vector (eIF4E-shRNA2) was selected to knock down eIF4E expression and investigate the effect of eIF4E-shRNA on eIF4E-regulated gene expression and cell proliferation both in vitro and in vivo. Followingly, the changes of cell cycle and apoptosis in the stably transfectants (MCF-7) were detected by flow cytometry and TUNEL methods, while we also explored possible apoptosis pathways. Finally, we investigated the effect of shRNA targeting eIF4E on the chemosensitivity of breast carcinoma cells to cisplatin in vitro and in vivo. *Results* Two survivin promoter-driven eIF4E-shRNA vectors were successfully constructed. eIF4E-shRNA2 but not eIF4E-shRNA1 efficiently downregulated the levels of eIF4E expression in the stably transfected MCF-7-s2 cells but not in the stably transfected MCF210-s2 cells, while MCF-7-s2 showed obvious proliferation suppression but MCF210-s2 did not. The downregulation of eIF4E expression significantly reduced the levels of VEGF, FGF-2 and cyclinD1 expression, suppressed cell growth, induced cell cycle arrest in G_0/G_1 phase and subsequent apoptosis by activating caspase 3 in MCF-7 cells. The results of FCM and TUNEL staining assays indicated that the classic apoptosis characters of the MCF-7 cells stably expressing eIF4E-shRNA2 manifested an apoptosis rate of 18.3%, significantly higher than those in the control groups ($P < 0.05$). Morever, we found that downregulation of c-IAP1, c-IAP2 and c-Myc but not Bcl-2 family proteins were involved in the apoptosis induced by eIF4E-shRNA2. In tumorigenicity assay, xenograft tumors developed from MCF-7-s2 cells in mice showed a significant slowdown in the growth speed and formation rate compared with control groups. Furthermore, we also testified that eIF4E-shRNA could synergistically enhance the cytotoxicity effects of cisplatin to MCF-7 cells both in vitro and in vivo. *Conclusion* Survivin promoter–driven RNA interference system could efficiently and specifically downregulate eIF4E expression in human breast carcinoma cells but not in normal human mammary epithelial cell cells. Thus, eIF4E might play an important role in chemosensitivity to cisplatin, and survivin promoter-driven RNAi targeting eIF4E can be used as adjuvant therapy for human breast carcinomas with tumor specificity and high efficacy.

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Introduction

Breast carcinoma is one of the most common malignancies affecting women around the world and one in seven women is predicted to develop invasive breast cancer during their lifetime [1]. Conventional chemotherapy regimens for the treatment of breast carcinoma have limited efficacy for their significant toxicity in the body [2]. To improve its horrible prognosis, we need to better understand the mechanism of breast carcinoma development and to find novel approaches to both diagnosis and treatment that are far more efficient than currently available techniques [3]. Thus, an understanding of the molecular mechanisms involved in breast carcinoma formation and progression would be helpful to develop more effective treatments for breast carcinoma.

Eukaryotic initiation factor 4E (eIF4E) is a 25-kDa cap binding protein that plays an important role in the initiation of protein translation. It is the limiting component of a complex including several members of the eIF4 group of eukaryotic protein synthesis initiation factors, which usually regulates cap-dependent protein synthesis by binding to the m7GpppX cap of mRNA and regulating the recruitment of mRNA to ribosomes [4–7]. The most frequently aberrant change in the translational apparatus is the upregulated levels of eIF4E expression, which selectively affects transport of specific transcripts, increases cap-dependent translation, suppresses apoptosis and induces malignant transformation [8–10]. Several research groups have reported that the overexpression of eIF4E gene is associated with tumor angiogenesis in breast cancer and the degree of eIF4E overexpression predicts cancer recurrence and outcome in stage I to III breast cancer patients [11, 12]. Morever, the eIF4E overexpression has been found in many other types of human malignancies, such as head and neck squamous carcinoma, colon carcinoma, bladder carcinoma, cervix carcinoma and lung cancer, suggesting that the upregulated level of eIF4E expression is associated with malignant transformation, tumorigenesis and metastasis of tumors [13–17]. It also has been reported that the upregulated levels of eIF4E expression greatly increase translation of some mRNAs encoding proteins contributing to angiogenesis and proliferation such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-2) and cyclin D1 [18, 19]. VEGF and FGF-2 are considered to be two important stimulators of migration and angiogenesis, which are related to the progression and metastasis of breast carcinoma. Additionally, the elevated level of cyclin D1 expression has been found to be involved in cisplatin chemoresistance of many tumor cells, including breast cancer [20]. Therefore, we hypothesized that eIF4E would be a good molecular target for breast carcinoma therapy. Other researchers reported that anti-sense or small interfering RNA (siRNA) targeting eIF4E or cyclin D1 could suppress oncogenic properties and enhance chemosensitivity to cisplatin in head and neck carcinoma cells [21, 22], but the use of this target via downregulating eIF4E expression in the treatment of human cancers still needs to find a method with high efficacy and tumor specificity, which can effectively inhibit eIF4E gene expression in tumor cells but can not affect the eIF4E activity in normal cells.

RNA interference (RNAi) is a post-transcriptional mechanism of gene silencing through chromatin remodeling, inhibition of protein translation or direct mRNA degradation. This technology is currently the most widely utilized technique in functional genomic studies and therapeutic gene regulation [23]. Specific gene silencing can be achieved in a variety of cell systems using an in vitro synthesized small interfering RNA (siRNA) or DNA vector-based shRNA [24]. siRNA-mediated silencing effect is transient, but DNA vector-based shRNA could achieve a stable supply of anti-cancer siRNA for gene therapy. To achieve a tumor-specific silencing of targeted gene, we explored the possibility of a tumor-specific promoter to drive shRNA expression. Survivin, a novel member of the inhibitors of apoptosis (IAP) protein family, is highly active in human cancers but is inactive in most somatic cells [25]. Recently, the survivin gene has been cloned by several independent groups and it has been found to be expressed at high levels in primary tumors and cancer cell lines but repressed in most somatic tissues [26]. The promoter of the suvivin gene has been subcloned by us, recently [27]. Deletion analysis of the survivin promoter identified a core promoter region of ~980 bp upstream of the transcription start site. Transient expression assays revealed that the core promoter is significantly activated in tumor cells but is repressed in normal primary cells [28]. Thus, we want to address whether the survivin promoter may be used for tumor-specific gene silencing.

In the present report, we initially developed a novel tumor-specific RNAi system targeting eIF4E directly under the control of survivin promoter. Following, we performed Western blot analysis on a panel of four human breast carcinoma cell lines, revealing eIF4E overexpression in all four breast carcinoma cells in comparison with normal human mammary epithelial cell line, MCF210. Furthermore, we established stably transfectants (eIF4E-shRNA2) which displayed minimal levels of eIF4E expression in MCF-7 cells but showed no obvious changes in MCF210 cells. In those stably transfected MCF-7-s2 cells, we detected the reduced expression of growth-related

genes such as VEGF, bFGF and cyclinD1, while we also observed that those stably transfected cells showed a significant enhancement in chemosensitivity to cisplatin besides a marked proliferation inhibition and apoptosis induction. Taken together, all these experimental data provided evidences of the potential use of survivin promoter-driven RNAi targeting eIF4E as adjuvant therapy for human breast carcinoma.

Materials and methods

Cell lines and culture conditions

A normal human mammary epithelial cell line (MCF210) and four human breast cancer cells (T47D, Bcap37, SKBR-3, MCF-7) were purchased from Cell Culture Center, Fourth Military Medicine University. All cells were cultured in RPIM-1640 medium (Gibico, USA) supplemented with 10% fetal calf serum and 1% penicillin–streptomycin. All cell lines were maintained at 37°C with 5% CO_2.

Plasmids construction

The pSUPER.retro vector was provided from OligoEngine (Seattle, WA, USA). The survivin promoter gene (GenBank NM U75285) including 980 bp upstream from the transcription initiation site was amplified from our previously obtained survivin promoter plasmid by polymerase chain reaction (PCR). The PCR product was then subcoloned into the *Bgl*II and *Eco*RI site of pSUPER.retro vector to replace H1-RNA promoter. A minimal poly (A) sequence (5′-*TTATTTCCTAGAAAATAAAAGTAACCTAGACACAC AACCAAAAAACATACGCCGGCGA*-3′) was used as a terminal sequence. DNA template oligonucleotides corresponding to eIF4E gene (GenBank NM BC035166) 1711–1729 bp and 2021–2039 bp were designed and synthesized as follows: eIF4E–shRNA1 (sense, 5′-*GATCCGCG GCTGATCTCCAAGTTTTTCAAGAGAAAACTTGGAGAT CAGCCGCAGA*-3′), eIF4E-shRNA2 (sense, 5′-*GATCT GCAATATGGACTACGAATGTTCAAGAGACATTCGTAG TCCATATTGCAGA*-3′) and a non-specific shRNA, NS (sense, 5′-*GATCTAAGCATTAGGTACAGCATTTCAAGA GAATGCTGTACCTAATGCTTAGA*-3′). All of the above sequences were inserted into the *Bgl*II and *Hind*III enzyme sites of pSUPER.retro vector, respectively. Those recombinant plasmids were named pSUPER-SP-eIF4EshRNA1, pSUPER-SP-eIF4EshRNA2, and pSUPER-SP-NS vector, respectively (Fig. 1). Those recombinant vectors were confirmed by the digestion analysis of restriction endonuclease and all inserted sequences were verified by DNA sequencing.

Transfection and selection of stable transfectants

Human breast carcinoma cells (MCF-7) and normal human mammary epithelial cells (MCF210) were seeded at 70–80% confluence in 6-well plates, respectively. Then, the cells were transfected with those recombinant pSUPER vectors containing a puromycin selection marker using LipofectAMINE Plus (Grand Island, NY) according to the manufacturer's protocols and were selected with 8 μg/ml of puromycin (Sigma, USA) for 14 days. Single clones were isolated and expanded for an additional two months in media containing 3μg/ml of puromycin. Name of stable transfectants were MCF-7-s1 or MCF210-s1(transfected with pSUPER-SP-eIF4EshRNA1), MCF-7-s2 or MCF210-s2 (transfected with pSUPER-SP-eIF4EshRNA2), MCF-7-NS or MCF210-NS (transfected with pSUPER-SP-NS), and MCF-7-pS.retro or MCF210-pS.retro (transfected with parental vector pSUPER.retro), respectively.

RT-PCR for eIF4E mRNA

Total RNA was extracted from untransfected or stably transfected MCF-7 cells and MCF210 cells using TRIzol reagent (Invitrogen, USA), respectively. RNA of 2 μl (1 μg/μl) was used to synthesize cDNA using Superscript First-Strand Synthesis Kit (Promega, USA) following the manufacturer's protocols. The cDNA was used to amplify the eIF4E gene, while the house keeping gene β-actin was also amplified as an internal standard. The corresponding primer sequences were as follows: eIF4E, forward: 5′-*ATG GCGACTGTCGAACCGG*-3′, reverse: 5′-*GCTATCTTAT CACCTTTAGC*-3′; β-actin, forward: 5′-*AGCAACCGGGAG CTGGTGG*-3′, reverse: 5′-*CATTTCCGACTGAAGAGTG*-3′. The cycling program was performed as follows: 1cycle of 94°C for 3 min; 35 cycles of 94°C for 40 s, 60°C for 40 s, 72°C for 90 s; followed by a final elongation step of 72°C for 10 min. Then, RT-PCR products were electrophoresed through a 1.5% agarose gel with ethidium bromide. Signals were quantified by densitometric analysis using the Labworks Image Acquisition (UVP, Inc., Upland, CA). The inhibitory rate of eIF4E mRNA expression was calculated as follows: inhibitory rate = [1 − (MCF-7-s1 or s2 eIF4E density/MCF-7-s1 or s2 β-actin density)/ (MCF-7 eIF4E density/MCF-7 β-actin density)] × 100%.

Western blot analysis

Western blot analysis was performed using a previously described procedure [29]. Those untransfected and stably transfected MCF-7 or MCF210 cells were collected and lysed (50 mmol/l of Tris–HCl, 150 mmol/l of NaCl, 50 mmol/l of EDTA, 1% NP-40, 0.5 mmol/l of PMSF, and 2 mg/ml of pepstatin A) on ice, respectively. Anti-eIF4E,
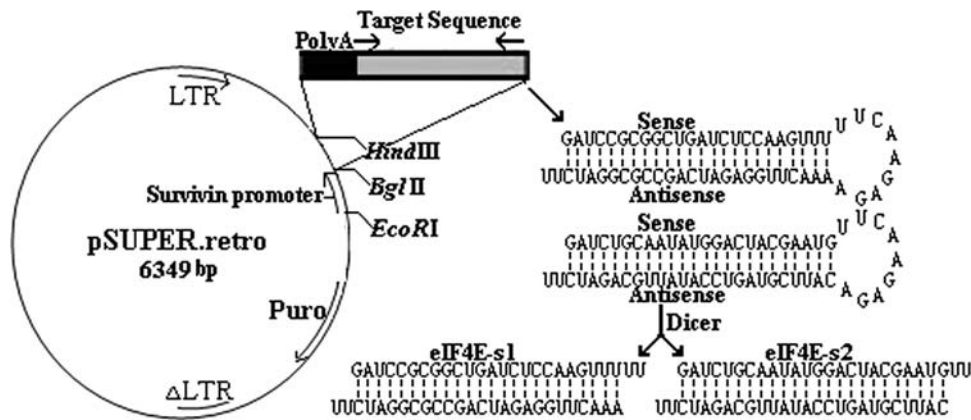
Fig. 1 Schematic diagram of the recombinant pSUPER.retro vector. The H1-RNA promoter was replaced by survivin promoter and shRNA encoding template was inserted between *Hin*dIII and *Bgl*II enzyme sites downstream of the survivin promoter and PolyA as a termination signal. The predicted secondary structure of the pSUPER-SP-shRNA targeting eIF4E is shown. The transcript, a short hairpin double-stranded RNA (dsRNA), can be further cleaved by Dicer to generate a 21-nucleotide siRNA binding and destroying eIF4E mRNA

anti-survivin, anti-VEGF, anti-FGF-2, anti-cyclinD1, anti-cleaved caspase 3, anti-c-IAP1, anti-c-IAP2, anti-c-Myc, anti-Bcl-2, anti-Bcl-xl, anti-Bad, anti-pBad and anti-actin (Santa Cruz Biotechnology, USA) antibodies were used for Western blot assay. Signals were detected by enhanced chemiluminescence (ECL; Amersham). The levels of eIF4E protein expression were determined densitometrically and normalized to β-actin. The inhibitory rate of eIF4E protein expression was calculated according to above mentioned formula. The cells that had higher inhibitory rate of eIF4E expression were harvested and used for further assays.

In vitro cell proliferation assay

Cell viability was measured by a 3-(4,5-dimethythazolz-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Four kinds of MCF-7 and MCF210 cells (cells without transfection, cells stably transfected with parental vector, non-specific shRNA or eIF4E–shRNA2), at 1.0×10^4 per well, were seeded into seven 96-well culture plates, with each plate having all four kinds of cells and each group consisted of 12 parallel wells. Every day, we selected one plate from those plates at random for MTT assay. Every well was added 10 µl MTT(5 mg/ml) to each well, incubated for 4 h, 100 µl DMSO was added, and lysed for 15 min. After 24-h cultivation, optical densities of one of seven culture plates were determined on a microreader (Bio-Rad Co.) at 490 nm. The inhibitory rate of cell growth was calculated as: inhibitory rate = $(1 - A_{\text{treated group}}/A_{\text{untreated group}}) \times 100\%$. All experiments were performed three times independently.

In vitro colony formation assay

For the colony formation assay, approximately 1.0×10^3 untransfected or stably transfected MCF-7 or MCF-210 cells were plated in 10-cm culture dishes, respectively. During this period, we fed them every 2–3 days with complete medium. After 18-day incubation, cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted. All experiments were performed in triplicate.

Analysis of cell cycle

Approximately 1.0×10^4 cells of each group (untransfected and stably transfected MCF-7 cells) were seeded into 100 ml flasks and incubated until there was 80–85% confluence in complete medium. Then, the cells were harvested, washed with ice-colded PBS twice, fixed with 70% ethanol overnight at 4°C, washed and resuspended in 100 ml of PBS containing final concentrations of 50 mg/ml RNase A for 30 min at room temperature. Finally, the fixed cells stained with propidium iodide (PI). PI-stained cells were analyzed by EPICS-ELITE-ESP Flow Cytometry (Coulter Biosciences). All of the samples were assayed three times, and the fraction of cell cycle distribution was calculated.

Flow cytometry for analysis of apoptotic cells

The untransfected or stably transfected MCF-7 cells were harvested, washed with ice-colded PBS twice, and resuspended in binding buffer (10 mM of Hepes, pH 7.4, 150 mM of NaCl, 2.5 mM of CaCl$_2$, 1 mM of MgCl$_2$, 4% bovine serum albumin). Using Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Oncogene Research Products, Boston, MA), cells were stained with Annexin V-fluorescein isothiocyanate and propidium iodide following the manufacturer's protocols. After a 15-min incubation in the dark at room temperature, stained cells were immediately analyzed on an Epics XL-MCL flow cytometer (Beckman Coulter, USA). All of

the samples were assayed in triplicate, and the cell apoptosis rate calculated using the following formula: apoptosis rate = $(N_{apoptotic\ cell}/N_{total\ cell}) \times 100\%$.

TdT-mediated dUTP nick end labeling (TUNEL) assay

MCF-7 cells stably transfected with siRNA duplex for 48 h, and then with adriamycin for 24 h; the effects on cell apoptosis were examined by TUNEL assay. Cells were fixed in 4% paraformaldehyde (pH 7.4), then stained and analyzed for apoptosis using an In Situ Cell Death Detection Kit, Fluorescein (Nanjing Keygen Biotech. Co., Ltd). Fixed cells were permeabilized using a mixture containing 0.1% sodium citrate and 0.1% TritonX100 and then incubated with TUNEL reaction mixture containing terminal deoxynucleotidyltransferase and fluorescein-dUTP at 37°C for 60 min.

Measurement of caspase-3 activity

Caspase-3 activity was measured using caspase colorimetric assay kit (Sigma, USA), according to the manufacturer's instructions. In brief, cells were scraped into PBS, pelleted at low speed, and resuspended in lysis buffer for 10 min at 4°C. Cell lysates were cleared by concentration and assayed for caspase-3 activity using a DEVDpNA peptide substrate and incubated for 6 h at 37°C. The activities were quantified spectrophotometrically at a wavelength of 405 nm. Caspase activity was calculated as the change in absorbance at 405 nm and divided by total protein concentration.

Murine Xenograft Model for tumorigenicity assay

The effect of eIF4E on tumorigenicity was assessed by subcutaneous injection of stably transfected MCF-7 cells into athymic nude mice. Each aliquot of 5.0×10^6 cells was injected into the back of BALB/c nude mice (Nu/Nu, female, 6–7 weeks old), which were maintained under pathogen-free conditions. The formation of subcutaneous tumors was monitored and measured with a digital caliper. The tumor volume formed was calculated by the following formula: $V = 0.4 \times D \times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter). At 42 days after inoculation, all mice were sacrificed, and s.c tumors were resected and fixed in 10%PBS. Animal experiments in this study were carried out in accordance with medicine institutional guidelines of Fourth Military Medical University.

In vitro chemosensitivity assay

In vitro, MCF-7 cell chemosensitivity to cisplatin (Sigma, USA) was evaluated by MTT analysis and apoptosis detection assay. Firstly, the IC_{50} of cisplatin to MCF-7 cells was examined by MTT assay according to the previously described methods. Then, the untransfected or stably transfected MCF-7 cells in the 96-wells were treated with various concentrations of cisplatin at 0, 2.0, 4.0, 8.0, 16.0, and 32.0μg/ml, respectively. After 48-h incubation, the cell viability and cell apoptosis was detected according to methods described above. The cell survival value index was calculated as: Survivial index = $[A_{490(5\text{-}FU}+) / A_{490(5\text{-}FU}-)] \times 100\%$

In vivo chemosensitivity to cisplatin in nude mice

Xenograft tumors were developed as above. In brief, suspensions of 2.0×10^6 cells (MCF-7-NS and MCF-7-s2) in N.S (50 μl) were injected s.c. in to the back of BALB/c nude mice (Nu/Nu, female, 6–7 weeks old) at day 0. The inoculation was conducted in mice (10/group), and mice were treated with cisplatin (5.0 mg/kg; thrice weekly) or with 0.1 ml N.S (PH 7.4; thrice weekly) as described previously with modifications. Tumor growth was calculated from the average volume of tumors, and tumor volume was calculated as already described. At 35 days after inoculation, all mice were sacrificed, and s.c tumors were resected and fixed in 10%PBS. Animal experiments in this study were carried out in accordance with medicine institutional guidelines of Fourth Military Medical University.

Statistical analysis

Results are expressed as Means ± standard deviation (SD). Statistical analyses were performed using SPSS statistical software. Student's t-test and one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests were adopted. Values of $P < 0.05$ were considered as significant and indicated by asterisks in the figures.

Results

The eIF4E and survivin expression in a panel of breast carcinoma cells and a normal mammary epithelial cell line

The levels of eIF4E and survivin expression in breast cancer cell lines were determined by comparing the levels of survivin protein and eIF4E protein in a panel of human breast carcinoma cells (T47 D, Bcap37, SKBR-3, MCF-7) to that in a normal mammary epithelial cell (MCF210) by Western blot analysis. After normalization to β-actin, the levels of eIF4E protein expression in the breast cancer cell lines varied from 3.5-fold (T47D) to 6.5-fold (MCF-7).
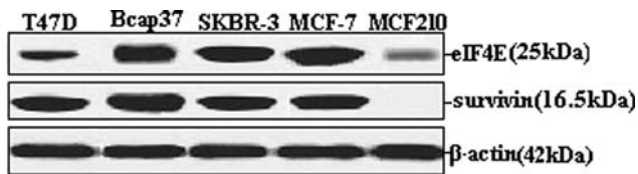
Fig. 2 Western blot analysis comparing eIF4E and survivin expression in various human breast carcinoma cells to MCF210, a normal mammary epithelial cell. The levels of eIF4E protein expression in four tumor cell lines varied from 3.5-fold (T47D) to 6.5-fold (MCF-7) compared to that in normal mammary cells. The high-level expression of survivin protein was detected in four tumor cell lines but not in normal cells. β-actin was used to normalize for any differences in protein loading between lanes

These values were obtained by comparing the normalized level of eIF4E in MCF210 cells to that of eIF4E in other breast carcinoma cells (Fig. 2). Considering higher levels of eIF4E expression in MCF-7 cells, so we selected MCF-7 cell line for following assays. We also detected the levels of survivin protein expression in those cells, as shown in Fig. 2, survivin was expressed at high levels in four breast carcinoma cells but not detected in normal mammary epithelial cell. All these data provided a basis for the survivin promoter-driven shRNA to silence eIF4E gene in breast carcinoma with tumor specificity and high efficacy.

Tumor-specific downregulation of eIF4E expression by survivin promoter-driven shRNA

To exclude off-target silencing effect mediated by specific-shRNA, we employed two different sequences of eIF4E-shRNAs in the present study. In order to evaluate the downregulation of eIF4E mRNA and protein expression, RT-PCR and Western blot analyses were performed. As shown in Fig. 3, the levels of eIF4E mRNA and protein expression in the MCF-7-s2 cells were significantly reduced in comparison with those in the untransfected MCF-7 cells, and the inhibitory rates were 68.5% and 59.6% ($P < 0.05$), respectively; However, the levels of eIF4E mRNA and protein expression in MCF-7-s1, MCF-7-NS and MCF-7-pS.retro cells showed no obvious difference ($P > 0.05$). In addition, we observed that there was no difference among MCF210-s1, MCF210-s2, MCF210-NS, MCF210-pS.retro and untransfected MCF210 cells ($P > 0.05$, Fig. 4). Morever, no effects of RNAi were observed on the expression of β-actin used as an internal control. All these results indicated that the survivin promoter–driven RNA interference system could specifically downregulate eIF4E expression in breast carcinoma cells but not in normal mammary epithelial cells. Following, we chose pSUPER-SP-eIF4EshRNA2 for further assays.
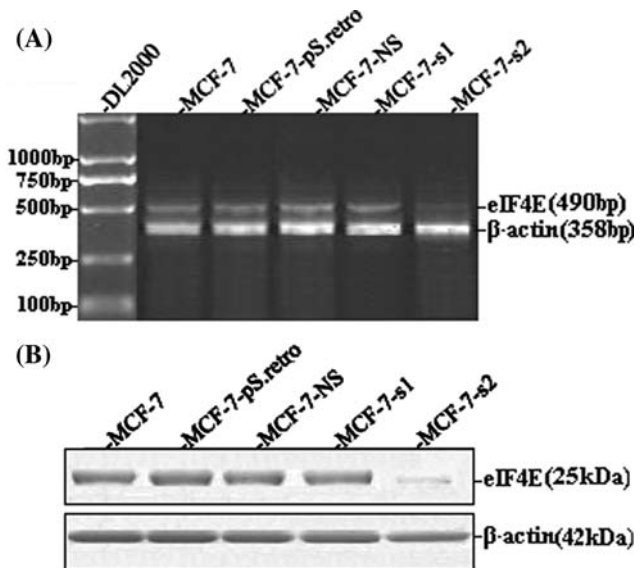

Fig. 3 (**a**) eIF4E mRNA and protein expression in MCF-7 cells detected by semi-quantitative RT-PCR and (**b**) Western blot analysis. When compared to untransfected MCF-7 cells, the eIF4E mRNA and protein expression in MCF-7-s2 obviously reduced by 68.5% and 59.6%, respectively ($P < 0.05$), but the eIF4E mRNA and protein expression in MCF-7-pS.retro, MCF-7-NS and MCF-7-s1 cells showed no difference ($P > 0.05$). β-actin was considered as an internal normalization standard. Marker: DL2000

Effects of eIF4E-shRNA on expression of other eIF4E-regulated genes

It has been reported that the overexpression of eIF4E gene might induce the upregulated expression of angiogenesis and growth-related genes such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-2) and cyclinD1. Thus, we want to determine the effects of specific-shRNA targeting eIF4E on the levels of these proteins. As shown in Fig. 5, when compared to untransfected MCF-7 cells, the expression of VEGF, four FGF-2 isoforms and cyclinD1 proteins in the eIF4E-downregulated MCF-7-s2 cells significantly decreased by 73.4%, 58.6% and 83.8%, respectively ($P < 0.05$). However, there was no difference in the protein expression level among MCF-7, MCF-7-pS.retro and MCF-7-NS cells ($P > 0.05$).

eIF4E-shRNA significantly and specifically inhibited in vitro cell growth

In order to determine the phenotypical changes, we first investigated the effect of RNAi-mediated downregulation of eIF4E expression on MCF-7 and MCF210 cells proliferation in vitro. In this experiment, cellular proliferation was monitored by MTT assay daily for 7 days. The cell growth curve showed that compared with untransfected MCF-7 cells, MCF-7-s2 cell proliferation was significantly inhibited in a
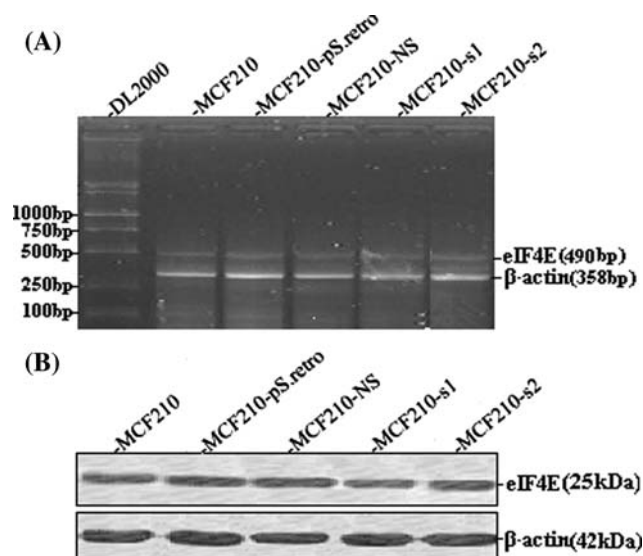
Fig. 4 (**a**) eIF4E mRNA and protein expression in MCF210 cells detected by semi-quantitative RT-PCR and (**b**) Western blot analysis. The eIF4E mRNA and protein expression showed no obvious difference among MCF210, MCF210-pS.retro, MCF210-NS, MCF210-s1 and MCF210-s2 cells. The results showed that survivin promoter-driven shRNA targeting eIF4E had no effect on the eIF4E mRNA and protein expression in normal MCF210 cells. β-actin was considered as an internal normalization standard. Marker: DL2000
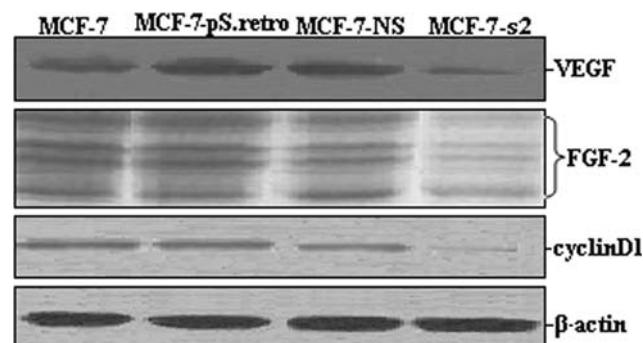

Fig. 5 Effects of eIF4E-shRNA on VEGF, FGF-2 and cyclinD1 protein expression in MCF-7 cells. The stably transfected MCF-7 cells were harvested and analyzed for the expression of VEGF, FGF-2 and cyclinD1 by Western blot analysis. The same blots were reacted with anti-β-actin antibody as loading control. When compared to untransfected MCF-7 cells, the expression of VEGF, FGF-2 and cyclinD1 proteins in MCF-7-s2 significantly decreased by 73.4%, 58.6% and 83.8%, respectively ($P < 0.05$), but the levels of those proteins expression in MCF-7-pS.retro and MCF-7-NS cells showed no significant difference ($P > 0.05$)

time-dependent manner and the highest inhibitory rate was $48.2 \pm 1.6\%$ on day 7 ($F = 873.216, P < 0.01$), while MCF-7-NS and MCF-7-pS.retro cells showed no obvious proliferation inhibition ($P > 0.05$; Fig. 6a). In those MCF210 cell models, we found that eIF4E-shRNA2 could not induce proliferation inhibition in MCF210-s2 cells ($P > 0.05$; Fig. 6b). All these further testified the tumor specificity of survivin promoter–driven RNA interference system.
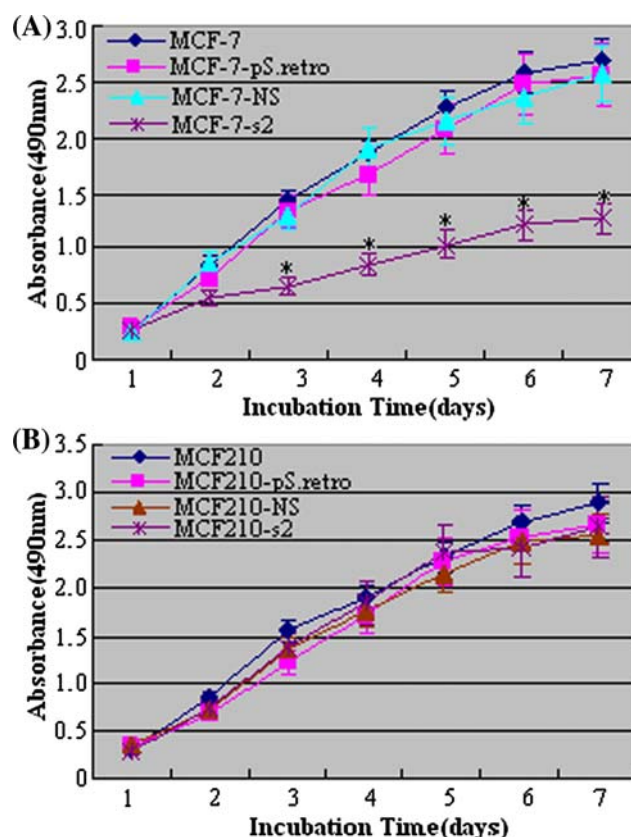
Fig. 6 Survivin promoter-driven eIF4E-shRNA specifically suppressed MCF-7 cell proliferation but not MCF210 cell proliferation. The cell proliferation of untransfected or stably transfected MCF-7 and MCF210 cells was measured by MTT assay. (**a**) The cell proliferation of MCF-7-s2 was obviously inhibited in a time-dependent manner, and the highest inhibitory rate was $48.2 \pm 1.6\%$ on day 7 ($F = 873.216, P < 0.01$); (**b**) The cell proliferation of MCF210-s2 (the highest inhibitory $= 8.65\%$ on day 7) showed no obvious changes compared to control cells ($F = 26.835, P > 0.05$). Data shown are the mean results $\pm$ SD of a representative experiment performed in triplicate. $* P < 0.01$

eIF4E-shRNA showed obviously reduced colony formation potential in vitro

To investigate whether the potential of shRNA targeting eIF4E mentioned above might result in suppression of tumor cell colony formation in vitro, we plated untransfected or stably transfected MCF-7 and MCF210 cells in vitro, respectively. Colony formation assay was assayed 18 days after plated. The mean values $\pm$ SD from three determinations are shown in Fig. 7. As expected from the results of MTT assay, the colony numbers obtained from MCF-7-s2 cells (averaged number $= 342$) were much lower than those obtained from MCF-7-NS, MCF-7-pS.retro and MCF-7 cells (averaged number $= 788, 776$ and 812; $P < 0.05$, Fig. 7a). However, as shown in Fig. 7b, there were no obvious differences among colony numbers obtained from MCF210, MCF210-pS.retro, MCF210-NS,
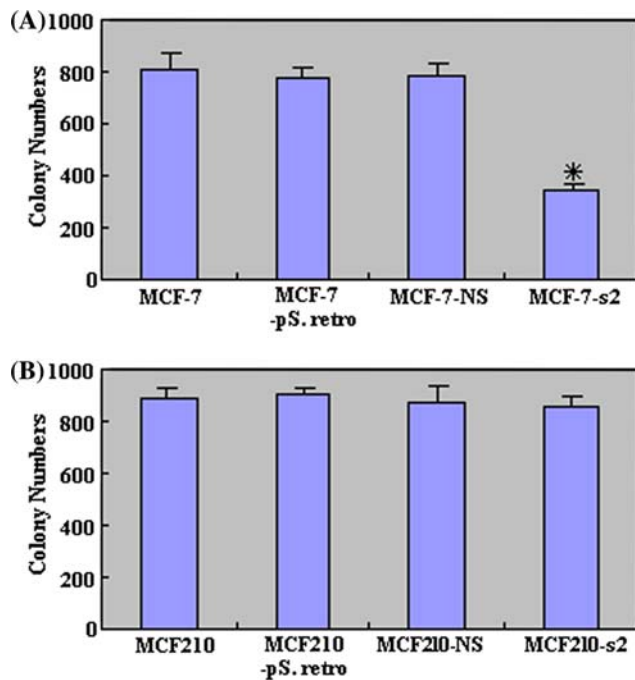
Fig. 7 Effects of eIF4E-shRNA on colony formation of MCF-7 cells in vitro. The untransfected or stably transfected MCF-7 and MCF210 cells were seeded onto six-well culture plates at a concentration of 3.0×10^2 cells/well and cultured for 17 days. The means ± SD of colony number was counted in three different wells at 18 days after seeded, and the averaged number was plotted. The number of colonies in the MCF-7-s2 cell group (averaged colony number = 342) was significantly reduced ($P < 0.05$) compared with that seen in the other cell groups. The experiments were repeated thrice and similar results were obtained. Representative data are shown, * $P < 0.05$
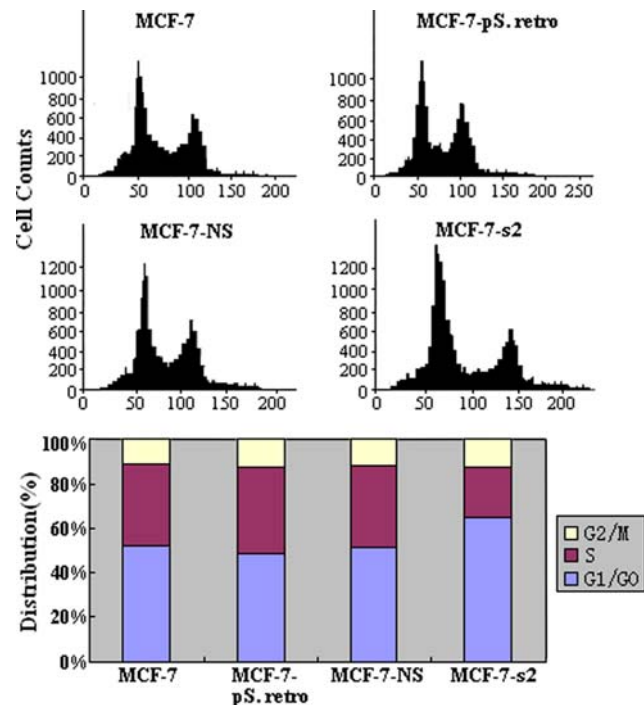

Fig. 8 Effects of eIF4E-shRNA on the cell cycle progression. The MCF-7-s2 and other control cells were fixed, stained and analyzed for PI fluorescence intensity by flow cytometry. Cell cycle distributions of the control and shRNA-transfected cells were determined from the flow cytometry data. It was shown that the percentage of MCF-7-s2 cells (G_1/G_0 phase) increased by 14.7 ± 1.1%, while the percentage of MCF-7-s2 cells (S phase) reduced by 13.2 ± 1.6% ($P < 0.05$)

and MCF210-s2 cells (averaged number = 886, 902, 874 and 856; $P > 0.05$).

The effect of eIF4E-shRNA on the cell cycle of MCF-7 cells

To elucidate the mechanisms underlying the siRNA-mediated growth inhibition, we performed cell cycle analysis to examine the effects of eIF4E-shRNA on the cell cycle of MCF-7 cells and each assay was performed three times. The mean values of triplicate experiments were shown in Fig. 8. Compared with that of untransfected MCF-7, MCF-7-pS.retro and MCF-7-NS cells, the percentage of MCF-7-s2 cells in the G_1/G_0 phase obviously increased by 14.7 ± 1.1%, while the percentage of MCF-7-s2 cells in S phase significantly reduced by 13.2 ± 1.6% ($P < 0.05$).

Enhancing apoptosis of MCF-7 cells by eIF4E-shRNA

We hypothesized that the growth suppression of MCF-7 cells by RNAi-mediated downregulation of eIF4E

expression was caused by apoptotic cell death. In order to explore this possibility, we measured levels of apoptosis in stably transfected MCF-7 cells and control MCF-7 cells using various apoptosis detection assays. Firstly, cell apoptosis detected by flow cytometry showed that the apoptosis rate of MCF-7-s2 significantly increased to 18.3 ± 1.7% ($P < 0.05$), while there were no differences in cell apoptosis among MCF-7, MCF-7-pS.retro and MCF-7-NS cells (2.4%, 3.2% and 4.6%; $P > 0.05$, Fig. 9a), which was 3.8 ± 0.6%, 4.2 ± 1.0% and 5.2 ± 0.7%, respectively. Secondly, the TUNEL assay for detection of apoptosis at a single cell level, based on labeling of DNA strand breaks, was performed. Results from analysis of the TUNEL assay revealed that eIF4E-shRNA treatments increased apoptosis of these cells because the majority of MCF-7-s2 cells showed intense brown staining when compared to the control cells (Fig. 9b).

The effect of eIF4E-shRNA on the activation of apoptotic pathways

To determine further insight into the mechanism of eIF4E-shRNA2-induced apoptosis, we first investigated
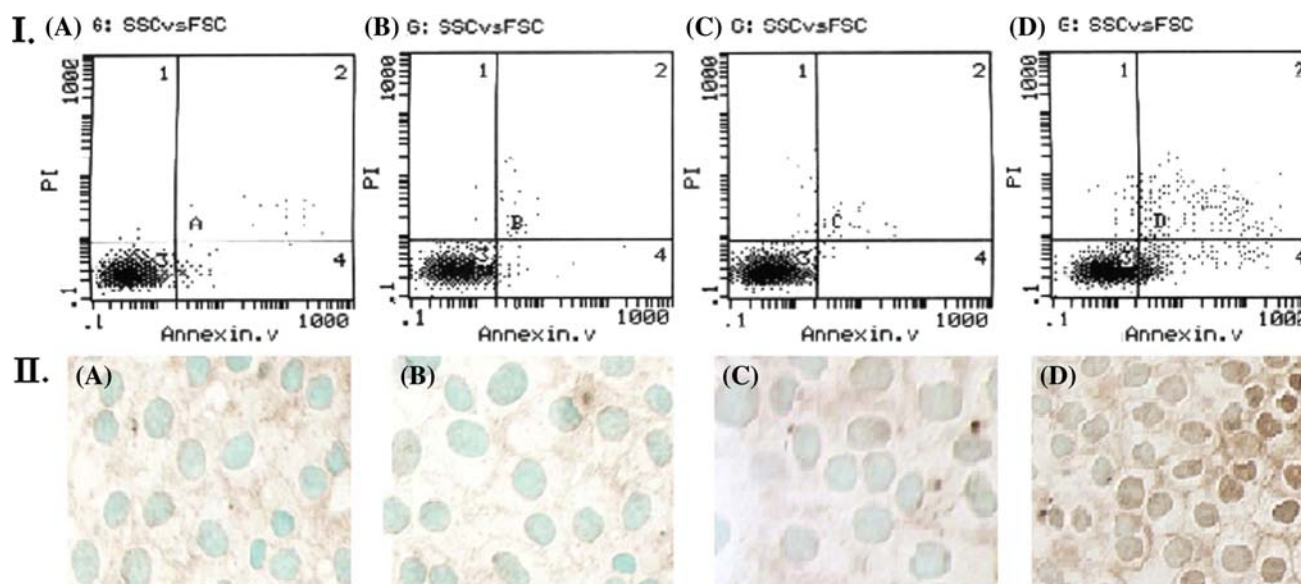
Fig. 9 eIF4E-shRNA significantly enhanced apoptosis of MCF-7 cells. (**I**) The apoptotic rate of MCF-7 cells was detected by flow cytometry. The FCM results showed that the apoptotic rate of MCF-s2 obviously increased by $18.3 \pm 1.7\%$ ($P < 0.05$); while there were no obvious differences in cell apoptosis among MCF-7-pS.retro, MCF-7-NS and untransfected MCF-7 cells (3.2%, 4.6% and 2.4%; $P > 0.05$). (**II**) TUNEL assay was performed to detect apoptotic cells. Compared to control cells, MCF-7-s2 cells showed an increasing dark brown-positive signal located in nucleus. It was shown that apoptotic rate of the eIF4E-downregulated MCF-7 cells was significantly higher than the controls. (**a**) MCF-7; (**b**) MCF-7-pS.retro; (**c**) MCF-7-NS; (**d**) MCF-7-s2

the involvement of caspase-3. We used anti-cleaved caspase 3 antibodies to perform Western blot analysis and detected caspase 3 activity on untransfected or stably transfected cells (MCF-7, MCF-7-pS.retro, MCF-7-NS and MCF-7-s2). As shown in Fig. 10a, b, the activation of caspase 3 could be observed and the caspase-3 activity increased by 221% in MCF-7-s2 cells. Followingly, in order to confirm that the activation of caspases culminates in the apoptosis of MCF-7 cells, we employed the MTT assay to detect the cell viability of MCF-7-s2 cells or the cell viability of MCF-7-s2 treated with a pancaspase inhibitor. We observed that a pancaspase inhibitor could partially restore the cell viability of MCF-7-s2 cells, confirming that the cell death of MCF-7 cells induced by eIF4E-shRNA2 was a caspase-dependent process (Fig. 10c). Next, Western blot analysis was performed to determine whether the levels of the antiapoptotic proteins (c-IAP1, c-IAP2, c-Myc, Bcl-2 and Bcl-xl) and the pro-apoptotic proteins (Bad and phosphorylated Bad^{Ser136}) were altered in the presence of eIF4E-shRNA2. MCF-7-s2 cells stably expressing eIF4E-shRNA2 had a significant reduction in the levels of c-IAP1 c-IAP2 and c-Myc but not Bcl-2 family proteins as compared with controls (Fig. 10d). These results showed that downregulation of c-IAP1, c-IAP2 and c-Myc but not Bcl-2 family proteins were involved in the apoptosis induced by eIF4E-shRNA2.

Downregulation of eIF4E in the MCF-7 cells retards tumorigenicity in nude mice

In order to confirm inhibition of eIF4E expression by shRNA treatment, tumor homogenates were subjected to Western blot analysis for eIF4E. Mean eIF4E expression in tumors formed from MCF-7-s2 cells was 64.5% lower than those in tumors formed from other control cells (Fig. 11a). To explore the possible effect of eIF4E-shRNA2 on tumor growth in vivo, s.c. tumor formation assay in nude mice was performed. As shown in Fig. 11b, the xenografts developed from MCF-7-s2 cells at 42 days (averaged size = 233.5 mm^3) were significantly smaller than the xenografts developed from MCF-7, MCF-7-pS.retro, and MCF-7-NS cells (averaged size = 413.7, 434.5 and 397.7 mm^3). All these experimental data showed that RNAi-mediated downregulation of eIF4E expression exerted a potent growth inhibitory effect on MCF-7 cells in vivo.

The specific downregulation of eIF4E expression enhances cisplatin cytotoxicity in vitro

Other reports have shown that the overexpression of cyclinD1 induced chemoresistance of human solid tumors to cisplatin. Our previous and others studies also showed that the upregulated levels of cyclinD1 expression are
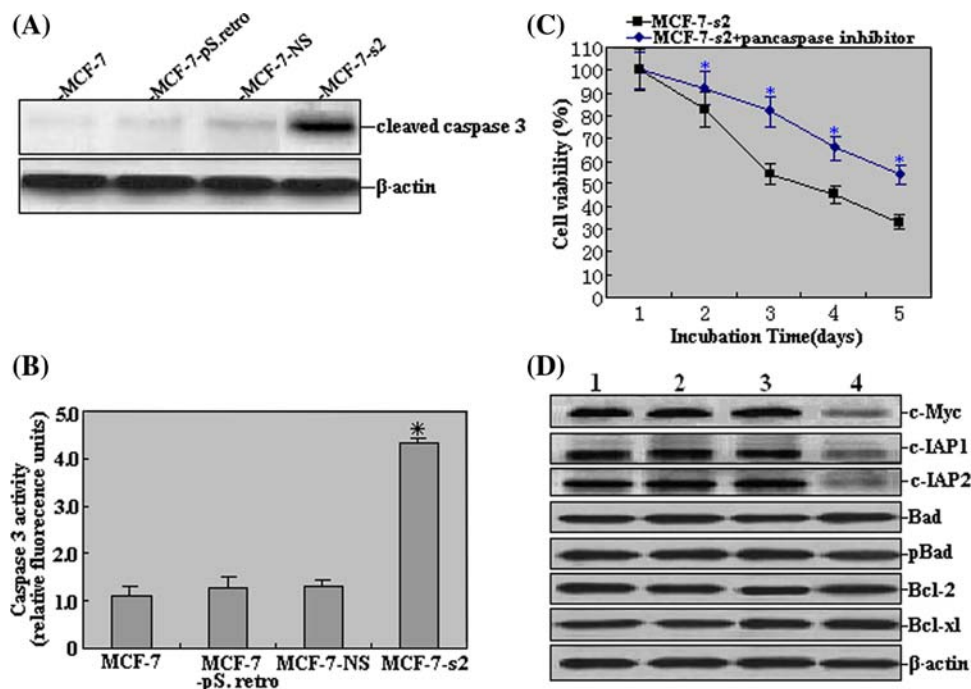
Fig. 10 Effects of eIF4E-shRNA on the activation of apoptotic pathways. (**a**) Immunoblot of untransfected and stably transfected MCF-7 cells. 60 mg of total protein from cell lysates was loaded per lane and blotted with anti-cleaved caspase-3 antibodies. Equal loading was confirmed by showing equal β-actin levels. (**b**) Caspase-3 activity of MCF-7-s2 cells increased by 221%. * $P < 0.05$ versus control cells. (**c**) The stably transfected MCF-7-s2 cells were treated with and without caspases inhibitor Z-VAD(OMe)-FMK. Cell viability was determined by the MTT assay (* $P < 0.05$). (**d**) Immunoblot of untransfected and stably transfected MCF-7 cells. 60 mg of total protein from cell lysates was loaded per lane and blotted with antibodies against c-Myc, c-IAP1, c-IAP2, Bad, phospho-Bad, Bcl-2 and Bcl-xl. Equal loading was confirmed by showing equal β-actin levels. (1) MCF-7; (2) MCF-7-pS.retro; (3) MCF-7-NS; (4) MCF-7-s2

associated with the overexpression of eIF4E gene, so we next explored whether the shRNA-mediated downregulation of eIF4E expression could affect the sensitivity of MCF-7 cells to the antitumor agent cisplatin. Firstly, we sought to determine the effect of eIF4E knockdown on the cisplatin IC_{50} of breast carcinoma cells in vitro. The stably transfected or untransfected MCF-7 cells were plated into 96-well trays and exposed to 0–15 μg/ml cisplatin for a further 48 h. IC_{50} (12.3 μg/ml) was determined by MTT assay (data not shown). Compared with control cells, the cisplatin IC_{50} significantly decreased by 61.1% in MCF-7-s2 cells (Fig. 12a). As shown in Fig. 12b, we observed that the survival index in MCF-7-s2 cells was obviously decreased with the addition of 4.0 $\sim$ 32.0 μg/ml cisplatin compared with controls ($P < 0.05$). Furthermore, following cisplatin treatment, the apoptotic fraction of MCF-7-s2 cells markedly increased in comparison with that of control cells (Fig. 12c).

The effect of eIF4E downregulation on chemosensitivity to cisplatin in vivo

In view of these findings in vitro, we wanted to test the efficacy of eIF4E-shRNA2 as an in vivo chemosensitizing strategy in a nude mouse xenograft model. To explore the possible effect of eIF4E downregulation on chemosensitivity to cisplatin in vivo, s.c tumors (MCF-7-NS or MCF-7-s2) were formed in nude mice followed by treatment with cisplatin or N.S. After the treatment with cisplatin, the proliferation of tumors developed from MCF-7-s2 cells was significantly inhibited compared with that of those developed from MCF-7-NS cells (Fig. 13a). At 35 days after inoculation, we monitored tumor size as described above. Following 5 weeks of treatment with cisplatin or N.S, the average tumor size of tumor developed from MCF-7-s2 and MCF-7-NS was 134.5 mm^3 and 208.9 mm^3, respectively (Fig. 13b). The RNAi-mediated downregulation of eIF4E expression led to a 35.6% suppression of tumor proliferation. Hence, eIF4E-shRNA2 along with chemotherapeutic cisplatin could produce a synergistic cytotoxicity effect on the cell proliferation of breast carcinoma in vivo.

Discussion

Dysregulation of translation is critical to malignant transformation and development [30]. Eukaryotic initiation
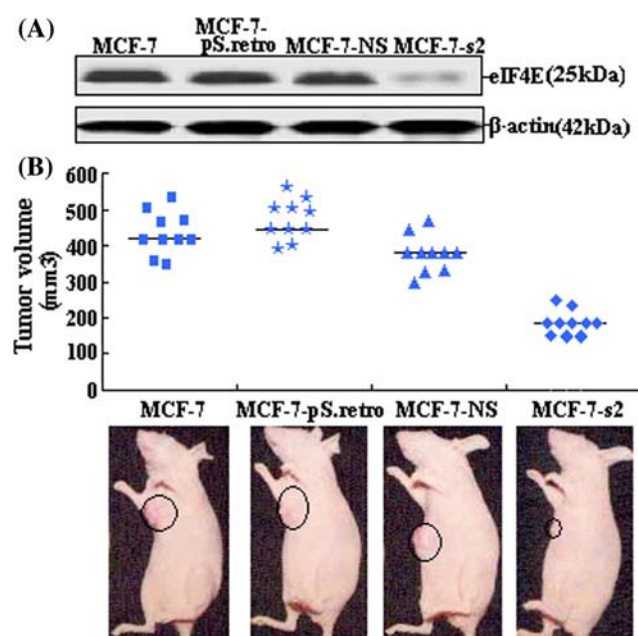
Fig. 11 Stable downregulation of eIF4E expression inhibited tumorigenicity in vivo. (**a**) Protein samples extracted from MCF-7 tumors of ten animals per group were analyzed using Western blot analysis for eIF4E expression levels. The level of eIF4E protein in MCF-7-s2 tumor tissues significantly decreased by 64.5%. β-actin was included as a loading control. (**b**) Tumorigenicity of shRNA stably transfected clones in xenograft model. Ten mice were included in each group. Tumor volumes were measured at 42 days after the inoculation. Representative pictures of tumors in each group were shown

Fig. 12 eIF4E-shRNA promoted cisplatin-induced cytotoxicity in vitro. (**a**) The IC_{50} of Cisplatin in MCF-7-s2 decreased by 61.1% compared with that in MCF-7 cells, * $P < 0.05$. (**b**) Stably transfected or untransfected MCF-7 cells were treated with various concentrations of cisplatin, respectively. Cell viability was determined by MTT chronometry. Cell survival index was calculated as $[A_{490(5-FU+)}/A_{490(5-FU-)}] \times 100\%$, * $P < 0.05$. (**c**) The apoptotic fraction of MCF-7-s2 cells following cisplatin exposure increased by 7.9-fold compared to that of MCF-7 cells following cisplatin, * $P < 0.05$

factor 4E (eIF4E) is the rate-limiting factor in cap-dependent translation, and its bioavailability is strictly controlled through its association with eIF4E-binding protein 1 (4E-BP1). The overexpression of eIF4E gene has been reported to induce the upregulation of cyclin D1 [31], a potent cell cycle regulator as well as VEGF and FGF-2, two angiogenic factors associated with tumor progression, invasion and metastasis [32, 33]. The eIF4E gene has been shown to be overexpressed in breast carcinoma but not in benign breast tissue from noncancer patients [11]. Morever, the degree of eIF4E overexpression appears be important and can predict cancer recurrence in stage I to III breast carcinoma patient. Cisplatin, a chemotherapeutic drug that was thought to form inter- and intra-strand DNA crosslinks, has been used as first-line therapy to treat breast carcinoma, but its activity is far from satisfactory [34]. Thus, in order to improve the prognosis of patients with type of refractory cancer, it is necessary to identify and target gene conductive to the treatment of breast carcinoma, such as enhancement of conventional chemotherapy. Chemosensitivity to cisplatin in human cancer cells has been reported to associate with the levels of cyclin D1 expression and inhibiting cyclin D1 expression could markedly decrease multiple chemoresistance of tumor cells [35]. Truely, the upregulated levels of cyclinD1 expression
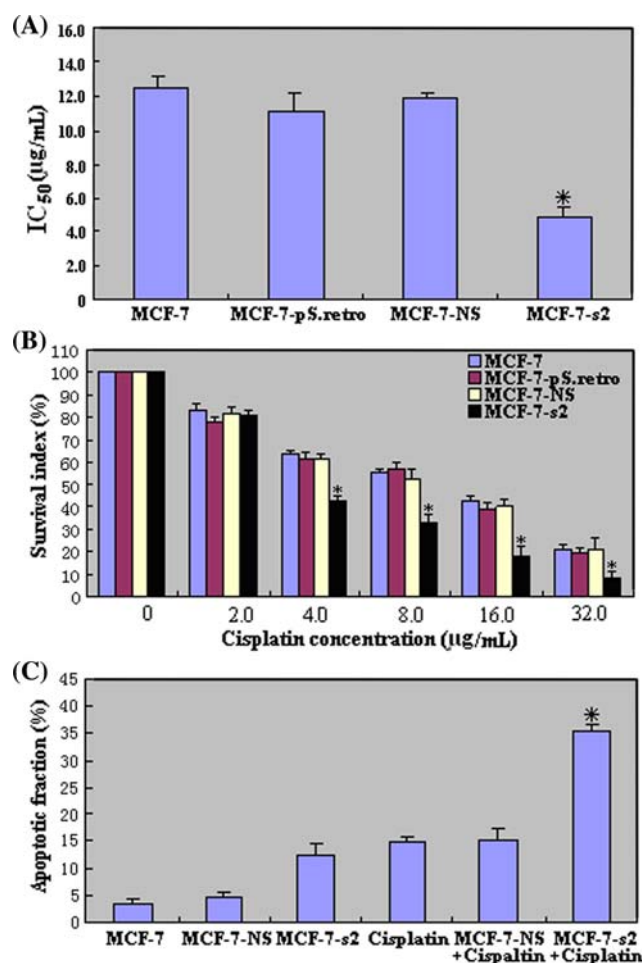
in many human cancers are related to the overexpression of eIF4E gene, so we hypothesized that reducing eIF4E expression would downregulate cyclinD1 expression and enhance chemosensitivity of cancer cells to cisplatin. Therefore, in an attempt to prove its possibility and determine the potential of eIF4E as a therapeutic target, we employed DNA vector-based shRNA technique to downregulate its expression and analyzed its phenotypical changes in MCF-7 cells.

Presently, the most commonly used plasmids for expressing shRNAs in cells contain CMV promoter and RNA polymerase-III-based promoters, including the U6 and H1-RNA promoters [36–38]. These promoters synthesize shRNA and elicit RNAi efficiently, but they have a major limitation: inhibition cannot be controlled in a
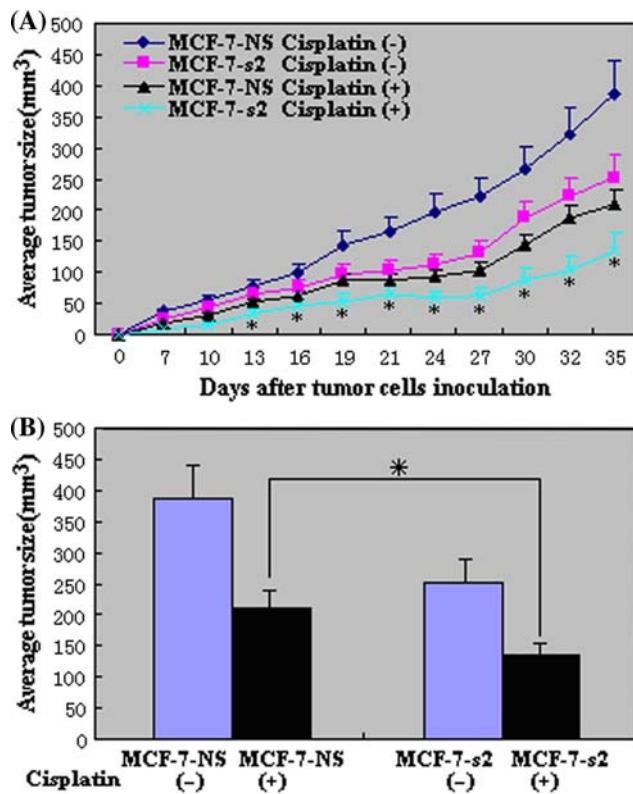
Fig. 13 Downregulation of eIF4E expression enhanced cisplatin-induced cytotoxicity in vivo. Mice were treated with cisplatin (5.0 mg/kg; thrice weekly) or with 0.1 ml N.S (pH 7.4; thrice weekly). (**a**) Tumor growth in the mice injected with MCF-7-s2 cells stably expressing eIF4E shRNA or MCF-7-NS cells stably expressing non-specific shRNA with or without cisplatin. The inoculation was performed in ten mice. (**b**) Tumor size at day 35 after the inoculation. Grey column, average tumor size at day 35 after the inoculation of MCF-7-NS or MCF-7-s2 cells in mice treated with N.S; Black column, average tumor size at day 35 after the inoculation of MCF-7-NS or MCF-7-s2 cells in mice treated with cisplatin (10/group). * $P < 0.05$

tissue-specific or cell-specific manner, because the CMV promoter and RNA Pol III promoters are constitutively expressed in all cell types. Thus, the use of these promoters to drive shRNA expression in vivo would silence a given gene in all cell types, and thus produce undesirable effect in non-target cells. Recently, a Pol II promoter-based shRNA construct has been reported as an alternate method to drive shRNA expression. Since some Pol II promoters possess cell- or tissue-specific property, and they can provide an attractive strategy for targeted gene silence in gene therapy to decrease the probability of off-target silencing and cellular toxicity [39]. Survivin promoter belongs to Pol II promoter and displays high activity in human cancer cells but not in differentiated cells. Our previous and some of others' reports have shown that survivin promoter employed to drive therapeutic genes could achieve tumor-targeted gene therapeutic effect [28, 40]. Unfortunately, there have been few reports about using survivin promoter

to drive shRNA in the anti-tumor study. Thus, we wanted to test a strategy to downregulate eIF4E expression in a tumor-specific manner by constructing survivin promoter-driven shRNA eukaryotic expression vector.

In the present study, we could specifically downregulate eIF4E expression in breast carcinoma cells (MCF-7) but not in normal mammary epithelial cells (MCF210) using DNA vector-based shRNA driven by survivin promoter. Morever, the downregulation of eIF4E expression significantly suppressed MCF-7 cell growth but had none effect on the MCF210 cell growth. All these results showed that the survivin promoter–driven RNA interference system can inhibit targeted gene expression with tumor specificity and high efficacy. To explore the mechanism of growth inhibition, we detected the changes of cell cycle in the MCF-7 cells stably expressing eIF4E-shRNA2. Results indicated that the eIF4E downregulation induced an accumulation of the cells in the G_0/G_1 phase, which led to proliferation suppression of breast carcinoma cells. Next, we used survivin promoter-driven shRNA vector targeting eIF4E to explore the antitumorigenic effect in vivo. From tumorigenicity assay in nude mice, we observed that the downregulation of eIF4E expression obviously inhibited the tumor growth in vivo. Furthermore, FCM and TUNEL stain assays indicated that shRNA targeting eIF4E induced the increased apoptosis of MCF-7 cells. In the eIF4E shRNA-induced apoptosis, we found that downregulation of c-IAP1, c-IAP2 and c-Myc but not Bcl-2 family proteins were involved in that course, but the exact mechanism of eIF4E shRNA-induced apoptosis needs to be further clarified. All experimental data showed that the downregulation of eIF4E expression could give rise to growth suppression and lead to apoptosis induction in breast carcinoma cells.

Since the overexpressed eIF4E exerts profound effects on cell growth and survival, and causes malignant transformation, we explored the therapeutic role of eIF4E in combination with chemotherapeutic drug. Cisplatin, a platinum-based chemotherapy drug used to treat various types of cancers, produces cell cytotoxicity by forming inter- and intra-strand DNA cross-links and the cytotoxic effect might result from replication inhibition by cisplatin-DNA adducts and induction of apoptosis [41]. Cisplatin has also been widely applied in clinic to treat human breast carcinoma, but the clinical therapeutic results are not satisfying [42, 43]. It has been reported that the overexpression of eIF4E induces the upregulated levels of cyclinD1 expression which confers many tumors cisplatin resistance. Thus, we believe that inhibition of eIF4E expression might abolish the multidrug resistance-related signaling pathways leading to decreased efflux of cisplatin from cells. In this study, as reported on other human malignancies by other researchers, results from

chemosensitivity tests showed that the RNAi-mediated downregulation of eIF4E expression synergistically enhanced the cytotoxicity of cisplatin both in vitro and in vivo, which made us believe that cisplatin chemotherapy could be more effective in combination with RNAi-mediated downregulation of eIF4E expression.

Taken together, our results suggest that the tumor-specific survivin promoter can drive shRNA expression to silence targeted gene in tumor cells but not in normal cell, and the use of DNA vector-based shRNA technology can effectively and specifically downregulate eIF4E gene expression, which leads to growth suppression of breast carcinoma cells and enhancement of chemosensitivity to cisplatin both in vitro and in vivo. Therefore, the combination of survivin promoter and shRNA will be a novel strategy in targeted cancer gene therapy and may become a promising approach for the chemosensitization of human breast carcinomas in the future. Further researches should focus on the delivery strategies that can direct eIF4E-shRNA vectors specifically into tumor cells with low toxicity and high efficacy.

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