Biochemical and Biophysical Research Communications 421 (2012) 396–402

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Short-hairpin RNA-mediated Heat shock protein 90 gene silencing inhibits human breast cancer cell growth *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 27 March 2012
Available online 10 April 2012

Keywords:
Heat shock protein 90
Short hairpin RNA
Breast cancer
MCF-7 cell

ABSTRACT

Hsp90 interacts with proteins that mediate signaling pathways involved in the regulation of essential processes such as proliferation, cell cycle control, angiogenesis and apoptosis. Hsp90 inhibition is therefore an attractive strategy for blocking abnormal pathways that are crucial for cancer cell growth. In the present study, the role of Hsp90 in human breast cancer MCF-7 cells was examined by stably silencing Hsp90 gene expression with an Hsp90-silencing vector (Hsp90-shRNA). RT-PCR and Western blot analyses showed that Hsp90-shRNA specifically and markedly down-regulated Hsp90 mRNA and protein expression. NF-*k*B and Akt protein levels were down-regulated in Hsp90-shRNA transfected cells, indicating that Hsp90 knockout caused a reduction of survival factors and induced apoptosis. Treatment with Hsp90-shRNA significantly increased apoptotic cell death and caused cell cycle arrest in the G1/S phase in MCF-7 cells, as shown by flow cytometry. Silencing of Hsp90 also reduced cell viability, as determined by MTT assay. *In vivo* experiments showed that MCF-7 cells stably transfected with Hsp90-shRNA grew slowly in nude mice as compared with control groups. In summary, the Hsp90-shRNA specifically silenced the Hsp90 gene, and inhibited MCF-7 cell growth *in vitro* and *in vivo*. Possible molecular mechanisms underlying the effects of Hsp90-shRNA include the degradation of Hsp90 breast cancer-related client proteins, the inhibition of survival signals and the upregulation of apoptotic pathways. shRNA-mediated interference may have potential therapeutic utility in human breast cancer.

1. Introduction

Breast cancer is the most common cancer in women worldwide, representing 22.9% of all cancers in women, and causing 458,503 deaths each year (13.7% of all cancer deaths in women) [1]. In the US, breast cancer has an incidence of 121.8 per 100,000 women, and is second only to lung cancer as a cause of cancer-related death [2]. While the incidence is still lower in China (estimated to be between 10 and 60 per 100,000), there is a projected rise to more than a 100 per 100,000 by 2021, which could result in a total of 2.5 million patients with breast cancer [3].

Heat shock proteins (HSP) are crucial factors in the heat shock response, and their levels are elevated in a wide range of cancers including mammary carcinoma [4]. Specifically, Hsp90 has been identified as a potential drug target, as it chaperones proteins involved in tumor progression and resistance to therapy, such as the estrogen receptor, receptor tyrosine kinases of the ErbB family, and p53 [5].

In breast cancer, Hsp90 stabilizes mutated and overexpressed oncoproteins, and can induce the activation of growth and transforming pathways in the absence of growth factors [6,7]. Most of the substrate proteins associated with Hsp90 are protein kinases or transcription factors that are important in cellular carcinogenesis [8]. In addition, Hsp90 may contribute to the evolution of treatment resistant cell populations by permitting the emergence of variant proteins that can overcome the selection stress of cancer therapy [9,10]. Given the crucial roles of Hsp90 in growth arrest and apoptosis, its inhibition in breast cancer cells might be an attractive therapeutic strategy.

RNA interference (RNAi) gene silencing can be achieved by delivering viral vectors expressing short hairpin RNA (shRNA), which is processed into small interfering RNA (siRNA) in target cells, thus knocking down the gene of interest [11].

In the present study, a shRNA plasmid vector targeting Hsp90 was constructed and transfected into the human breast cancer cell line MCF-7, where it persistently generated siRNA against Hsp90.

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http://dx.doi.org/10.1016/j.bbrc.2012.04.032

We then investigated the effect of Hsp90 silencing on cell proliferation, cell cycle distribution, and cell death *in vitro*. We also established a xenograft mouse model to determine the effect of RNAi against Hsp90 on the growth of xenografts *in vivo*.

2. Materials and methods

2.1. Construction of plasmids expressing Hsp90-targeted shRNA

The Hsp90-specific shRNA was designed as a 57-mer containing a hairpin-loop and 21 nucleotides derived from the Hsp90 gene (Genbank accession number NM_003299). The target sequence used to generate the shRNA construct against Hsp90 was 5′-CCT GTG GAT GAA TAC TGT ATT-3′ (nucleotide 1816–1836). The double-stranded oligodeoxyribonucleotides were: 5′-CCG GCC TGT GGA TGA ATA CTG TAT TCT CGA GAA TAC AGT ATT CAT CCA CAG GTT TTT-3′ and 5′-AAA AAC CTG TGG ATG AAT ACT GTA TTC TCG AGA ATA CAG TAT TCA TCC ACA GGC CGG-3′ The lack of homology to known genes was confirmed by conducting an NCBI blast query. The candidate shRNA sequence was identified using the Ambion siRNA algorithm. A negative control shRNA containing a scrambled siRNA target sequence was prepared. The oligonucleotides were ligated into the *AgeI* and *EcoRI* sites of the pLKO.1-puro plasmid to generate recombinant plasmids (Fig. 1).

2.2. Culture of MCF-7 cells and stable Hsp90-shRNA vector-expressing cells

The MCF-7 cell line, which is one of the most widely used breast cancer cell lines [12], was cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, USA). Cultures were maintained at 37 °C in a humidified incubator with 5% CO_2. MCF-7 cells were seeded at 5×10^4 cells per well in 24-well plates 24 h prior to transfection. Transfection was performed at 70–80% cell confluence with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were transfected with empty plasmid, Hsp90-shRNA, and negative control shRNA vector. Stably transfected cells were selected using 5 μg/mL puromycin for 1 month, and the resulting three sublines were used for all further experiments.

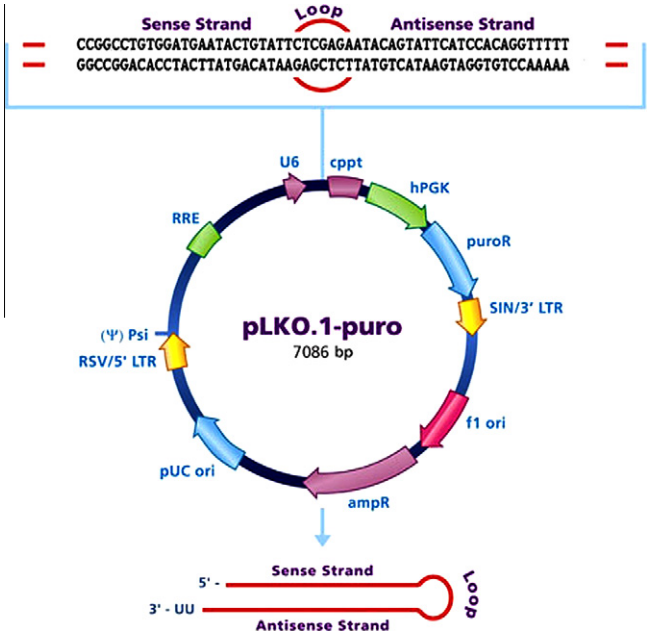

Fig. 1. Map of the pLKO.1-puro-based plasmid vector containing a shRNA insert. The original pLKO.1 TRC cloning vector has a 1.9 kb stuffer that is released by digestion with AgeI and EcoRI. shRNA oligos are cloned into the AgeI and EcoRI sites in place of the stuffer. The puro R resistance gene is used for selection of the pLKO.1 plasmid in mammalian cells. The human U6 promoter drives RNA polymerase III transcription for generation of shRNA transcripts.

2.3. Western blot analysis

Proteins from the parental MCF-7 cells (mock) and transfected sublines (empty vector, Hsp90 shRNA, and negative control shRNA) were extracted in radio-immunoprecipitation assay buffer (Beyotime, Shanghai, China). The protein concentration of whole cell lysates was determined using the BCA Protein Assay Kit (Beyotime). Equal amounts of protein samples from whole-cell lysates were loaded onto SDS–PAGE gels (8–15%). After electrophoresis, the proteins were transferred to nitrocellulose membranes (Amersham Bioscience, Piscataway, NJ) and incubated with the indicated antibodies (Hsp90, NF-*k*B, Akt and GAPDH, all purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.4. Real time-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using the Trizol™ reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA (0.2 μg) was reverse transcribed using the High Capacity cDNA synthesis kit (Applied Biosystems, California, USA) according to the manufacturer's instructions. RT-PCR was performed using the QuantiFast Sybr Green PCR kit (Qiagen, USA) and QuantiTect primers (Hsp90 and 18S rRNA) according to the manufacturer's instructions. For analysis of Hsp90 mRNA levels, we used 5′-GAG ATC AAA GAC TAC AGT CCC-3′ as the upstream primer and 5′-GTT CGT GCT CAT ACT TGG TC-3′ as the downstream primer. Analysis of samples was performed on the Light Cycler 480 real-time PCR machine (Roche Diagnostics, USA).

2.5. Assessment of apoptosis and cell-cycle analysis by flow cytometry

Apoptosis was measured by staining with annexin V-fluorescein isothiocyanate (FITC, an apoptotic cell marker) and propidium iodide (PI, a necrotic cell marker) [13]. The degree of apoptosis was expressed as the percentage of cells stained with annexin V-FITC/PI determined by flow cytometry analysis using a FACScan flow cytometer (Becton–Dickinson FACScan) and WinList software (Verity, Topsham, ME).

Cell-cycle analysis was performed by propidium iodide DNA staining [14]. Cells were harvested by trypsinization and centrifugation, washed twice with cold PBS, and fixed with cold 75% ethanol at 4 °C overnight. The fixed cells were collected, washed twice with PBS and resuspended in PBS containing 10 μg/mL PI (Sigma, USA) and 100 μg/mL RNase A, then incubated at 4 °C for at least 30 min avoiding light to eliminate intracellular RNA. The percentage of cells in each phase of the cell cycle was measured by flow cytometry.

2.6. Cell proliferation assay

The cell viability of the MCF-7 cell line was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, MCF-7 cells were seeded in 96-well plates at a density of 1×10^4 cells per well in 100 μL medium. The plates were incubated in a 37 °C humidified incubator for adherence overnight. Cells were harvested at 24, 48, 72, and 96 h. The MTT assay was performed according to the manufacturer's manual (Sigma).

2.7. Mouse xenograft model of human MCF-7 tumors

Nude mice (4–6 weeks old, 18–22 g of body weight) were randomly assigned to four groups (n = 5 per group), and then injected subcutaneously with 3×10^6 cells in 100 μL saline. Tumor growth was monitored by measuring the largest (a) and smallest (b) two perpendicular diameters with a caliper, and calculating the tumor volume (V) = $a \times b^2 \times 0.5$. Tumors were allowed to grow until they reached a diameter of 5–7 mm.

2.8. Immunohistochemistry

Mouse xenograft tumor tissues were harvested at the end of the treatment period and fixed in 10% neutral buffered formalin, processed in paraffin, and sectioned into 5 μm thick slices. Tissue sections were then subjected to immunohistochemical staining with antibodies against proliferating cell nuclear antigen (PCNA) and developed using the avidin–biotin complex (ABC) procedure as recommended by the manufacturer (Vector).

2.9. Statistical analysis

The results were expressed as mean ± SD. The Student's t-test was used to determine statistical significance. For medians showing a non-normal distribution, we used non-parametric tests (e.g., Mann–Whitney test) to compare differences. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS 12.0 software.

3. Results

3.1. Hsp90 knockdown by shRNA-expressing vectors

Hsp90 shRNA significantly reduced Hsp90 expression at both protein and mRNA levels as shown in Fig. 2(A–C). The mRNA expression was reduced by 79.81 ± 7.63% by Hsp90 shRNA compared to negative control shRNA, mock, and empty vector controls ($P < 0.01$), while no difference was found between the control groups ($P > 0.05$).

3.2. Effect of Hsp90 shRNA on cell proliferation and cell cycle distribution

The results of MTT assays showed a gradual increase in cell number in the three control groups (mock, empty vector, and negative control shRNA) from 24 h (1.03 ± 0.025, 1.11 ± 0.081 and 1.05 ± 0.084, respectively) to 96 h (2.76 ± 0.066, 2.64 ± 0.133 and 2.58 ± 0.078, respectively). On the other hand, the number of cells in the Hsp90 shRNA transfected group remained relatively unchanged (1.15 ± 0.117) in the same time period. Statistical analysis indicated that the shRNA against Hsp90 significantly inhibited the proliferation of MCF-7 cells compared with the mock ($P < 0.001$), vector control ($P < 0.001$), and negative control shRNA groups ($P < 0.001$). There were no differences between the control groups ($P > 0.05$, Fig. 3A).

To investigate the mechanism of growth inhibition of MCF-7 cells by Hsp90-shRNA, cell cycle analysis was performed using PI staining and flow cytometry to determine the number of cells in G0/G1, G2/M and S phases. In comparison to the mock, vector control and negative control shRNA transfected cells, Hsp90-shRNA transfection resulted in the accumulation of cells in the G0/G1 phase with a concomitant decrease in cells progressing to the S and G2/ M phases, indicating a block of the G1/S transition. The total number of cells in the S and G2 phases was greater than that of the corresponding cells in mock ($P < 0.01$), vector ($P < 0.01$) and negative control shRNA ($P < 0.01$). Again, no statistically significant difference was observed between the control groups (Fig. 4). These results suggested that RNAi-mediated silencing of Hsp90 resulted in the inhibition of cell growth in MCF-7 cells, which might be due to a defect in the G1 to S phase transition.

3.3. Hsp90-shRNA enhances apoptosis in vitro

Furthermore, to investigate whether Hsp90-shRNA induces apoptosis of breast cancer cells, apoptotic cell death was assessed using flow cytometric analysis of annexin V stained cells. The rate of apoptosis was expressed as the sum of the percentages of apoptotic cells. The apoptotic rates of Hsp90-shRNA, negative control shRNA, vector control, and mock were 18.12% ± 2.49%,

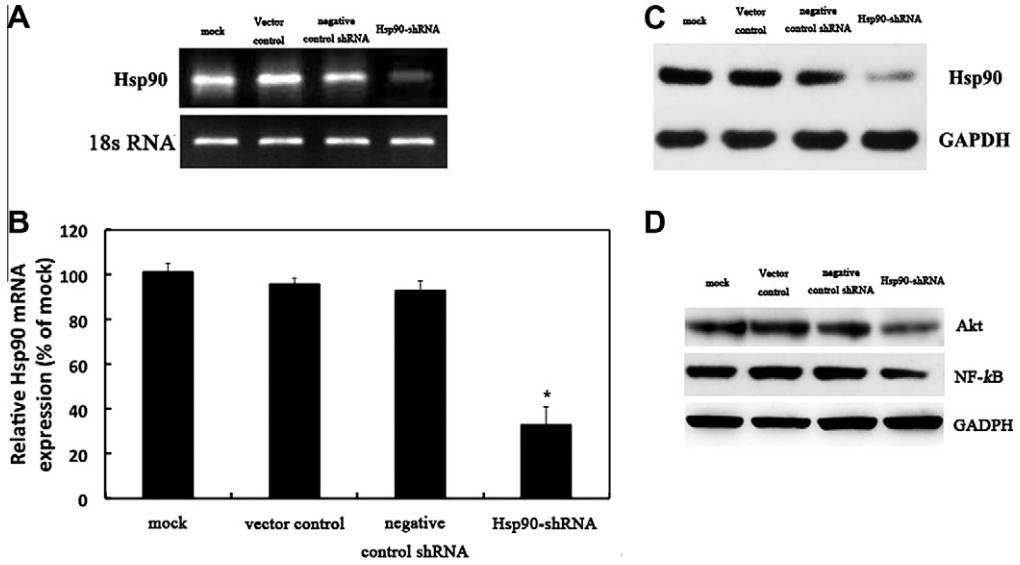

Fig. 2. Stable silencing of Hsp90 gene in MCF-7 cells. The pLKO.1-puro plasmids containing either the specific targeted-sequence for Hsp90-shRNA or a negative control shRNA were transfected into MCF-7 cells. Mock indicates parental cells, vector control indicates transfected with empty vector. (A) RT-PCR electrophoretogram of Hsp90 and 18s RNA in the four groups; (B) relative Hsp90 mRNA Expression by RT-PCR: transfection of Hsp90 with siRNA for Hsp90 significantly decreased Hsp90 mRNA expression (*$p < 0.01$ vs. mock, vector control and negative control shRNA), while 18s RNA was not affected by the Hsp90 siRNA (n = 3). (C) Western blot analysis of Hsp90 and GADPH in the four groups; (D) Western blot analysis of Akt, NF-kB and GADPH in the four groups.

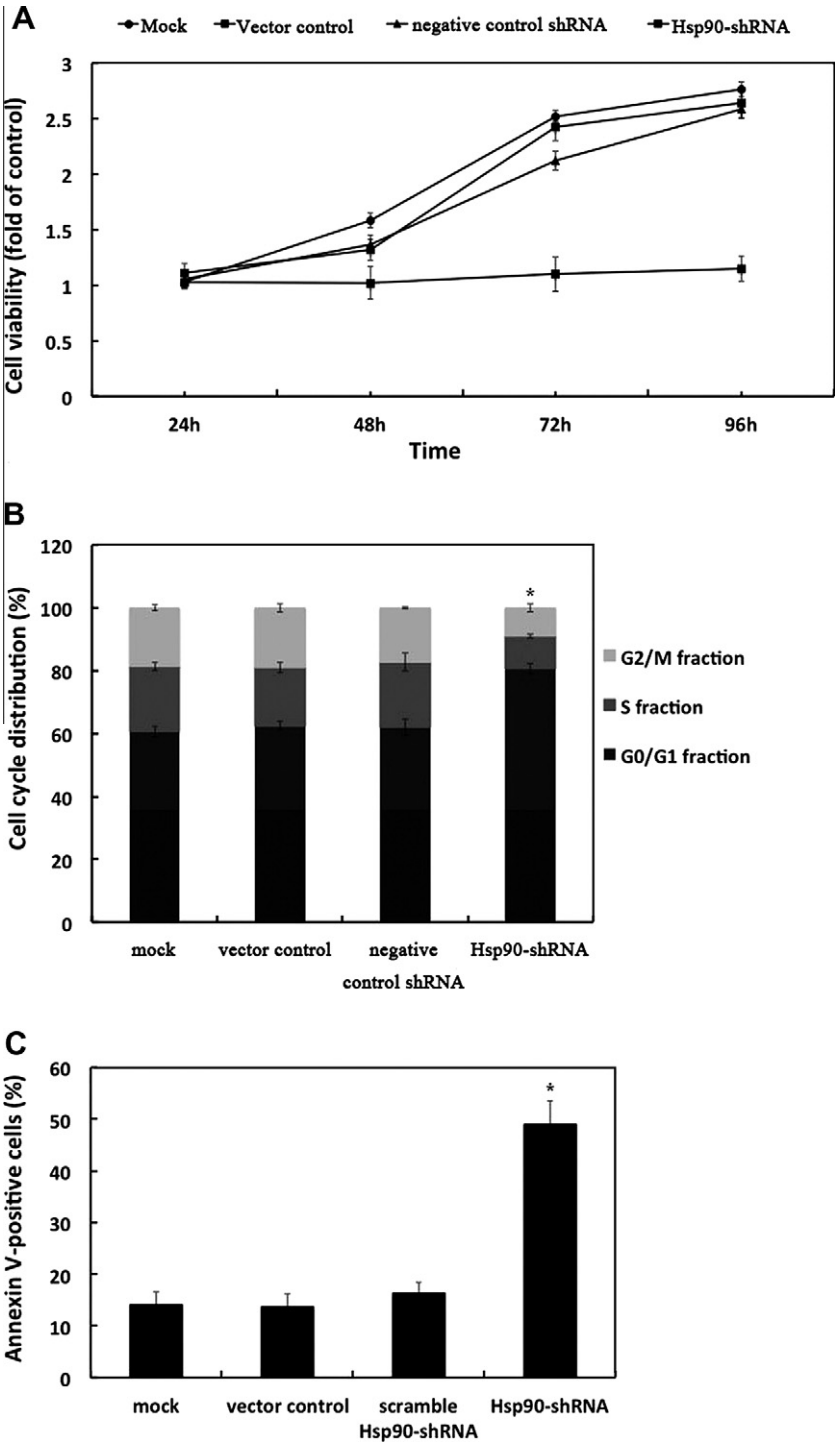

Fig. 3. (A) Viability of cells transfected with mock, vector control, negative control shRNA, and Hsp90-shRNA. Data were expressed as mean ± SD ($n = 3$); (B) Cell cycle detected by flow cytometry. The fraction of cells in G2/M + S phases in the Hsp90-shRNA group was lower than that of mock, vector control, and negative control shRNA groups. Data were expressed as mean ± SD of the three experiments. (*$p < 0.01$ vs. mock, vector control and scrambled Hsp90-shRNA); (C) MCF-7 cells were stably transfected with the vector control, negative control shRNA, and Hsp90-shRNA constructs. Apoptotic cell death was determined using the annexin-V binding assay.

2.36% ± 0.56%, 3.13% ± 0.66% and 3.22 ± 0.46%, respectively, indicating a significant increase in apoptotic cell death in Hsp90-shRNA expressing cells compared to negative control shRNA ($P < 0.001$), vector control ($P < 0.001$) and mock groups ($P < 0.001$). There were no significant differences between the control groups (Fig. 3B).

To further explore the molecular mechanisms involved in the induction of apoptosis by Hsp90-shRNA, Akt and NF-*k*B expression levels were assessed by Western blotting. Indeed, Akt and NF-*k*B were downregulated in MCF-7 cells transfected with Hsp90-shRNA (Fig. 2D), suggesting that Hsp90-shRNA induces apoptosis in MCF-7 cells through the downregulation of survival signals.

3.4. Hsp90 shRNA inhibits breast cancer growth in vivo

To examine the effect of Hsp90 silencing on tumor growth *in vivo*, we established xenografts in nude mice. Tumor size was monitored every 2 days with a caliper. Tumor growth of xenografts

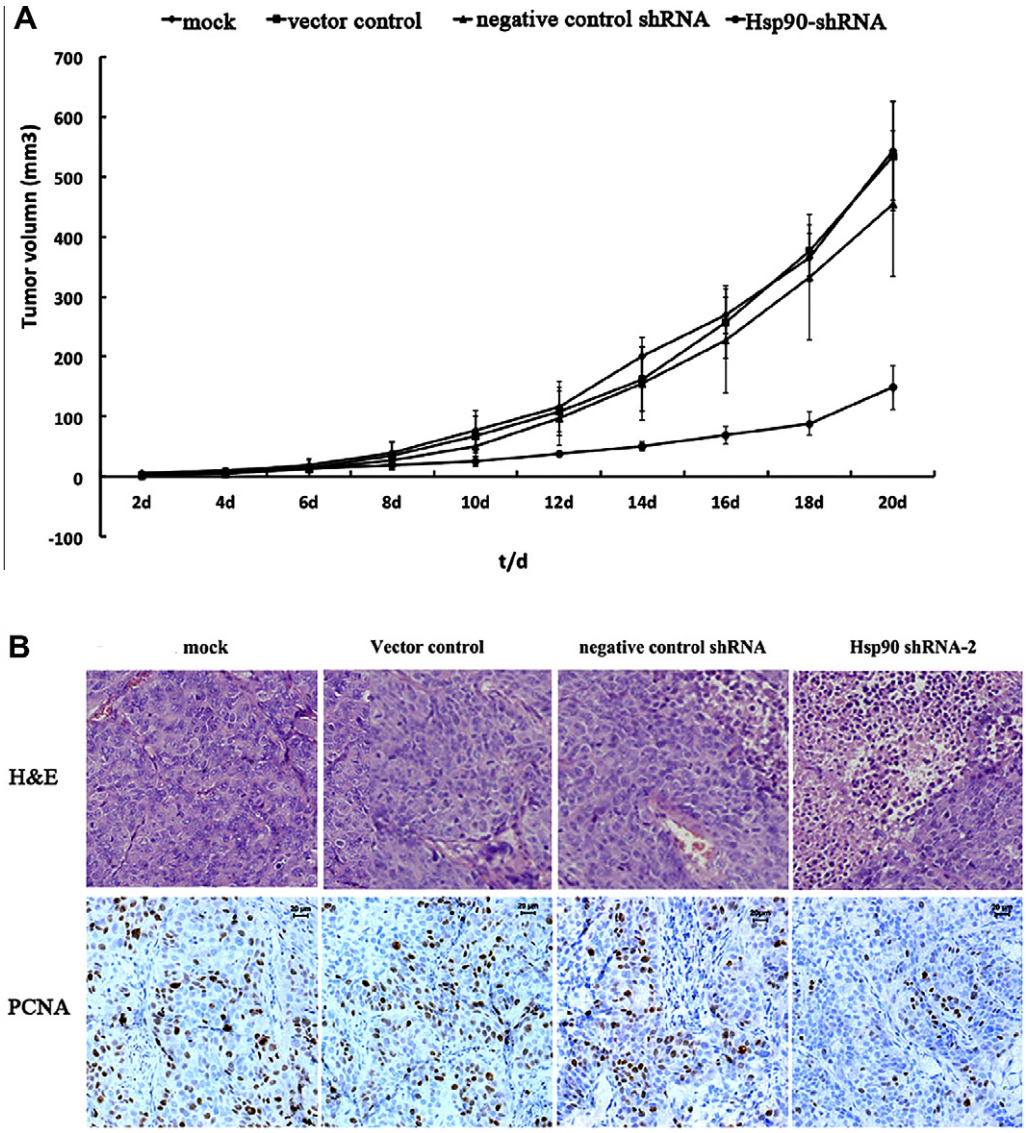

Fig. 4. Effect of Hsp90 shRNA on breast cancer growth *in vivo*. (A) Growth curves of MCF-7 tumors in nude mice of the different groups; (B) The Hsp90 shRNA group displayed suppression of breast cancer growth *in vivo*. (A) Paraffin-embedded xenograft tumor sections of the different groups were subjected to HE staining (original magnification ×200). Microscopy analysis showed massive necrotic areas in Hsp90 shRNA tumors, while the mock, vector control and negative control shRNA groups showed only subtle areas of necrosis; (B) Immunohistochemical staining with anti-PCNA antibody. Dark brown color indicates positive immunostaining (original magnification ×200). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

derived from negative control shRNA and vector control cells was comparable to that of the mock group, showing a marked increase in tumor volume 10 days after tumor cell inoculation in these three groups. In contrast, xenografts derived from Hsp90-shRNA transfected cells showed a slow growth pattern, indicating that the silencing of Hsp90 resulted in growth inhibition of MCF-7 tumors *in vivo* (Fig. 4A, $P < 0.01$). H&E staining was performed to elucidate the mechanism of xenograft tumor growth inhibition by Hsp90-shRNA, which revealed that Hsp90-shRNA tumors were pale, with a massive necrotic center. Immunohistochemical analysis of tumor sections using antibodies against PCNA, a well known marker of cell proliferation, showed a dramatic reduction of the PCNA signal in Hsp90-shRNA derived tumors compared to the mock, vector control and negative control Hsp90 tumors (Fig. 4B).

4. Discussion

In the present study, we demonstrated that RNAi knockdown of Hsp90 in the human breast cancer cell line MCF-7 mediated by transfection with a self-constructed shRNA-containing viral vector reduced the expression of Hsp90 by approximately 70%, resulting in slower cell proliferation *in vitro*, and reduced tumor growth *in vivo*. These effects were most likely caused by a block in the progression of the cell cycle at the G1 to S transition, and by downregulation of the key survival signaling molecules Akt and NF-*k*B. Xenografts of Hsp90-shRNA transfected MCF-7 cells showed a necrotic center, reduced proliferation, and markedly slower tumor growth.

Heat shock proteins are a family of molecules that have been highly conserved through evolution. Initially described as proteins organizing protein folding, they have been implicated in numerous cellular functions [15]. The role of Hsp27, Hsp70, and Hsp90 in carcinogenesis has been extensively investigated *in vitro* and *in vivo* [16–19]. Hsp90, in particular, interacts with many proteins that are overexpressed and/or mutated in cancer and are involved in cancer progression and resistance to therapy [20]. Its functions are essential for important cell processes such as proliferation, cell cycle control, angiogenesis and apoptosis [21]. Hsp90 inhibition is

therefore an attractive strategy to simultaneously block multiple abnormal pathways that are crucial for the growth of several tumor types, including breast cancer [22]. The inhibition of Hsp90 is predicted to result in the loss of the mutant pool and inability to respond to changes in the microenvironment as well as to anticancer treatment. At present, there are several methods of targeting Hsp90, including indirect inactivation of Hsp90 by pharmacological inhibitors, and direct blocking by antisense oligonucleotides and RNAi [23,24]. The latter is a very potent tool to silence genes with high specificity and low toxicity. However, to the best of our knowledge, downregulation of Hsp90 expression by RNAi in human breast cancer MCF-7 cells has not been reported to date.

The present shRNA-mediated silencing strategy for the reduction of Hsp90 expression in human breast cancer lines proved highly effective. As demonstrated by RT-PCR and Western-blotting, we achieved a reduction of gene expression of approximately 70%, which was substantial enough to robustly affect the multiple roles of Hsp90 in MCF-7 cells.

Hsp90 promotes cell survival through its involvement at different steps of the NF-*k*B activation pathway [25], and is essential to maintain NF-*k*B activity [26]. Phosphorylation of Akt leads to the activation of NF-*k*B and induces the phosphorylation of Bad [27], resulting in its inability to translocate to the mitochondria to initiate downstream apoptotic events [28]. In addition, Hsp90 plays an important role in maintaining Akt kinase activity by directly interacting with the kinase and preventing its dephosphorylation [29,30]. Consistent with these functions, we observed that RNAi knock down of Hsp90 decreased NF-*k*B and Akt protein levels in MCF-7 cells and increased apoptosis as determined by flow cytometric assessment using annexin-V and PI.

Furthermore, cell cycle arrest at the G1 phase in response to Hsp90 inhibition has been reported previously [31], and this was also confirmed by our work, in which the results of flow cytometry analysis showed cell cycle arrest in the G1/S phase in Hsp90-shRNA transfected MCF-7 cells. The effects of Hsp90 inhibition on apoptosis and the cell cycle were confirmed by the results of MTT assays, which showed the inhibition of cell proliferation caused by Hsp90 silencing, and the analysis of xenograft tumors in mice, which demonstrated reduced tumor growth and a sizeable necrotic center visible on histology.

The most important shortcoming of our work is that our approach was not used to treat mice *in vivo*, which would more closely resemble a clinical scenario. However, this proof-of-concept study suggests that silencing of Hsp90 – if substantial enough – might be of value for the treatment of breast cancer. For RNAi, the main problem is to find an effective vehicle to transport the therapeutic gene sequence into the cells of interest, and to achieve transfection of the desired duration [32]. In addition, potential adverse events caused by off-target effects and interference with endogenous micro-RNAs should be considered [33]. Nevertheless, recent advances in the fields of bioengineering and nanotechnology suggest that these issues can be resolved, and a number of promising clinical trials have already been conducted using shRNA technology [32,33].

In summary, the present results demonstrated that RNAi targeted specifically against Hsp90 resulted in gene silencing with high efficacy in breast cancer cells, leading to the inhibition of cell proliferation *in vitro* and *in vivo*. Possible molecular mechanisms underlying this effect include cell cycle arrest at the G1/S transition, and the induction of apoptosis by blocking important survival signaling molecules such as Akt and NF-*k*B, with the subsequent upregulation of apoptotic pathways. This method – if translated to the clinic – may have potential therapeutic use in human breast cancer.

Acknowledgment

The present study was funded by National Natural Science Foundation of China (No. 30901770), the International (Regional) Cooperation and Exchange Program (No. 81150110493), Shanghai Municipal Science and Technology Commission (No. 10JC1412700).

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