Effect of liposome-mediated HSP27 transfection on collagen synthesis in alveolar type II epithelial cells

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Abstract. To investigate the effect of liposome Lipofectamine® 2000-mediated HSP27 plasmid transfection in A549 human alveolar type II epithelial cell line on collagen synthesis during transforming growth factor-β1 (TGF-β1)-induced type II epithelial cell transition to myofibroblasts. Cells were transfected with varying ratios of the Lipofectamine® 2000-mediated heat shock protein 27 (HSP27) plasmid and the transfection efficiency was determined using flow cytometry. The maximum transfection efficacy was confirmed by laser confocal microscopy. HSP gene expression and the most efficient HSP27 plasmid were determined using reverse transcription-quantitative polymerase chain reaction. Western blot analysis was used to examine HSP27 and collagen expression levels. With a transfection efficiency of 83%, the 8 μg:20 μl ratio of liposome: Plasmid had the highest transfection levels. Among the four different interference sequences in the HSP27 plasmid, the D sequence had the highest interference effect with 70% silencing of the *HSP27* gene. The expression of type I and III collagen in TGF-β1-induced transition of A549 human alveolar type II epithelial cell line to myofibroblasts was significantly downregulated by the successful transfection with HSP27-interfering plasmid. The expression of type I and III collagen in the TGF-β1-induced transition of A549 cells to myofibroblasts was significantly downregulated by transfection of A549 cells with HSP27 plasmid D-interfering sequence and optimal ratio of Lipofectamine® 2000 and HSP27 plasmid.

Introduction

Heat shock protein 27 (HSP27) is a differential protein isolated from silicosis tissue (1). HSP27 belongs to a small molecular weight HSP family, whose proteins contain a conservative C-terminal domain, termed the α-lens protein domain. Previous studies revealed that HSP27 may antagonize oxidative stress, inhibit cell apoptosis and act as a chaperone in inflammatory reactions, cell signal transduction, cell differentiation, and proliferation by regulating actin cytoskeleton structure (2-7). HSP27 is involved in the epithelial-mesenchymal transition (EMT) in cancer (8) and in the development and progression of organ fibrosis (9). However, previous studies have also revealed that HSP27 may exert dual regulation during EMT in organ fibrosis (9,10). Transforming growth factor-β1 (TGF-β1) is an important transforming growth factor associated with fibrosis *in vitro* and *in vivo* (11,12). A previous study by the authors revealed that TGF-β1 may induce A549 human alveolar type II epithelial cells to differentiate into myofibroblasts (13). In order to investigate the effect of HSP27 on the differentiation of A549 human alveolar type II epithelial cell line into myofibroblasts and collagen synthesis, the current study used liposome transfection to transfect A549 human alveolar type II epithelial cell line and determined the optimal liposome:plasmid ratio and the best interference sequence. Lipofectamine® 2000 was used in the current study as the protocol was established, safe and reliable with high transfection efficacy. Following successful transfection of the HSP27 interfering plasmid, the effect on collagen expression during TGF-β1-induced differentiation of A549 human alveolar type II epithelial cells into myofibroblast was examined.

Materials and methods

Reagents and materials. The following reagents were used in the study: A549 human alveolar type II epithelial cell line derived from human lung carcinoma (cat. no. TCHu150, Cell Bank of Chinese Academy of Science, Shanghai, China); high-glucose Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA); 10% fetal bovine serum (A15-101, PAA, New Bedford, Massachusetts, USA; www.openfos.com/supply/3298452-PAA-LAB-in-New-Bedford-MA/); TGF-β1 (cat. no. 100-21; Peprotech, Inc., Rocky Hill, NJ, USA), mouse anti-HSP27 monoclonal antibody (cat. no. ab114067; Abcam, Cambridge, UK), rabbit polyclonal anti-type I/type III collagen antibody (cat. nos. BA0325/0326, GeneTex, Inc., Irvine, CA, USA), Platinum® SYBR®-Green qPCR SuperMix-UDG kit (cat. no. C11733038, Invitrogen;

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Thermo Fisher Scientific, Inc.), Lipofectamine® 2000 transfection reagent (1.5 ml/U; Invitrogen; Thermo Fisher Scientific, Inc.); HSP27-interfering kit including fragments A, B, C and D (Shanghai GenePharma Co., Ltd., Shanghai, China); Opti-MEM medium (500 ml/bottle, Gibco; Thermo Fisher Scientific, Inc.); and polyclonal anti-GAPDH antibody (cat. no. sc-25778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Cell culture and experimental groups. Specific *HSP27* gene fragment was introduced into A549 cells by transfection. Cells were divided into three groups: i) Blank plasmid control group; ii) blank plasmid + TGF-β1 group; and iii) HSP27-interfering plasmid + TGF-β1 group.

Transfection efficacy determined by flow cytometry. Cells were seeded into 10 cm diameter dishes and transfected at 90-100% confluency. Initially, two aliquots of 3 ml Opti-MEM medium was added into two 5 ml Eppendorf micro test tubes, Lipofectamine® 2000 and HSP27 plasmid were added individually into the two tubes and mixed with OPTI-MEM medium. A series of Lipofectamine® 2000 volumes (20, 25, 30, 20, and 20 μl) and HSP27 plasmid (8, 8, 8, 9 and 10 μg) were correspondingly tested. The reagents were individually incubated at 37˚C for 10 min prior being mixed and incubated at 37˚C for an additional 20 min. The transfection mixture was added into dishes with 15 ml OPTI-MEM and mixed by gentle shaking. After 72 h incubation at 37˚C, cells were trypsinized with 0.25% trypsin, harvested, and centrifuged at 201 x g on 4˚C for 5 min. Transfection efficacy of each combination of Lipofectamine® 2000 and plasmid was determined using fluorescent intensity via flow cytometry.

Examination by laser confocal microscopy. Cells were seeded at $6x10^3$ cells/well in the small dish for confocal observation. HSP27 plasmid and Lipofectamine® 2000 were added at 8 μg:20 μl ratio for transfection as aforementioned. The fluorescence marker green fluorescent protein (GFP) in the plasmid cells were observed under laser confocal microscope at a magnification of x800.

Screen optimal interfering HSP27 plasmid by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were seeded at a density of $6x10^3$ cells/well in 6-well plate and transfected when cells reached 90-100% confluency. For transfection, 500 μl OPTI-MEM medium was added into 1.5 ml Eppendorf tube and 12 tubes were prepared for 6 transfection groups, including 4 different HSP27 interfering sequences, HSP27 positive control and negative control plasmids; 5 μl Lipofectamine® 2000 transfection reagent or the plasmid was mixed with OPTI-MEM and incubated for 10 min. Then the transfection reagent mixture was added into plasmid/OPTI-MEM mixture and incubated at 37˚C for 20 min. RNA was isolated with TRIzol (Invitrogen, Thermo Fisher Scientific, Inc.) after 72 h and the concentration and purity of total RNA was measured with deionized water as control by micro nucleic acid analyzer. The primer of target gene was designed and synthesized (Invitrogen; Thermo Fisher Scientific, Inc.). HSP27: Forward, 5'-GCTTCACGC GGAAATACACG-3' and reverse, 5'-GTGATCTCGTTG GACTGCGT-3'; β-actin forward, 5'-GTCACCTTCACCGTT CCAGTTTT-3' and reverse, 5'-CTTAGTTGCGTTACACCC TTTCTT-3'. qPCR amplification was performed as follows: Pre-denaturation at 95˚C for 15 sec followed by 40 cycles at 60˚C for 20 sec. And data were analyzed to determine the optimal HSP27 interfering sequence by the method of ΔΔCq (14).

Immunoblotting. Protein was isolated from cells with RIPA (Beijing Solarbio Science & Technology Co., Ltd. Beijing, China) following the designated transfection period. After determining the protein concentration by BCA assay, 50 μl samples per lane were loaded on 10% SDS-PAGE. The blots were subjected to standard transfer methods and PVDF membranes were blocked with 10% BSA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and then incubated with primary antibodies (HSP27, type I and III collagen all at 1:200 dilution and GAPDH at 1:100 dilution) at 4˚C overnight. The membranes were incubated with goat anti-rabbit IgG-AP (BA1011; Boster Biological Technology, Pleasanton, CA, USA) and goat anti-mouse IgG-AP (BA1010; Boster Biological Technology) secondary antibodies diluted at 1:5,000 at 37˚C for 2 h and then with RCIP/NBT chromogenic reagent for 1 min. The optical density of protein band was determined by ImageJ software (National Institutes of Health, Bethesda, MD, USA). Relative expression of protein was calculated by the ratio of optical density of target protein band to internal control band. Statistical analysis was performed on relative protein expression.

Statistical analysis. SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis which included Bartlett's, Brown-Forsythe and Levene's tests, to test for equal variance followed by Student-Newman-Keuls multiple-range test and one-way analysis of variance was used to compare differences between groups.

Results

Transfection efficacy of Lipofectamine® 2000 and HSP27 assessed by flow cytometry. Five different combinations of HSP27 plasmid and transfection reagent Lipofectamine® 2000 were assessed, including 8 μg:20 μl; 8 μg:25 μl; 8 μg:30 μl; 9 μg:20 μl; 10 μg:20 μl. Flow cytometry data indicated in Fig. 1 revealed that transfection efficacy was 83, 76, 76, 70, 74%, respectively. The 8 μg:20 μl ratio led to a 83% transfection efficiency and was considered to be the optimal liposome to plasmid ratio and used for subsequent experiments.

Laser scanning confocal microscope detection for the optimal ratio of Lipofectamine® 2000 and HSP27 with best transfection efficacy. Flow cytometry results were confirmed with microscopy and after successful transfection at 8 μg:20 μl of HSP27: Lipofectamine® 2000, GFP positive expression was visible in the cytoplasm (Fig. 2).

Optimal interfering HSP27 plasmid identified using RT-qPCR. Data from the RT-qPCR are presented in Fig. 3 and indicated that the interference effect of the four different sequences was 48, 55, 59 and 30% with respect to the positive control group

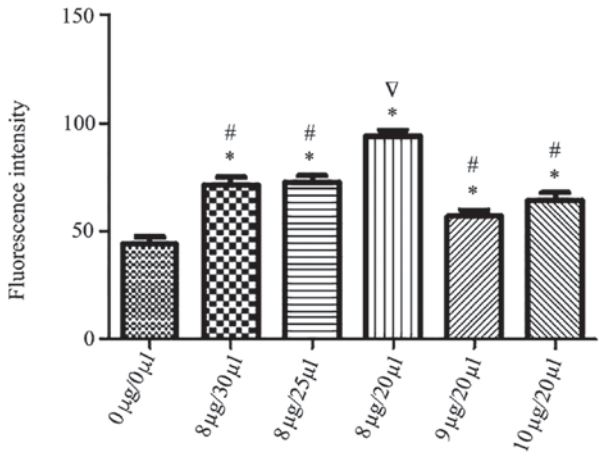

Figure 1. Comparison of transfection efficacy with different ratios of plasmid concentration to transfection reagent. Cells were transfected with different ratios of plasmid concentration: Lipofectamine® 2000 reagent and fluorescence density was examined by flow cytometry. With ratios of 8 μg:30 μl; 8 μg:25 μl; 8 μg:20 μl; 9 μg:20 μl; 10 μg:20 μl, the transfection efficacy was respectively 76, 76, 83, 70, 74% when compared with the 0 μg/μl plasmid control group. At 83%, the most effective transfection ratio was 8 μg:20 μl. *P<0.05 vs. control group, #P<0.05 vs. 8 μg:20 μl group. ▽Indicates most efficient plasmid/transfection ratio.

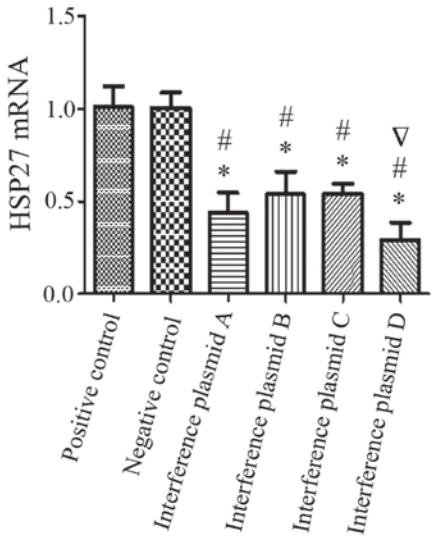

Figure 3. Comparison of silencing efficacy with four different interference fragments in alveolar type II epithelial cells. The optimal ratio of plasmid to transfection reagent was used (8 μg:20 μl) and four different interference plasmids (A, B, C, D) were transfected into A549 alveolar type II epithelial cells. HSP27 mRNA expression was detected using reverse transcription-quantitative polymerase chain reaction. The interference effect of each interference plasmid (A, B, C, D) was 48, 55, 59 and 30% when compared with the positive control group, and 48, 54, 58 and 30% when compared with the negative control group. From the four plasmids, plasmid D had the highest gene silencing effect with 70% HSP27 gene silencing. *P<0.05 vs. positive control, #P<0.05 vs. negative control. ▽Indicates most efficient interference fragment plasmid. HSP27, heat shock protein 27.

and 48, 54, 58 and 30% relative to negative control group. The fourth interfering sequence (D) achieved the most efficient gene silencing of HSP27 with 70% of the HSP27 gene being silenced, which was statistically significant compared with positive control and negative control group.

Transfection efficacy and the expression of type I and III collagen. Data from western blot experiments revealed that compared with the HSP27 negative control group, the expression of HSP27 and phosphorylated HSP27 in transfection of HSP27-interfering plasmid group was downregulated (Fig. 4; P<0.05). The difference was statistically significant compared with the transfection of GAPDH in the positive control group, the expression of GAPDH was significantly upregulated in transfected with HSP27 interfering plasmid and the HSP27 negative control group (P<0.05). This indicated that the transfection of HSP27 interference plasmid was successful. Additionally, findings from the western blot analysis revealed that compared with the blank plasmid transfection group, the expression of HSP27 and pHSP27 increased, which was accompanied by the increased expression of type I and III collagen in blank plasmid group, which was induced with TGF-β1 (Fig. 5), which was statistically significant (P<0.05).

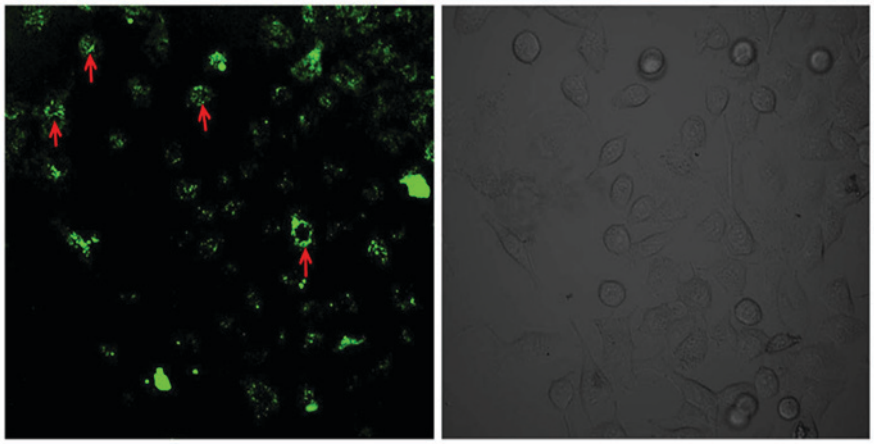

Figure 2. Alveolar type II epithelial cell transfection using the 8 μg:20 μl as a ratio of plasmid to transfection reagent. Laser scanning confocal microscope imaging indicated that following transfection with 8 μg:20 μl as a ratio of heat shock protein 27 plasmid to transfection reagent Lipofectamine® 2000, green fluorescent protein-positive expression was observed in the cytoplasm (red arrows, left panel). The right panel corresponds to the transfected cells under a bright-field microscope. Magnification, x800.

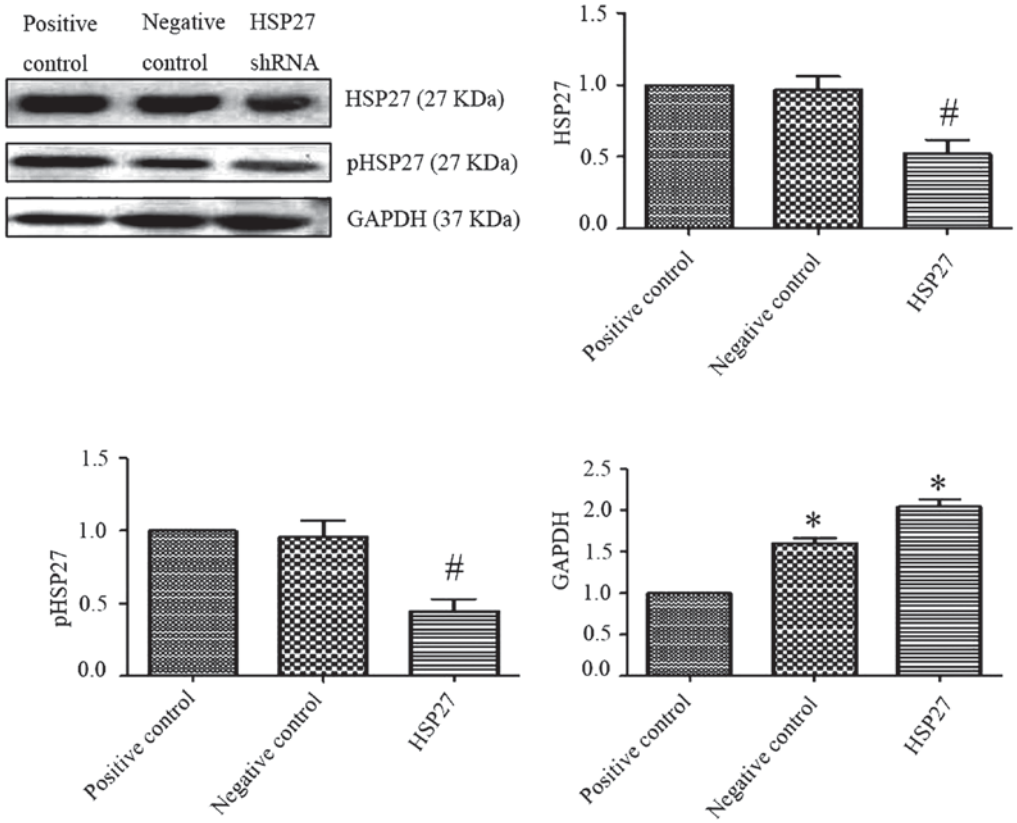

Figure 4. Transfection of HSP27-interfering plasmid into A549 alveolar type II epithelial cells. Western blotting revealed that under optimal ratio of plasmid to Lipofectamine® 2000, the expression of HSP27 and p-HSP27 was downregulated in the HSP27shRNA D-interfering fragment transfected A549 cells when compared with HSP27 plasmid negative control group. The expression of GAPDH in HSP27-interfering plasmid group and negative control group was upregulated when compared with the GAPDH positive control group. This indicated that the transfection was successful. *P<0.05 vs. HSP27shRNA positive control, #P<0.05 vs. HSP27shRNA negative control. shRNA, short hairpin RNA; HSP27, heat shock protein 27; p-HSP27, phosphorylated-HSP27.

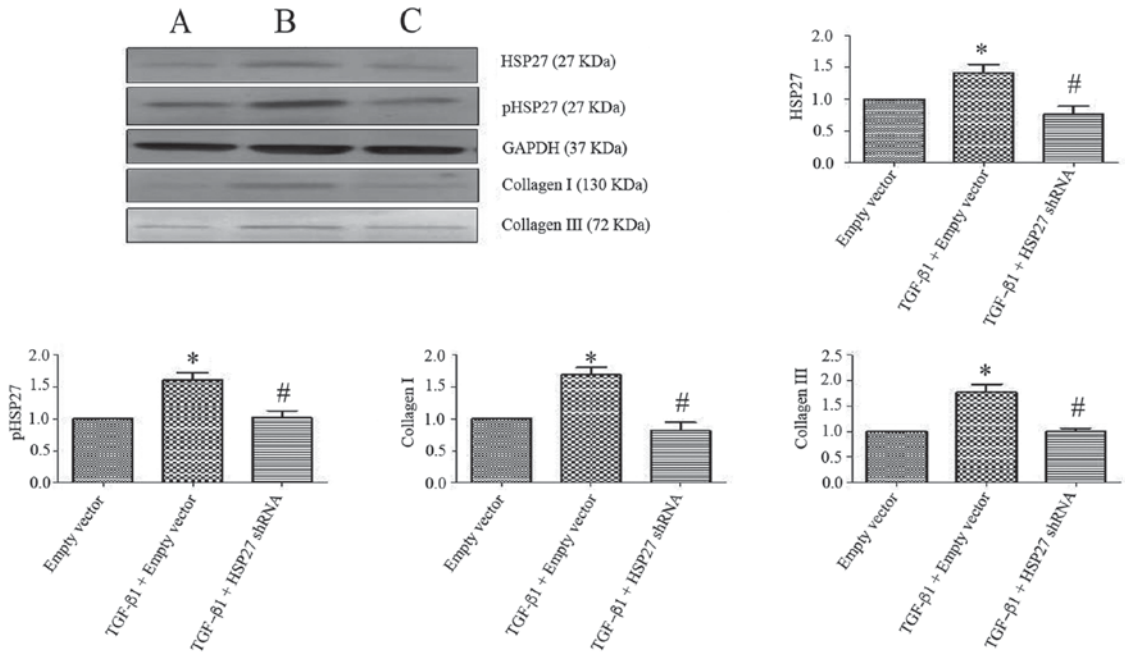

Figure 5. Effect of HSP27 interfering plasmid on the expression of type I and III collagen in TGF-β1-induced alveolar type II epithelial cell. Western blotting revealed that compared with the empty vector transfection group, the expressions of HSP27, pHSP27, and type I and III collagen were upregulated in TGF-β1-induced empty vector transfection group. Following transfection with an HSP27-interfering plasmid, cells were treated with TGF-β1. The expression levels of HSP27, pHSP27, type I and III collagen were downregulated in HSP27-interfering plasmid group when compared with the TGF-β1-induced empty vector group. (A) Vector transfection group. (B) TGF-β1-induced empty vector transfection group. (C) HSP27-interfering plasmid group. The difference was statistically significant. P<0.05. TGF-β1, transforming growth factor-β1; HSP27, heat shock protein 27; p-HSP27, phosphorylated-HSP27; shRNA, short hairpin RNA.

The expression of HSP27 and pHSP27 was significantly downregulated along with the reduced expression of type I and III collagen in the TGF-β1-induced HSP27-interfering plasmid group, compared with the TGF-β1-induced blank plasmid transfection group ($P<0.05$).

Discussion

Cationic liposome is a promising non-virus gene vehicle (15,16), whose membrane property confers various advantages in application terms of therapy and study of gene transfection, including maintaining the biological activity of genes, protecting genes from degradation by lysosomes. It is also non-immunogenic, easy to apply, with high repeatability and it is naturally degraded (17-25). The current study used Lipofectamine® 2000 to transfect A549 human alveolar type II epithelial cell line with HSP27-interfering plasmid; the procedure was easy, safe and reliable with high transfection efficacy, and met the requirement of subsequent experiments. Therefore, this method provides a reference in the application of Lipofectamine® 2000-mediated transfection of other cell lines.

HSP27 is a small molecular weight protein in heat shock protein family (26). Previous studies have previously reported that HSP27 is involved in the tumor cell EMT (27-32). In breast cancer, tumor cells that undergo EMT exhibit a high expression of HSP27 protein, which indicated that HSP27 protein may be closely associated with breast cancer cell EMT and may be involved in tumor migration and drug resistance (32). When MET-5A pleural mesothelial cells were stimulated *in vitro* with TGF-β1, the expression of HSP27 was significantly upregulated and accompanied by the increased expression of α-smooth muscle actin (α-SMA) and reduced the expression of E-cadherin, whereas cell morphology changed from flat cobblestones to long spindle-shapes. When HSP27-specific inhibitor OGX-427 (antisense oligonucleotides) or HSP27 siRNA transfection were used the increased expression of HSP27 and α-SMA protein by TGF-β stimulation was effectively reduced (9). Additionally, the downregulation of E-cadherin expression was also inhibited and cell morphology was maintained as flat or round-like. This indicated that HSP27 may be important for fibrosis and involved in pleural mesothelial cell EMT (9). In NRK52E tubular epithelial cells, TGF-β1 stimulation led to the upregulation of HSP27 expression, accompanied by the upregulation of α-SMA and vimentin, as well as fibronectin expression and led to downregulation of E-cadherin expression. This suggested that HSP27 has a protective role in tubular epithelial cell EMT (10). Additionally, HSP27 had a protective effect on myocardial cells in ischemic heart disease and adriamycin-induced myocardial injury due to this anti-apoptotic effect (33,34). Therefeore, it is important to investigate the effect of HSP27 on A549 human alveolar type II epithelial cell EMT. Extracellular matrix (ECM) deposition is a direct cause of fibrogenesis (35-39). Type I and III collagen are essential ECM components that reflect the differentiation extent of myofibroblasts (40-43). In our previous studies, we found that 5 ng/ml TGF-β1 was able to successfully induce the transition of A549 human alveolar type II epithelial cells to myofibroblasts, accompanied with upregulation of type I and III collagen expression (13). In A549 cells transfected with HSP27-interfering plasmid, the expression of type I and III collagen was downregulated by TGF-β1 stimulation, which suggested that HSP27 is a factor that may induce fibrosis in the transition of TGF-β1-induced A549 human alveolar type II epithelial cells to myofibroblasts. The present study provided the experimental basis and theoretical evidence for further investigation of the effect of HSP27 *in vivo* on EMT in silicosis or fibrosis.

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Availability of data and materials

The analyzed data generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

FY conceived and designed the experiments. HJD, XMG, HBP and XLH performed the experiments. HJD, HX and JW analyzed the data. FY wrote the manuscript and HJD provided support on reagents/materials/analysis tools.

Ethics approval and consent to participate

A549 human alveolar type II epithelial cell line derived from human lung carcinoma purchased from Cell Bank of Chinese Academy of Science, Shanghai, China which was received ethics approval. The manuscript involves no other human participants, human data or human tissue.

Consent for publication

All authors consent for publication.

Competing interests

The authors declare that they have no competing interests.

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