

Down-regulation of angiotensin II by shRNA reduces collagen synthesis in hepatic stellate cells

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Abstract. The angiotensin-converting enzyme 2 (ACE-2), angiotensin II type I receptor (ATIR) antagonists and angiotensin-converting enzyme inhibitors (ACEI) were explored to block the renin-angiotensin-aldosterone system (RAAS). The experimental results were still not satisfactory, mainly due to excessive level of angiotensin II (AngII) in gene expression. RNA interference (RNAi) is a mature gene blocking technique, able to block target gene expression efficiently, specifically and continuously. In this study, we observed the effect of short hairpin RNA (shRNA) expression vectors targeting rat AngII on collagen synthesis in hepatic stellate cells (HSCs). According to rat AngII gene sequences, three AngII targeted shRNA expression vectors were designed and constructed. Using liposomes as transfection reagents, they were transfected into HSC-T6 cells. Enzyme digestion confirmed that the transfected shRNA target gene segment was successfully cloned to the vectors. Compared with the control group, AngII mRNA expression examined in shRNA1, shRNA2 and shRNA3 groups was inhibited by about 37, 30 and 61%, respectively. AngII protein expression in all three groups was also reduced by about 21, 24 and 59%, respectively. Furthermore, we revealed that the inhibitory effect exhibited a dose- and time-dependent relationship. In shRNA3 group, TGF- β 1 mRNA expression was reduced by about 51%. The levels of PIIIP, HA and LN were decreased by about 53, 47 and 58%, respectively. In conclusion, shRNA expression vectors targeting rat AngII can decrease collagen synthesis, which would hopefully serve as a foundation for RNAi study of liver fibrosis *in vivo*.

Introduction

Liver cirrhosis is the late stage of various chronic liver diseases. The 5-year mortality of decompensated cirrhosis is 70-86%,

seriously endangering human health. So far, the only effective treatment of advanced cirrhosis is liver transplantation. But more post-operative complications, high costs of surgery and severe shortage of donor livers restrict the launching of liver transplantation (1). Liver fibrosis is the intermediate link in the progression from chronic liver diseases to cirrhosis. Therefore, early prevention or reversion of liver fibrosis is of great significance in improving the prognosis of various chronic liver diseases, deferring the development of cirrhosis and reducing the economic burden of patients (2).

Angiotensin II (AngII) has a wide spectrum of biological effects, which can not only regulate blood pressure and water-salt metabolism, but also promote cell proliferation, regulate extracellular matrix (ECM) metabolism and increase expression of cytokines (3). AngII is involved in the occurrence and development of cardiac and renal fibrosis (4,5). Currently, angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II type I receptor (ATIR) antagonists have been widely used in the clinical treatment of cardiac and renal fibrosis (6,7). However, there are few studies on liver fibrosis.

A recent study found that there was a local renin-angiotensin-aldosterone system (RAAS) in the liver (8). In a study of cultured rat hepatic stellate cells (HSCs), AngII promoted the contraction of HSC in a dose- and time-dependent manner, thereby promoting HSC proliferation and DNA synthesis (9). Therefore, AngII is considered as an important profibrogenic factor involved in initiation and maintenance of fibrogenesis in the liver.

Strategies aimed at disrupting RAAS are being extensively investigated for treating liver fibrosis and various animal studies have demonstrated an antifibrotic effect (10-12). Currently, angiotensin-converting enzyme 2 (ACE-2), ACEI and ATIR antagonist were explored to block RAAS. But the experimental results were still not satisfactory, which was mainly due to excessive level of AngII in gene expression. These inhibitors worked only at the protein level. Therefore, a new synthesis of AngII was still an endless stream of additional loss of AngII. RNA interference (RNAi) technique is a promising therapeutic strategy because of its potent knock down of the target gene with high sequence specificity (13,14). There is evidence that RNAi technique can be used to treat a number of diseases, such as hereditary diseases, viral hepatitis and certain cancers (15-17).

It is generally believed that activation of HSCs is the intermediate link of initiating the development of liver fibrosis

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(18). TGF- β 1 is generally accepted as the strongest factor that activates HSCs (19). In this study, AngII-targeted short hairpin shRNA expression vectors were tested in HSC-T6 cells for gene silencing and therapeutic efficacy, which would hopefully provide a potent tool for the prevention and treatment of liver fibrosis via an RNAi technique.

Materials and methods

Materials. Materials used in the present study included plasmid pGenesil1.1, *E. coli* DH-5 α , restriction endonucleases and T4 DNA ligase (Wuhan Genesil Biotechnology Co., Ltd., Wuhan, China), HSC-T6 cell strain (Institute of Tumor Research of the Chinese Academy of Medical Sciences, Beijing, China), Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA), fetal bovine serum (FBS) (HyClone, Logan, UT), RPMI-1640 medium and 0.25% trypsin (Gibco, Carlsbad, CA), goat anti-rat AngII polyclonal antibody (Santa Cruz, Santa Cruz, CA), HRP labeled rabbit anti goat secondary antibody (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China), StarPrep Plasmid Miniprep Kit, DNA maker, RT-PCR assay kit and RNAiso™ Plus extraction reagents (Takara, Dalian, China). PIIP, HA and LN ELISA kits were purchased from Shanghai Hai Institute of Medical and Biological Technology Co., Ltd. (Shanghai, China).

Design of small interfering RNA (siRNA). Target gene NM_134432.2 was searched from GenBank. According to Ambion's principles of siRNA design, three sequences of 21 nucleotides beginning with AA and containing 30-50% GC were selected and used as target sites. They were shRNA1, 5'-CUGAGAAUAAAUGAUGAGCtt-3' (sense), 5'-GCUC AUCAUUUAUUCUCAGtt-3' (antisense); shRNA2, 5'-UGG ACCAAACAACUGUUUGtt-3' (sense), 5'-CAAACAGUUG UUUGGUCCAtt-3' (antisense); and shRNA3, 5'-ACAACU GUUUGUGAAUAAAtt-3' (sense), 5'-UUUUUUCACAAA CAGUUGUtt-3' (antisense). Another unrelated sequence was used as control. No homology sequence was found by Blast analysis. The sequence structure was as follows: Eco31I + sense + loop + antisense + stop signal + Sal + Eco31I. Single-stranded DNA oligonucleotide was synthesized and annealed to form double strands by Wuhan Genesil Biotechnology Co., Ltd.

Construction of AngII targeted shRNA expression vectors. Plasmid pGenesil1.1 was digested by Eco31I, and large segments were harvested by 1% agarose. The gene segment and the linearized plasmid were connected to recombine a new target plasmid. Competent bacterium DH-5 α was then transformed and cultured in LB medium containing anti-Kana activity overnight. The plasmid was extracted with the plasmid extraction kit, and positive clones were analyzed by SacI digestion.

HSC-T6 cell culture. HSC-T6 cells were seeded to 25 cm² flasks, to which 5 ml RPMI-1640 mixed medium containing 10% FBS and 100 U/ml penicillin, 100 μ g/ml streptomycin, 4 mmol/l glutamine and 1 mol/l HEPES was added, and incubated in a 37°C, 5% CO₂ thermostatic incubator. Cells were grown in confluence, and passaged with 0.25% trypsin at 2-3 day intervals.

Plasmid transfection. HSC-T6 cells were digested and dispersed with 0.25 g/l trypsin, and seeded in 6-well plates. When the density of the cell slide reached ~87%, they were transfected in five groups as follows, (i) control group; (ii) negative group (shRNA non-related group); (iii) shRNA1 group; (iv) shRNA2 group; and (v) shRNA3 group. Each group was composed of 4 wells. Transfection was performed according to the protocol of the kit. Briefly, HSC-T6 cells were seeded in 6-well plates at a density of 1.8x10⁵ cells/well and cultured for 24 h to reach 87% confluence. ShRNA expression vectors were mixed with Lipofectamine 2000 in Opti-MEM I medium for 20 min at room temperature to allow complex formation. The transfection mixture was then added to each well with 2 ml of FBS free DMEM medium. After 5 h of incubation, 0.2 ml of FBS was added and incubated for another 48 h.

RNA extraction and semi-quantitative RT-PCR. After 48 h later, total RNA was extracted by addition of RNAiso™ Plus in each group and dissolved with 30 μ l dermase to determine the concentration by UV spectrophotometry. The quality of RNA was testified by agarose electrophoresis. RT-PCR was performed by the two-step method. Reverse transcription used random 9mers as random primers and BcaBest polymerase as reverse transcriptase under the condition of tranquilization at 30°C for 5 min, polymerization at 65°C, extension for 30 min, inactivation of the RT at 98°C for 5 min, and stabilization at 5°C for 5 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference in PCR. AngII up- and downstream primer sequences were as follows, forward, 5'-CTCCCAGAGCCAACCTTT-3'; reverse, 5'-ACACTTCT CCCTCTGACG-3'; the amplification length was 327 bp. TGF- β 1 up- and downstream primer sequences were as follows, forward, 5'-CAAAGACATCACACACAGTA-3'; reverse, 5'-AGGTGTTGAGCCCTTTCCAG-3', the amplification length was 410 bp; GAPDH up- and downstream primer sequences were as follows, forward, 5'-CAG TGCCAGCCTCGTCTCAT-3'; reverse, 5'-AGGGGCCATC CACAGTCTTC-3'; the amplification length was 595 bp. The primers were designed and synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China). PCR conditions were as follows, degeneration at 94°C for 1 min, annealing at 55°C for 50 sec and extension at 72°C for 1 min, totaling 30 cycles. The PCR products were electrophoresed with 1.5 g/l agarose, photographed and analyzed with the gel imaging system (Bio-Rad, Hercules, CA) to determine the absorbance (A) of the respective target genes and GAPDH amplification products, and ratios were calculated.

Protein extraction and Western blot assay. After 48 h, cells were washed with ice-cold phosphate buffered saline (PBS). Total protein was extracted by addition of 50 μ l protein lysis buffer per 5x10⁶ cells, and centrifuged at 12,000 rpm at 4°C for 20 min. The supernatant was harvested, and protein concentration was determined by UV spectrophotometry and Bradford method, and kept at -70°C. The above cell protein extraction solution was electrophoresed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresed protein was electrically transferred to the nitrocellulose membrane

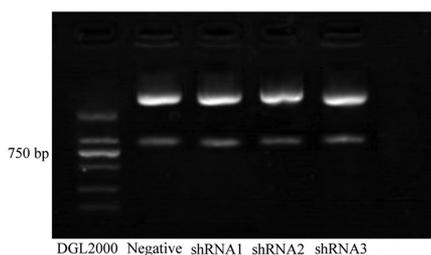


Figure 1. Identification of AngII targeted shRNA expression vector digestion. Plasmid AngII and plasmid vector pGenesil1.1, in each only one SacI restriction site, was digested by SacI. DNA (1000 bp) band was digestible, indicating that the target gene segment AngII had been inserted into pGenesil1.1.

and sealed in 50 g/l skimmed milk in PBS for 3 h, to which goat anti-rat AngII polyclonal antibody was added and wet cultured at 4°C overnight. The nitrocellulose membrane was washed with 3 g/l Tween-PBS for 15 min x3. The washed membrane was placed in HRP labeled rabbit anti-goat secondary antibody and wet cultured by oscillation for 2 h, and then washed with 3 g/l Tween-PBS for 15 min x3. GAPDH was used as the control. The antigen-antibody complex was developed by enhanced chemiluminescence, exposed in the dark room and analyzed for integral absorbance (IA) of the protein bands using quantitative software, Quantity One 4.4.

Optimization of the dosage and time of shRNA expression vector delivery. The shRNA expression vector that exhibited the most potent inhibitory effect was selected and transfected into HSC-T6 cells at different doses (1, 2, 4, 6 and 8 µg). After 48 h, cells were harvested and AngII mRNA expression was analyzed by RT-PCR to determine the optimal dosage of the shRNA expression vector. The selected shRNA expression vector with the maximum inhibitory effect on AngII was transfected at the optimal dosage into HSC-T6 cells. The AngII mRNA expression was analyzed at 12, 24, 48 and 72 h after transfection to determine the best time point for inhibition.

Effect of shRNA expression vector on TGF-β1 mRNA expression. The shRNA expression vector that exhibited the most marked inhibitory effect was selected and transfected into HSC-T6 cells at the optimal dosage. After the best time point for inhibition, total RNA was extracted by addition of RNAiso™ Plus. RT-PCR was performed by the two-step method.

ELISA of PIIP, HA and LN. The shRNA expression vector that exhibited the most potent inhibitory effect was selected and transfected into HSC-T6 cells at the optimal dosage. After the best time point for inhibition, we collected the supernatant of the cultured cells and detected the levels of PIIP, HA and LN according to the protocol of ELISA kits.

Statistical analysis. Data are expressed as means ±SD, and statistical treatment was performed by least significant difference (LSD).

Results

Identification of enzyme digestion. AngII targeted shRNA expression vectors were digested by SacI. DNA band (1,000 bp)

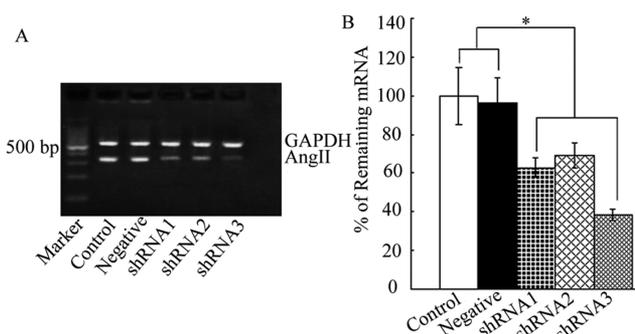


Figure 2. Effect of three pairs of shRNA expression vectors on AngII mRNA expression. Three pairs of shRNA expression vectors were transfected into HSC-T6 cells for 48 h. Compared to the control group, AngII mRNA expression were inhibited in the shRNA1, shRNA2 and shRNA3 groups ~37, 30 and 61%, respectively. (A) Results of RT-PCR. (B) Statistical analysis. Data are presented as the mean ±SD (n=4). *p<0.05.

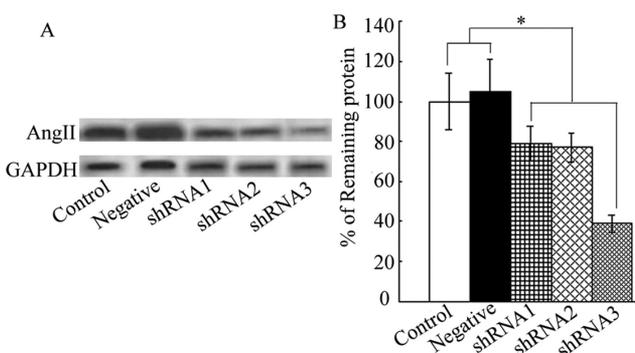


Figure 3. Effect of three pairs of shRNA expression vectors on AngII protein expression. Three pairs of shRNA expression vectors were transfected into HSC-T6 cells for 48 h. Compared to the control group, AngII protein expression was decreased in the shRNA1, shRNA2 and shRNA3 groups was ~21, 24 and 59%, respectively. (A) Results of Western blotting. (B) Statistical analysis. Data are presented as the mean ±SD (n=4). *p<0.05.

was digestible, indicating that the target gene segment AngII had been inserted into pGenesil1.1 (Fig. 1).

Transient transfection of HSC-T6 cells. Using Lipofectamine™ 2000 as the transfection reagent, shRNA expression vectors were transfected into HSC-T6 cells. After 48 h, green fluorescence was seen in transfected cells under the fluorescence microscope, the transfection rate being about 40%.

AngII mRNA expression of HSC-T6 cells in different groups. The result of RT-PCR showed that there was no decline in AngII mRNA expression in the negative group as compared with the control group, and that the three shRNA groups showed varying degrees of inhibitory effect, of which the shRNA3 group exhibited the strongest inhibitory effect (Fig. 2).

AngII protein expression of HSC-T6 cells in different groups. Western blotting showed that there was no decline in AngII protein expression in the negative group as compared with the control group, and that the three shRNA groups showed varying degrees of inhibitory effect, of which shRNA3 group exhibited the strongest inhibitory effect (Fig. 3).

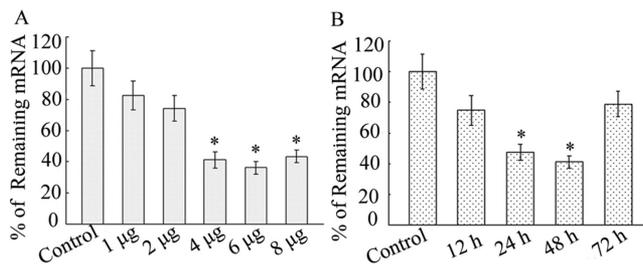


Figure 4. Dose- and time-dependent down-regulation of AngII mRNA by shRNA expression vector. (A) Effect of shRNA expression vector dose on AngII silencing. The inhibitory effect of shRNA expression vector in shRNA3 group on AngII increased concomitantly with increased doses. Compared with the control group, the inhibitory rates at 1, 2, 4, 6 and 8 μg dosage were 18, 26, 58, 64 and 57%, respectively. The maximum inhibitory effect was observed at 6 μg dosage. Data are presented as the mean \pm SD (n=4). * p <0.05. (B) Persistence of shRNA expression vector gene silencing. The time points were 12, 24, 48 and 72 h. Compared with the control group, the inhibitory rates were 25, 52, 59 and 21%, respectively. The maximum inhibitory effect was observed at 48 h. Data are presented as the mean \pm SD (n=4). * p <0.05.

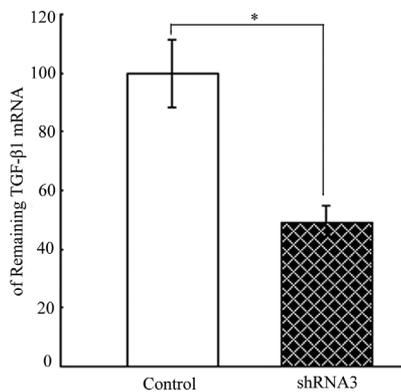


Figure 5. Effect of shRNA expression vector on TGF- β 1 mRNA expression. shRNA expression vector in shRNA3 group was transfected into HSC-T6 cells at 6 μg for 48 h. Compared with the control group, TGF- β 1 mRNA expression was inhibited by ~51%. Data are presented as the mean \pm SD (n=4). * p <0.05.

Dose- and time-dependent down-regulation of AngII by shRNA expression vector. As shown in Fig. 4, there was a significant increase in the AngII silencing effect as we increased the doses of shRNA expression vector from 1 to 8 μg , showing that the inhibitory effect of shRNA expression vector on AngII was dose-dependent. AngII mRNA expression was examined at 12, 24, 48 and 72 h post-transfection. It was significantly decreased with the time prolonging, the lowest being at 48 h post-transfection.

Effect of shRNA expression vector on TGF- β 1 mRNA expression. TGF- β 1 is the key profibrogenic cytokine in response to chronic liver injuries. It not only increases ECM synthesis but also suppresses ECM degradation. We investigated whether shRNA expression vector could lower TGF- β 1 mRNA expression. We designed TGF- β 1 mRNA specific primers to determine the effect of AngII gene silencing on TGF- β 1 mRNA expression. As seen in Fig. 5, compared with the control group, shRNA expression vector in the

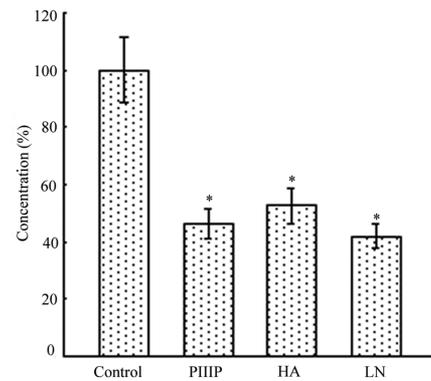


Figure 6. Effect of shRNA expression vector on PIIP, HA and LN secretion. shRNA expression vector in shRNA3 group was transfected into HSC-T6 cells at 6 μg for 48 h. Compared with the control group, the levels of PIIP, HA and LN were reduced ~53, 47 and 58%, respectively. Data are presented as the mean \pm SD (n=4). * p <0.05.

shRNA3 group exhibited a marked inhibition of TGF- β 1 mRNA expression, which was decreased by 51%.

Effect of shRNA expression vector on PIIP, HA and LN. To evaluate whether shRNA expression vector could enhance the degradation of ECM, we detected the levels of PIIP, HA and LN secreted in culture media. As shown in Fig. 6, compared with the control group, levels of PIIP, HA and LN in the shRNA3 group were reduced by 53, 47 and 58%, respectively.

Discussion

Liver fibrosis is the consequence of imbalanced synthesis and degradation of the hepatic ECM due to continuous action of various detrimental factors, which include chronic hepatitis, alcohol abuse and toxic agents (20). It was reported that activated HSC expressed ATIR (21). After AngII combining with ATIR to form a complex, AngII promoted HSC proliferation and ECM synthesis by activating phospholipase C, guanylyl cyclase, tyrosine kinase, mitogen-activated protein kinase and other means (22). Therefore, RAAS plays an important role in the process of liver fibrosis. Currently, strategies aimed at blocking RAAS are being extensively explored.

AngII is excessive in gene expression, whereas inhibitors such as ACE-2, ACEI and ATIR antagonists worked only at the protein level. Therefore, these traditional treatments are still not satisfactory. RNAi technique is a relatively mature gene blocking technique at present, able to block intracellular target gene expression efficiently, specifically and continuously. The reason that RNAi has the significant inhibitory effect is its mechanism. siRNA of 21-23 nt in length binds with a ribozyme complex, forming so-called RNA-induced silencing complex (RISC). The activated RISC is localized to homogeneous mRNA by base pairing, and digests mRNA at the place of 12 bases from the siRNA3' end.

Selection of a potent siRNA sequence targeting a specific gene is a crucial step in developing its therapeutic applications. Using Lipofectamine™ 2000, we transfected shRNA expression vectors into HSC-T6 cells. The results showed



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AngII targeted shRNA expression vectors were effectively inhibit the expression of AngII mRNA.

Compared with the control group, AngII mRNA expression examined in the shRNA1, shRNA2 and shRNA3 groups were inhibited by about 37, 30 and 61%, respectively. Therefore, the shRNA3 group exhibited the maximum inhibitory effect. AngII protein expression was also significantly reduced in all three groups, 21, 24 and 59%, respectively, relative to that of the control group. While there was no inhibitory effect observed in the negative group. Studies on siRNA silencing effect revealed that the target mRNA secondary structure region combined with the siRNA antisense strand had a strong influence on siRNA activity (23). If the mRNA secondary structure region was complex, siRNA was difficult to combine with this region. The effect of RNAi would be affected or even completely non-functional. In this experiment we successfully constructed three AngII targeted shRNA expression vectors. At the same time, the use of RNA structure 4.5 software predicted the secondary structure of the target gene mRNA and found three pairs of siRNA position in the secondary structure. Semi-quantitative RT-PCR and Western blotting test results showed that siRNA located in the relatively simple region in the secondary structure of the target gene inhibited ~60%, yet siRNA located in the relatively complex region inhibited <60%. It showed that the secondary structure of target gene mRNA is an important factor affecting the effectiveness of RNAi.

As shown in Fig. 4, shRNA expression vector in shRNA3 group showed dose-dependent inhibition of AngII mRNA. Even though the dose increased to 8-fold, from 1 to 8 μ g, the expression of AngII mRNA did not increase much. The inhibitory ability of shRNA expression vector had no linear relationship with the dose. One of two reasons was that low-dose shRNA expression vector could also exert an obvious inhibitory effect because of its efficient action (24). The other reason was that lipidosome was also increased with the elevation in shRNA dose, causing poisoning to HSC-T6 cells (25). The level of AngII mRNA was significantly lowered with time, which was the lowest at 48 h post-transfection compared to the control group. The reason might be the relatively higher stability and consistent expression ability of the shRNA expression vector inside HST-6 cells (26).

HSCs are a major source of ECM. Activation of HSCs is the intermediate link of initiating the development of liver fibrosis. Many cytokines are involved in activation, proliferation and secretion of HSCs. Among these complex cytokines, TGF- β 1 is generally accepted as the strongest factor activating HSCs. This study showed that AngII targeted shRNA expression vector significantly decreased TGF- β 1 mRNA expression in HSCs, which was consistent with the result of Li *et al* (27). The inhibition mechanism may be achieved through blocking the protein kinase c pathway and its downstream protein tyrosine kinase pathways (28). The signaling pathways can induce cells to produce c-fos and c-jun that are combined into AP-1 complex, which is a *trans*-acting factor with leucine zipper structure. TGF- β 1 gene transcription starts after it has combined with AP-1.

Due to the complexity of ECM components, we must take joint detection indicators. PIIP can reflect collagen synthesis of HSCs. HA can reflect liver damage. LN, the main component

of the basement membrane, can reflect hepatic sinusoidal capillarization. The present findings showed that AngII targeted shRNA expression vector could reduce effectively the levels of PIIP, HA and LN. One of the reasons was that AngII targeted shRNA expression vector was able to reduce TGF- β 1 gene expression.

In summary, we successfully constructed AngII targeted shRNA expression vectors that inhibited TGF- β 1 mRNA expression and decreased collagen synthesis, thus laying a solid foundation for RNAi study of liver fibrosis *in vivo*.

Acknowledgements

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