Effects of small interfering RNA-mediated downregulation of the Krüppel-like factor 4 gene on collagen metabolism in human hepatic stellate cells

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Received August 21, 2014; Accepted May 1, 2015

DOI: 10.3892/mmr.2015.3848

Abstract. The nuclear transcription factor Krüppel-like factor 4 (KLF4) has an important role in cellular biological processes. However, the influence of KLF4 on collagen metabolism remains to be elucidated. In the present study, the effects and underlying mechanism of action of KLF4 on collagen metabolism was investigated in human hepatic stellate cells (HSC), by downregulating KLF4 expression using small interfering RNA (siRNA). The effects of KLF4 silencing by three predesigned siRNAs (siRNA1-3) were evaluated using both reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting in the human LX2 HSC line. The mRNA expression levels of KLF4 were decreased by ~34, 40, and 69% in the siRNA1, siRNA2, and siRNA3 groups, respectively, as compared with the control group. These results were concordant with the protein expression levels of KLF4, as determined by western blot analysis. In the siRNA3 group, the quantity of type I and type III collagen, and the expression levels of collagen metabolism proteins including matrix metalloproteinase-1 (MMP-1) and tissue inhibitors of metalloproteinases-1 (TIMP-1), were determined using both RT-qPCR and western blotting. Both the mRNA and protein expression levels of type I and type III collagen were significantly decreased in the siRNA3 group, as compared with the control group. The mRNA and protein expression levels of TIMP-1 were also significantly reduced in the siRNA3-treated cells, whereas the mRNA and protein expression levels of MMP-1 were significantly upregulated. Furthermore, KLF4 gene silencing significantly decreased the expression levels of numerous cytokines, including transforming grow factor-β1, tumor necrosis factor-α, and interleukin-1β. The results of the present study provide evidence of siRNA-mediated silencing of KLF4 expression, which may promote extracellular matrix (ECM) degradation, and inhibition of ECM synthesis. Therefore, KLF4 may be a promising target for the development of novel antifibrotic therapies.

Introduction

Hepatic fibrosis results from chronic injuries to the liver caused by chronic hepatitis, alcohol abuse, toxic agents, metabolic diseases involving an overload of iron or copper, autoimmune diseases, or congenital abnormalities (1,2). Hepatic fibrosis, which may ultimately lead to cirrhosis, is the pathological basis of all chronic hepatic diseases, and is characterized by an imbalance between the excessive synthesis and decreased degradation of extracellular matrix (ECM) components, specifically type I and type III collagen (3,4). The pathophysiology of ECM formation during fibrosis is multifaceted and complex, and is associated with alterations in the expression levels of both ECM proteases, such as matrix metalloproteinases (MMPs), and ECM protease inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs) (5). ECM formation also requires an increase in the synthesis of fibronectin and collagens (5). The process of ECM formation is maintained by growth factors, such as transforming growth factor-β1 (TGF-β1), and connective tissue growth factor, as well as pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 (6,7). Activated hepatic stellate cells (HSCs) are known to participate in ECM remodeling by producing various types of collagen, MMPs, TIMPs, and TGF-β1, thus deeply influencing fibrotic progression and regression (8,9). Numerous studies have indicated that the activation of HSCs is the cytological basis and the main initiator of hepatic fibrosis (10-13). Consequently, the inhibition of HSC proliferation has become an important antifibrotic therapeutic strategy.

Krüppel-like factors (KLF) are a subclass of the zinc-finger family of transcription factors. They are characterized by a DNA-binding domain that contains a conserved sequence, CX2CX3FX5LX2HX3H (14). The KLF family
consists of ≤16 members, which are in turn separated into structurally associated subgroups (15). KLFs regulate gene expression and are responsible for cell proliferation, apoptosis, differentiation, embryonic development, and somatic cell reprogramming (16,17). KLFs are also important regulators in the pathogenesis of various diseases, including ECM remodeling (18-21). Human KLF4 was initially identified in 1998 from a human umbilical vein endothelial cell cDNA library, using a DNA probe containing the zinc-finger region of human erythroid KLF (22). KLF4 has many important functions, including the regulation, proliferation, and differentiation of various epithelial and endothelial tissues (23,24).

Recent studies have shown that KLF4 regulates the expression of certain genes, including MMP-1, MMP-13, TIMP-1, and TGF-β1, and is responsible for ECM remodeling in Sprague-Dawley rat and mouse aortic vascular smooth muscle cell lines (25,26). However, the effects of KLF4 on HSCs and hepatic fibrosis remain unknown. In the present study, KLF4 expression was inhibited by transfecting chemically synthesized KLF4-specific small interfering (si)RNA into human LX2 HSCs, with the aim of ascertaining the effect of KLF4 on the ECM and its associated genes.

Materials and methods

Materials. The LX2 human HSC line was donated by Professor D.X. Sun (Division of Liver Diseases, Bethune International Peace Hospital, Shijiazhuang, China) and was originally sourced from the Institute of Tumor Research of the Chinese Academy of Medical Sciences (Beijing, China). Lipofectamine® 2000 and TRIzol® reagent were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS), Dulbecco’s modified Eagle medium (DMEM), and Opti-MEM were purchased from GE Healthcare Life Sciences (Logan, UT, USA). Rabbit anti-human KLF4 monoclonal antibody (cat. no. ab51733), rabbit anti-human collagen type I monoclonal antibody (cat. no. ab138492), rabbit anti-human collagen type III monoclonal antibody (cat. no. ab7778), and rabbit anti-human TIMP-1 monoclonal antibody (cat. no. ab109125) were purchased from Epitomics, Inc. (Burlingame, CA, USA). Rabbit anti-human MMP-1 polyclonal antibody (cat. no. 10371-2-AP) was purchased from ProteinTech Group, Inc. (Chicago, IL, USA). IRDye800-conjugated monovalent goat IgG secondary antibody (cat. no. 61-113-002) was purchased from Rockland Research Corp. (Rockland, MA, USA). The reverse transcription (RT) reagents were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The RT-quantitative polymerase chain reaction (RT-qPCR) assay kit was purchased from BioTeke Corporation (Beijing, China). The TGF-β1, TNF-α, and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Shanghai ExCell Biological Products Co., Ltd. (Shanghai, China).

Design of siRNAs. Using the Homo sapiens KLF4 mRNA nucleotide sequence from GenBank (GI: 194248076; http://www.ncbi.nlm.nih.gov/nuccore/NC_004235.4), and referring to the standard design strategy for siRNAs (27), three pairs of 21 bp reverse repeat sequences targeting KLF4 mRNA were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China); siRNA1 sense, 5’-UCAUUAACAAAGC UCAUTT-3’, antisense, 5’-AUGAGCCUCUGGUAUUG ATT-3’; siRNA2 sense, 5’-GGUCAUCAGCGUCAGCA ATT-3’, antisense 5’-UUUGCUAGCCGCAUGACCTT-3’; and siRNA3 sense, 5’-GGACUUUAUCUCCAAUTT-3’, and antisense, 5’-UUUGGAGAAGAAGUCCTT-3’. An unrelated sequence was used as a control, sense, 5’-UCUCCC GAAGCGUCAGGUTT-3’ and antisense, 5’-ACGUGACAC GUUCGAGAATT-3’.

Cell culture. The LX2 cells were cultured in DMEM supplemented with 12% FBS, 100 U penicillin, and 100 µg streptomycin (Sangon Biotech Co., Ltd., Shanghai, China), at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed every 2 days. The cells were subsequently digested with 0.25% trypsin (Gibco Life Technologies, Carlsbad, CA, USA) once the cells had reached 80-90% confluence.

siRNA transfection. The LX2 cells were digested and dispersed with 0.25% trypsin, prior to being seeded in 6-well plates. Once the cells had reached 70-80% confluence, they were separated into four groups and transfected with various siRNAs as follows: Control group; siRNA1 group; siRNA2 group; and siRNA3 group. Triplicate wells were established for each group. The cells were transfected with siRNA using Lipofectamine® 2000, according to the manufacturer’s instructions. Briefly, the LX2 cells were seeded into 6-well plates at a density of 1.8×10⁵ cells/well, and cultured for 24 h until they reached ~80% confluence. The 5 µl siRNAs (20 µM) were subsequently mixed with 5 µl Lipofectamine® 2000 in 250 µl Opti-MEM medium for 20 min at room temperature to allow complex formation. The transfection mixture was then added to each well with 2 ml FBS-free DMEM. Following a 6 h incubation, 200 µl FBS was added to the mixture and incubated for an additional 24 h or 48 h, prior to RNA harvesting and protein isolation.

RNA purification and RT-qPCR. Following an additional 24 h incubation period, the total RNA was isolated using TRIzol® reagent, and reverse transcribed into cDNA according to the manufacturer’s instructions. The cDNA (10 ng) was then used as the template for qPCR. Using the primer design software Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA), the specific primers for each gene were synthesized by BGI (Beijing, China) as follows: KLF4 forward, 5’-ATCTTT CTCCAGCTTGCCTG-3’, reverse, 5’-GGAGTCTGGCT ATGTTGGA-3’; type I collagen forward, 5’-CGAGGCCG CAAAGGTCTCATC-3’, reverse, 5’-TCATGTAACCCG GCGTT-3’; type III collagen forward, 5’-CGCCTCTCT CATTGGTCAAGG-3’, reverse, 5’-TCTCTGGAGAGCA GCTGA-3’; MMP-1 forward, 5’-CATGCTTTTCAAGCA GCC-3’, reverse, 5’-GGTACATCAAGCCCGAT-3’; and TIMP-1 forward, 5’-ACTTTCAAGCTCCCAAC-3’, and reverse, 5’-GCAATTCCTACAGCCCAACA-3’. GAPDH was used as an internal control and had the following primer sequence: Forward, 5’-TGTATCTGAGGACGACTA-3’, and reverse 5’-CCAGTACGAGGCGGATGAT-3’. RT-qPCR was performed in a Corbett rotor real-time cycler (Qiagen China Co., Ltd., Shanghai, China). The PCR consisted of an
initial denaturation step of 5 min at 95°C, 40 cycles of 10 sec at 95°C, 15 sec at 55°C and 15 sec at 72°C, followed by a heating step that involved the passage from 70°C to 99°C at a rate of 0.1°C/sec, allowing the acquisition of sufficient data to produce the denaturing curve of the amplified products. The comparative threshold method was used to calculate the relative levels of mRNA in the treated samples, as compared with the amount in the control group samples (28, 29). Each treatment was performed in triplicate, and the results were presented as the mean ± standard deviation.

Protein extraction and western blotting. After 48 h, the cells were washed with ice-cold phosphate-buffered saline (PBS). The total protein was subsequently extracted using 50 µl protein lysis buffer (Sangon Biotech Co., Ltd.) per 5x10^6 cells, prior to centrifugation of the cells at 12,000 x g for 20 min at 4°C. The supernatant was harvested, and the protein concentration was determined using a bicinchoninic acid assay (Sangon Biotech Co., Ltd.) and then stored at -80°C. The protein samples (60 µg) were then subjected to 8 or 12% SDS-PAGE, prior to being electrically transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). The membranes were then blocked with 5% non-fat milk in PBS containing 0.1% Tween® 20 for 1 h at room temperature, and were incubated with either anti-KLF4 (1:1,000), anti-collagen I (1:200), anti-collagen III (1:250), anti-MMP1 (1:300), or anti-TIMP1 (1:200) antibodies in tris-buffered saline containing 0.05% Tween® 20 at 4°C overnight. The washed membranes were then incubated with IRDye800-conjugated secondary antibody (1:20,000) for 1 h at 37°C, prior to being scanned with the Odyssey Infrared Imaging system (Li-COR Biosciences, Lincoln, NE, USA). The integrated intensity for each detected band was determined using ImageJ 1.46 software (National Institutes of Health, Bethesda, MA, USA). β-actin was used as the control (cat. no. ab197161; polyclonal rabbit IgG; 1:4,000; Abcam, Cambridge, UK).

ELISA of TGF-β, TNF-α, and IL-1β. At 48 h post-transfection the supernatant of the cultured cells was collected and the concentrations of TGF-β1, TNF-α and IL-1β were measured using ELISA kits according to the manufacturer’s instructions. The absorbance was measured using a microplate spectrophotometer (XMark; Bio-Rad Laboratories, Inc.) at 450 nm. TGF-β1, TNF-α and IL-1β levels were calculated based on a standard curve.

MTT cell viability assay. The cells in the exponential growth phase were seeded into 96-well plates at a density of 5x10^3/ml with 200 µl added to each well. Six repeated wells and negative control wells were used for each group. The HSCs were subsequently transfected with siRNA3 and cultured in DMEM supplemented with 12% FBS in 96-well plates for 12, 24, and 48 h, and 20 µl MTT solution (5 mg/ml; Sangon Biotech Co., Ltd.) was added to each well. The cells were then cultured for a further 4 h, and the solution was replaced with 150 µl dimethyl sulfoxide (Sangon Biotech Co., Ltd.). The absorbance value (A) was measured at 492 nm using an enzyme-labeling instrument (XMark; Bio-Rad Laboratories, Inc., Hercules, CA, USA), and subsequently used to calculate viability rate. Viability rate=(Aexperimental/Acontrol) x 100%.

Table I. Effects of KLF4 on LX2 hepatic stellate cell viability, as determined by an MTT assay.

<table>
<thead>
<tr>
<th>Group</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
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<tbody>
<tr>
<td>Control</td>
<td>101.25±1.35</td>
<td>100.69±1.22</td>
<td>101.56±2.31</td>
</tr>
<tr>
<td>KLF4</td>
<td>75.56±3.56a</td>
<td>52.35±2.34a</td>
<td>50.14±2.41a</td>
</tr>
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Results are presented as the mean ± standard deviation (n=6). *P<0.05, vs. the control group; KLF4, Krüppel-like factor 4.

Figure 1. Effects of the three siRNAs on the mRNA expression levels of KLF4. The three siRNAs were transfected into the LX2 hepatic stellate cells for 24 h. KLF4 mRNA expression was inhibited in the siRNA1, siRNA2, and siRNA3 groups by ~34, 40, and 69%, respectively, as compared with the control group. The results are presented as the mean ± standard deviation (n=3). *P<0.05, vs. the control group. siRNA, small interfering RNA; KLF4, Krüppel-like factor 4.

Statistical analysis. The data are expressed as the mean ± standard deviation, and were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Independent t-tests, one way analysis of variance, and least significant difference tests were subsequently carried out. P<0.05 was considered to indicate a statistically significant difference.

Results

Silencing of KLF4 expression with synthetic siRNAs. Using Lipofectamine® 2000 as the transfection reagent, siRNA1, siRNA2, and siRNA3 were transfected into the LX2 cells. The results of the RT-qPCR indicated that the mRNA expression levels of KLF4 were decreased by ~34, 40, and 69% in the siRNA1, siRNA2, and siRNA3 groups, respectively (Fig. 1). Western blot analyses showed that the protein expression levels of KLF4 were also inhibited to various extents in the three siRNA groups. siRNA3 exhibited the strongest inhibitory effect (Fig. 2), and was therefore selected to determine the effects of KLF4 gene silencing.

Effects of KLF4 siRNA on collagen synthesis in LX2 cells. The mRNA and protein expression levels of type I and type III collagen were determined by RT-qPCR and western blotting, respectively. The results indicated that the mRNA and protein expression levels of type I and type III collagen were significantly decreased in the siRNA3 group, as compared with the
Effects of KLF4 siRNA on collagen degradation in LX2 cells. Following the transfection of KLF4-specific siRNA into the LX2 cells, the results of the RT-qPCR and western blot analyses indicated that the mRNA expression levels of MMP-1 were significantly upregulated after 24 h in the siRNA3 group, and that the protein expression levels of MMP-1 were also significantly upregulated after 48 h, as compared with the control group. Furthermore, knockdown of KLF4 expression by KLF4 siRNA significantly reduced the mRNA and protein expression levels of TIMP-1 (Fig. 4).

Effects of KLF4 siRNA on TGF-β1, TNF-α, and IL-1β. The expression levels of TGF-β1, TNF-α, and IL-1β were measured using an ELISA 48 h post-transfection. The expression levels of the four cytokines were markedly decreased, as compared with the control group (178±20.3 pg/ml TGF-β1, vs. 52±8.1 pg/ml TGF-β1; 48±5.4 pg/ml TNF-α, vs. 35±4.1 pg/ml TNF-α; and 80±6.2 pg/ml IL-1β, vs. 41±5.7 pg/ml IL-1β; P<0.05) (Fig. 5).

KLF4 siRNA inhibits the viability of LX2 cells. KLF4 siRNA3 significantly inhibited the growth of LX2 cells, as compared with the control group. The viability rates of LX2 cells were determined to be 75.56% at 12 h, 52.35% at 24 h, and 50.14% at 48 h post-transfection (Table 1).

Discussion

RNA interference is an advanced gene blocking technique that permits the efficient, specific, and continuous inhibition of intracellular gene targets. The selection of a potent siRNA sequence targeting a specific gene is one of the most...
important steps in order to allow sufficient inhibition of gene expression. Numerous studies on the silencing effects of siRNA have revealed that the binding of the target mRNA secondary structure region to the siRNA antisense strand has a strong influence on the level of siRNA activity (30,31). If the mRNA secondary structure is complex, the siRNA will combine with less efficiency to the region. In the present study, three synthetic siRNAs were designed to target the coding regions of KLF4 mRNA, located between 909 -1702 bp. Using Lipofectamine® 2000, the siRNAs were subsequently transfected into LX2 cells. The results indicated that all three KLF4 siRNAs were able to effectively inhibit the mRNA and protein expression of KLF4, and siRNA3 exhibited the maximum inhibitory effect. The variations in the inhibitory effects of the siRNAs may be due to differences in the local secondary structures of the KLF4 mRNA, and to variations in the accessibility of the 1682-1702 bp region.

A substantial alteration in liver fibrosis or cirrhosis is due to the deposition of ECM, which is predominantly composed of type I and type III collagen, which accounts for ~80-90% of increased total collagen (32). The increases in type I and type III collagen is an important indicator of liver fibrosis (33). Although numerous hepatic cell types are able to synthesize ECM proteins, HSCs are unequivocally the primary cells involved in the production of excessive ECM detected in liver fibrosis (34). Therefore, in the present study, KLF4 siRNAs were transfected into HSCs in order to investigate the mRNA and protein expression levels of type I and type III collagen. The results indicated that the expression levels of type I and type III collagen were significantly decreased in the siRNA3 group, as compared with the control group. Furthermore, the results of an MTT cell viability assay indicated that KLF4 siRNA significantly inhibited the growth of HSCs. These data demonstrated that KLF4 siRNA was able to effectively

Figure 4. Effects of KLF4 gene silencing on MMP1 and TIMP1 expression levels. (A) siRNA3 was transfected into the LX2 hepatic stellate cells for 24 h. As compared with the control group, TIMP1 mRNA expression was inhibited, whereas MMP1 mRNA expression was increased. The results are presented as the mean ± standard deviation (n=3). (B) The protein expression levels of MMP1 and TIMP1 were determined using western blotting, and the extent of protein inhibition was compared against that of the control. (C) The data are presented as the mean ± standard deviation (n=3). *P<0.05, vs. the control group. MMP1, matrix metalloproteinase-1; TIMP-1, tissue inhibitors of matrix metalloproteinase-1; siRNA, small interfering RNA; KLF4, Krüppel-like factor 4.

Figure 5. ELISA measurements of TGF-β1, IL-1β and TNF-α. KLF4 siRNA3 was transfected into the LX2 hepatic stellate cells for 48 h. As compared with the control, the levels of (A) TGF-β1, (B) IL-1β and (C) TNF-α were significantly reduced. The data are presented as the mean ± standard deviation (n=3). *P<0.05, vs. the control group. siRNA, small interfering RNA; KLF4, Krüppel-like factor 4; siRNA; TGF, tumor growth factor; TNF, tumor necrosis factor; IL, interleukin.
suppress the synthesis of collagen by HSCs, and that KLF4 gene silencing may be a promising target for novel antifibrotic therapies.

The present study also assessed the role of KLF4 in the regulation of ECM metabolism in HSCs. MMPs are a class of calcium-dependent enzymes that have a major role in ECM degradation (35). There are currently eight subcategories of MMPs that have been identified in the liver. Current evidence indicates that, except for the granulocyte MMP-8, MMP-1 is the only collagenase with specificity for native interstitial type I and type III collagens produced in the liver (36). Previous studies have demonstrated that a negative correlation exists between MMP-1 and the degree of liver fibrosis, and that MMP-1 expression is inhibited in liver fibrosis (37-39). MMP-1 activity is regulated by TIMP-1, interacting at a 1:1 stoichiometry ratio (40). During fibrosis, the mRNA and protein expression levels of TIMP-1 are markedly increased. Therefore, the imbalance between MMP-1 and TIMP-1 is a principal feature of hepatic fibrosis (41,42). The present study showed that the silencing of KLF4 expression was able to increase MMP-1 expression, and reduce TIMP-1 expression in HSCs. Thus KLF4 gene silencing may promote ECM degradation.

Numerous cytokines are involved in the activation, proliferation, and secretion of HSCs. Among these complex cytokines, TGF-β1 is widely accepted as the strongest activating factor for HSCs (43). Based on data from HSCs and liver damage in animal models, a conclusive statement regarding liver fibrosis may be drawn: TGF-β1 is required for liver fibrosis and the reduction of TGF-β1 signaling reduces fibrogenesis (44-46). A recent study revealed that elevated KLF4 binds to the TGF-β1 promoter region and activates TGF-β1 transcription, which leads to ECM synthesis in myofibroblasts (26). The results of the present study were consistent with these previous findings, and demonstrated that the downregulation of KLF4 by siRNA decreased the expression levels of TGF-β1 in HSCs. In addition, the process of liver fibrosis development is accompanied by inflammation, and a close association between pro-inflammatory cytokines such as TNF-α, and liver fibrosis and cirrhosis has been reported (47). The results of the present study showed that KLF4 gene silencing significantly decreased the expression levels of TNF-α, suggesting that KLF4 gene silencing also decreases liver inflammation. IL-1β is another important pro-inflammatory cytokine known to promote local inflammatory responses, and consequently promote chronic liver fibrosis (48). In addition, IL-1β protects TGF-β1 and TNF-α from proteolysis by modulating its bioactivity and bioavailability. In this microenvironment, the cytokines may have a key role in the onset of fibrosis, and in the continued inflammatory response (49). The results of the present study also indicated that there was a significant decrease in the amount of secreted IL-1β following KLF4 gene silencing.

In conclusion, knockdown of KLF4 expression significantly inhibited ECM synthesis and proliferation of HSCs, most likely through MMP-1 and TIMP-1 activity, as well as cytokine modulation. The inhibition of KLF4 by siRNA may be an efficient and specific approach for the development of novel therapeutic methods in the treatment of liver fibrosis. Further studies are required in order to clarify the underlying mechanism of action of KLF4 on the production of cytokines and proteolytic enzymes in HSCs, as well as in other cell types.

Acknowledgements

The present study was supported by the Key Project of Medical Science Research of the Hebei Provincial Bureau of Health (grant no. 20110558).

References


