Research Article

COX-2 Regulates the Proliferation and Apoptosis of Activated Hepatic Stellate Cells through CDC27

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Cyclooxygenase-2 (COX-2) is an important rate-limiting enzyme in the synthesis of prostaglandins (PGs), which can be upregulated by various stimuli. COX-2 has been shown to be involved in the occurrence and development of hepatic fibrosis by regulating the proliferation and apoptosis of hepatic stellate cells (HSCs) in previous studies. The aims of the study are to study the mechanism of how COX-2 regulates the proliferation and apoptosis of HSCs and to provide new targets for the prevention and treatment of hepatic fibrosis. A short hairpin RNA targeting COX-2 was constructed, and the changes in proliferation and apoptosis of liver tissue cells and HSCs were observed, respectively. COX-2-shRNA-1 significantly suppressed the proliferation of HSCs in vivo. Moreover, knockdown of COX-2 significantly suppressed cell proliferation and accelerated cell cycle arrest and apoptosis in vitro. Among those differential genes related to cell proliferation and apoptosis, CDC27 and Sh3kbp1 were upregulated, but Plcd4 was suppressed. Mechanistically, the influence of COX-2 on HSCs partly depends on upregulating CDC27. Our results demonstrated that COX-2 regulates the proliferation and apoptosis of activated hepatic stellate cells through the CDC27 pathway. This study contributes to our understanding of the effect of COX-2 for the treatment of hepatic fibrosis.

1. Introduction

Hepatic fibrosis, a common pathophysiological process in many chronic liver diseases, is manifested by the excessive and abnormal deposition of extracellular matrix components in the liver. It is the necessary stage for the development of chronic liver disease to cirrhosis. It is now believed that liver fibrosis may be reversed to normal, while cirrhosis is not. However, there is no effective method for the treatment of liver fibrosis.

Cyclooxygenase-2 (COX-2) is an important rate-limiting enzyme in the synthesis of prostaglandin (PGs). COX-2 is hardly expressed in normal tissues, but can be upregulated when stimulated by various factors. Extensive research has shown that COX-2 participates in some pathological processes, such as cell malignant transformation, inflammatory

response, and catalyzing the biosynthesis of arachidonic acid (AA) to affect disease processes. Research in the area of hepatic fibrosis showed that COX-2 is an important molecule that affects the development of it. Celecoxib, a COX-2 inhibitor, suppresses the hepatic fibrosis induced by bile duct ligation (BDL) and thioacetamide (TAA) [1]. Other researchers used CCL4 to replicate the model of hepatic fibrosis and found that COX-2 knockout mice had less necrosis, degeneration, and connective tissue hyperplasia than the wild-type mice [2]. These mice also had lower expression of α -smooth muscle actin (α -SMA) in the liver tissue and less serum hyaluronic acid (HA), collagen IV (IV-C), and collagen III (PC III). Short hairpin RNA was used to silence COX-2 in nonalcoholic fatty liver disease (NAFLD) rats, and the liver tissue steatosis, inflammatory degree, and hepatic fibrosis were significantly reduced in COX-2 silencing rats [3].

TABLE 1: The sequences and products of amplified gene primer.

Gene	Primer	Sequences	Length (bp)
COX-2	Forward	ATCTAGTCTGGAGTGGGAGG	420
	Reverse	AATGAGTACCGCAAACGCTT	
α -SMA	Forward	TGTGCTGGACTCTGGAGATG	292
	Reverse	GATCACCTGCCCATCAGG	
Serpib2	Forward	GTAGATGTGAACGAGGAGG	142
	Reverse	GTATGGTGC GGTAATGT	
Cdc27	Forward	ACTGGCGACCTGTTACTA	359
	Reverse	CTGGCTTCTCACCTATTT	
Sh3kbp1	Forward	AGAAATGGACAGCAGGAC	271
	Reverse	TTGTTTGATGACAGGAGC	
Dusp1	Forward	TTGTTTGATGACAGGAGC	171
	Reverse	TGGAGACAGGGAAAGTTGAAGA	
Dusp6	Forward	CACGGTGACAGTGGCTTAC	215
	Reverse	AGACATTCTGGTTGGAGG	
TP53	Forward	GGAGTGCAAAGAGAGCACTG	134
	Reverse	CTCATTACAGCTCTCGGAACA	
Cxcl10	Forward	TTCCTGCAAGTCTATCCTG	170
	Reverse	TGTTCTTCTTCATTGTGGC	
Plcd4	Forward	TTTTGGCAACATTAGCAC	438
	Reverse	GACAACCAGGGCAGATAG	
GAPDH	Forward	TATCGGACGCCTGGTTAC	852
	Reverse	GCATCAAAGGTGGAAGAAT	

CDC27 is a core subunit of anaphase-promoting complex/cyclosome (APC/C). APC/C is a type of E3 ligase complex that regulates chromosome segregation and mitotic exit [4]. APC/C reportedly participates in the TGF- β signaling pathway, and TGF- β activates CDC27, which promoted liver fibrosis via the TGF- β pathway in both HSC and hepatocyte [5]. Recent evidence suggests that COX-2 may be involved in the development of hepatic fibrosis by regulating the proliferation and apoptosis of hepatic stellate cells (HSCs). SC-236, a selective COX-2 inhibitor, had an antihepatic fibrosis effect and significantly promoted the apoptosis and growth inhibition of isolated cultured HSCs [6]. Celecoxib, another COX-2 inhibitor, has been reported to suppress the formation of hepatic fibrosis in rats by inhibiting the proliferation of platelet-derived growth factor- (PDGF-) induced HSCs and promoting the apoptosis of these HSCs [1].

However, the mechanism of how COX-2 regulates the proliferation and apoptosis of HSCs remains unclear. Therefore, this study silenced the expression of COX-2 in hepatocytes and hepatic stellate cells in liver fibrosis rats by using short hairpin RNA. Then the changes of proliferation and apoptosis of HSCs and related gene expression profiles after COX-2 silencing were observed. Based on our experimental results, we found that COX-2 regulated the proliferation and apoptosis of activated hepatic stellate cells through the CDC27 pathway.

2. Materials and Methods

2.1. Animal Model. 48 male SD rats (Nanjing Junke Bioengineering Co., Ltd.) were randomly divided into four groups by body weight: control group (control), liver fibrosis model group (model), null plasmid control group (sh-NC), and

COX-2-shRNA-1 group (COX-2-shRNA-1). Except for the control group, the other 3 groups were fed a high-fat diet for 12 weeks after a week of adaptive feeding. Rats in the COX-2-shRNA-1 group and the sh-NC groups were injected with adenovirus plasmids and adenovirus no-load physique grains (PBS dilution) through the caudal vein from the beginning of the high-fat diet, respectively. The dose and time of injection were 1×10^9 pfu/each and once weekly. Rats in the control group and the model group were given an equal volume of PBS through the caudal vein. After 12 weeks of rearing, the rats were sacrificed by caesarean section to take 0.5~1.5 g of the left lateral lobe of the rat liver and immediately put it into ice-cold William E medium filled with a mixed gas of 95% oxygen and 5% CO for subsequent experiments. This experiment had been approved by the Animal Management Committee of our hospital.

2.2. Reverse Transcription-PCR. Reverse transcription-PCR (RT-PCR) was used to detect the mRNA expression of COX-2, α -SMA, CDC27, Sh3kbp1, Serpinb2, cxcl10, Dusp6, Dusp1, TP53, and Phlcd4. Total RNA from each liver sample and HSCs was extracted by Trizol reagent (Generay Biotech, China), and cDNA was synthesized by using Revert Aid First Strand cDNA synthesis Kit (Fermentas, Germany), according to the manufacturer's instructions. PCR was performed under the manufacturer's manual. Band intensity was measured by a densitometer and was normalized for comparison. RT-PCR was performed on target gene using specific primers (Table 1). GAPDH was selected as the internal reference gene. The expression level of gene was analyzed using the equation $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta CT = \Delta CT_{\text{experimental group}} - \Delta CT_{\text{control group}}$; $\Delta CT_{\text{experimental group}} = CT_{\text{target gene, experimental group}} -$

$$\frac{CT_{\text{internal reference gene, experimental group}}}{T_{\text{target gene, control group}} - CT_{\text{internal reference gene, control group}}} \times 2^{-\Delta\Delta CT_{\text{control group}}} = C$$
 and $2^{-\Delta\Delta CT}$ represents the multiple expression of gene in the experimental group relative to the control group.

2.3. Immunohistochemistry. Liver tissues were fixed in 4% formaldehyde solution for 3 to 5 days. Then, tissues were removed from the fixative solution and trimmed to an appropriate shape and thickness. Tissues were dehydrated, transparented, and embedded in wax, then dewaxed with xylene and rehydrated with gradient alcohol. Three of the most important steps were blocking and inactivating endogenous peroxidase and performing antigen retrieval. Primary antibodies anti COX-2 and α -SMA were used and incubated overnight in a refrigerator at 4°C (PBS buffer solution instead of primary antibody as a negative control). The samples were transferred to room temperature and equilibrated for 30 minutes and rinsed with PBS for 3 × 5 minutes before adding a secondary antibody. The secondary antibody was incubated at 37°C for 15 minutes and rinsed with PBS for 3 × 5 minutes. DAB staining was used.

2.4. Culture of HSC Cell Lines. HSC cell lines (HSC-T6) were obtained from the American Type Culture Collection (ATCC) and were cultured at 37°C with 5% CO₂ in a cell incubator. Cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) plus 1% 100 µg/ml penicillin/streptomycin. Cells were seeded in 6-well plates and grown to reach 70% confluence before transfection. For transfecting cells, cells were transfected with different plasmids using FuGENE HD Transfection reagent (Roche, Switzerland) following the manual instruction. Then, transfected cells were harvested for molecular assays.

2.5. Cell Transfection. shRNA targeting COX-2 (COX-2-shRNA) or CDC27 (sh-CDC27) were synthesized by Win Run Biotechnology Co, Ltd. (Changsha, China). The applied shRNA sequences for COX-2 were as follows: COX-shRNA-1, 5'-CCTCGTCCAGATGCTATCTTT-3'; COX-shRNA-2, 5'-AGTTCCAGTATCAGAACCGCATTGCCTCT-3'; and COX-shRNA-3, 5'-GACACCTTCAACATTGAAGACCAG GAGTA-3'. The applied shRNA sequences targeting CDC27 were 5'-CCGGGCCTATAACAGTGACTTGATTC TCGAGAATCAAGTCACTGTTATAGGCTTTTGTG-3'. According to the manufacturer's protocol, HSC-T6 cells (5 × 10⁵) were seeded in 6-well plates, incubated for 24 h, and then 200 ng/ml specific shRNA or negative control (NC) was transfected into the cells by using Lipofectamine®2000 (Invitrogen; USA). Effects of the shRNA were evaluated by western blotting and RT-PCR. A fluorescence microscope was used to observe the expression of green fluorescent protein in cells to evaluate the fluorescence density.

2.6. MTT Assay. Cells were treated for 24 h, 48 h, and 72 h. Then, 5 mg/ml MTT solution (in PBS) was added into the basal medium at 1:10 ratio. After 4-hour incubation, the remaining MTT solution was removed, and cells were solubilized with dimethyl sulfoxide. Optical density (OD) values were measured at the wavelength of 490 nm to reflect cell via-

bility: cell proliferation rate = (experimental group OD – control group OD)/(NC group OD – control group OD).

2.7. Flow Cytometry Assay. HSC-T6 cells were harvested after 48 h posttransfection. The culture medium was then changed into serum-free Hank's balanced salt solution. Then, cells were rinsed in pre-cold PBS. Fluorescein isothiocyanate-(FITC-) conjugated Annexin V and propidium iodine (PI) staining buffer were sequentially added. Cell apoptosis was then measured on a flow cytometry (BD, USA).

2.8. Cell Cycle Assay. Cell cycle of HSC-T6 cells was monitored by flow cytometry. After 48 h posttransfection, all groups of HSC-T6 cells were digested by trypsin and were washed in precold PBS. Cells were fixed by 70% cold ethanol overnight and were resuspended in 100 µg/ml RNase A in PBS for 30 min incubation at 37°C. Cell nuclei were stained by 50 µg/ml PI for 30 min. Cell cycle was then measured on flow cytometry using fluorescent-assisted cell sorting (FACS).

2.9. DNA Microarray Assay and Data Analysis. Microarray-based oligonucleotide hybridization approach was used to quantify expression levels of target gene [4]. In brief, total RNA was obtained as mentioned above. Then, the obtained sample RNA was labeled by fluorescence and turned into DNA. The labeled DNA was dissolved in 80 µl hybridization solution (3 × SSC, 0.2% SDS, 5 × Denhart's, and 25% formamide) and hybridized at 42°C overnight. After the hybridization is completed, it was first washed in a liquid containing 0.2% SDS and 2 × SSC at 42°C for 5 min and then washed in a 0.2 × SSC medium for 5 minutes in room temperature. After the slide is dried, it can be used for scanning. LuxScan 3.0 software (CapitalBio Corp., China) was used firstly to transform images into digital signals. Linear correction across chips was performed based on global mean values of cy5 and cy3 signals for normalization of means. All data from this experiment can be divided into three groups: (1) all data include the whole signal data set containing those with or without differential expressions, and ratio values were defined after Lowess normalization. (2) Checked genes include those genes with effective signals after assay. (3) Differentially expressed genes were screened out and annotated by pathway and GO database for functional assay and statistical analysis using Capital Bio software.

2.10. Western Blotting. Western blotting was used to quantify the expression of COX-2 and CDC27. In brief, rat hepatic tissues or cultured HSCs were lysed in RIPA buffer and proteins were quantified by BCA kit. About 20–40 µg protein samples were loaded onto SDS-PAGE gel for electrophoresis separation and were transferred to PVDF membrane. After blocking, the membrane was incubated with primary antibody including rabbit anti-COX-2 (#12282, CST, USA), rabbit anti-CDC27 (#9499, CST, USA), and rabbit anti-β-actin (#4970, CST, USA) overnight. On the next day, HRP-conjugated secondary antibody was added for room temperature incubation for 2 h. The membrane was then developed by ECL substrate, and images were captured by a computerized system.

FIGURE 1: COX-2 expression in each group after knocking down COX-2. (a) Green fluorescent protein expression was observed by a fluorescent microscope after transfection with COX-2-shRNA-1, COX-2-shRNA-2, and COX-2-shRNA-3 (magnification 100x). (b) RT-PCR was used to measure the efficiency of shRNA-mediated gene knockdown in 24, 48, and 72 hours. (c) The protein levels of COX-2 were significantly inhibited by COX-2-shRNA-1. * $p < 0.05$; *** $p < 0.001$; and ns: no significant difference, compared with the sh-NC group and control group.

2.11. Statistics. All data were presented as the mean $\pm$ standard deviation (SD). The differences between two groups were analyzed using unpaired Student's t -test. One-way ANOVA was used for multiple groups. A statistical significance was defined when $p < 0.05$. All statistical analyses were performed using SPSS18.0 software.

3. Results

3.1. COX-2 Expression in Each Group after Knocking Down COX-2. To evaluate the transfection efficiency of COX-2-shRNA, the fluorescence density of cells exceeded 70–85%, as observed by the fluorescent microscope (Figure 1(a)). No

FIGURE 2: (a) Western blotting for the protein expression of COX-2 after shRNA injection in hepatic tissues. (b) Western blotting for the protein expression of α -SMA after shRNA injection in hepatic tissues. (c) Detection the expression of COX-2 and α -SMA by immunohistochemistry in the liver tissue in rats (magnification 200x). * $p < 0.05$, compared with the sh-NC group and COX-2-shRNA-1 group.

fluorescent signal was observed in the control group, indicating a successful cell transfection. All the three-specific shRNA of COX-2 downregulate the COX-2 expression after transfection of 48 hours and 72 hours, and the most significant effect was caused by COX-2-shRNA-1 ($p < 0.001$, Figure 1(b)). After transfection of 24 hours, COX-2 mRNA decreased in the COX-2-shRNA-1-transfected group ($p < 0.05$). Further analysis of the results showed that a remarkably decreased COX-2 protein expression in the COX-2-shRNA-1-transfected group at 48 h ($p < 0.05$) while the other two groups showed no major difference (Figure 1(c)). What emerges from the results reported here is that the construction of shRNA targeting COX-2 is successful and can be used for further experiments.

3.2. Knockdown of COX-2 Suppresses Proliferation of HSCs in Liver Tissue of Rats with Hepatic Fibrosis. To investigate the effect of COX-2 on proliferation and apoptosis of HSCs in the liver tissue of rats with hepatic fibrosis, we firstly

generated a rat model in which COX-2 nanomedicine-shRNA-1 was injected. As the first evidence to support COX-2 nanomedicine for liver fibrosis, western blotting showed that the expression level of COX-2 nanomedicine in liver fibrosis model rats was significantly increased, and it can be effectively inhibited by transfection with COX-2 nanomedicine-shRNA-1 ($p < 0.05$, Figure 2(a)). As the indicator of HSC proliferation, the expression patterns of α -SMA showed similarity with COX-2 nanomedicine, as shown by the elevated expression in the model group and the downregulated expression in the COX-2 nanomedicine-shRNA-1 group ($p < 0.05$, Figure 2(b)). Detection of COX-2 nanomedicine and α -SMA in liver tissues by immunohistochemistry indicated that the expression trends of COX-2 nanomedicine and α -SMA were consistent with the results of western blotting (Figure 2(c)). Taken together, these results suggest that knockdown of COX-2 nanomedicine suppresses proliferation of HSCs in the liver tissue of rats with hepatic fibrosis.

(a)

(b)

FIGURE 3: Continued.

FIGURE 3: Knockdown of COX-2 suppressed stellate cell proliferation and induced cell apoptosis. (a) MTT assay for relative cell proliferation rate of HSCs after COX-2 knockdown. (b) Cell cycle analysis for cultured HSCs after COX-2 knockdown. (c) Flow cytometry approach for measuring changes in apoptotic rate after COX-2 knockdown. * $p < 0.05$; ** $p < 0.01$; and ns: no significant difference, compared with the sh-NC group.

3.3. Cox-2-shRNA-1 Suppressed Proliferation and Induced Apoptosis of HSCs In Vitro. Next, we investigated the effect of COX-2 knockdown on hepatic cell proliferation and apoptosis in vitro. Cell proliferation was remarkably inhibited after transfection at 72 h as demonstrated by the MTT assay ($p < 0.05$, Figure 3(a)). Further analysis of the cell cycle revealed that knockdown of COX-2 increased the percentage of cells arresting at G1 phase (Figure 3(b)). Moreover, cell apoptotic rate in the COX-2-shRNA-1 group was found significantly higher by FACS ($p < 0.01$, Figure 3(c)). Overall, these results suggest that knockdown of COX-2 effectively suppressed hepatic stellate cell proliferation and accelerating their apoptosis.

3.4. Changes in Expression of Dynamic Genes of Hepatic Stellate Cells after Knockdown of COX-2. To further investigate the mechanism of COX-2 in regulating proliferation and apoptosis of hepatic stellate cells, gene chip assay was used to compare differential between the untreated and treated cells. Gene chip results showed that 37 genes were upregulated and 8 genes were downregulated after shRNA transfection with 48 hours (Table 2). Analysis of cells after 72 hours transfection showed that 50 genes in HSCs showed increased, while 46 genes showed decreased (Table 3). We also performed GO annotation on those differentially expressed genes, among which three proliferation-associated genes were upregulated (Il6, Csf2, and Atf3) at 48 h, but without any downregulated gene. At 72 h, three proliferation genes (CDC27, TP53, and Cxcl10) were unregu-

lated and three genes (DUSP1, Serpinb2, and Plcd4) were downregulated. For all 8 genes associated with cell apoptosis, we found four of them (Il1b, Il6, Ccl2, and Phlda3) were upregulated at 48 h and without any gene down-regulated. At 72 h, Tp53 was upregulated while Sh3kbp1, Pea15, and Plcd4 were suppressed (Tables 1–3). Those genes with significant changes were further validated by RT-PCR. As the results demonstrated that COX-2-shRNA-1 elevated CDC27, but decreased the Plcd4 gene transcripts. However, opposite trends were observed as Sh3kbp1 was upregulated, plus DUSP1 upregulated, in sharp contrast with DNA chip assay (Figures 4(a) and 4(b)). No significant change was found in other candidate genes (Figure 4(c)). In summary, COX-2-shRNA-1 modulated the cell proliferation and apoptosis related to the change in the expression of CDC27 and Plcd4.

3.5. COX-2-shRNA-1 Regulated the Proliferation and Apoptosis of HSCs through Regulating CDC27. CDC27 has been demonstrated in modulating cell proliferation [7] and apoptosis of tumor cells [8]. Therefore, we speculate that COX-2 may affect cell proliferation and apoptosis by affecting CDC27. Our conjecture was confirmed by the results that knockdown of COX-2 significantly elevated the protein level of CDC27, and this can be attenuated by knockdown of CDC27 (Figure 5(a)). The role of CDC27 on hepatic stellate cell proliferation was further investigated. Compared with the COX-2-shRNA-1 group, increased cell proliferation in the COX-2-shRNA-1 + sh-CDC27 group indicated the

TABLE 2: Differential gene expression profiles between COX-2-shRNA-1 and sh-NC at 48 hours posttransfection.

	Oligo_id	Name	Ratio	Description
1	Rn30002068	Ppef2	7.8223	Protein phosphatase
2	Rn30009092	—	3.1274	Immunoresponsive gene 1
3	R001746_01	Cxcl10	2.4468	Small inducible cytokine B10 precursor
4	Rn30015483	Gbp4	2.4271	Similar to guanylate nucleotide-binding protein 4
5	Rn30001057	Oasl1	2.4164	2'-5'-Oligoadenylate synthetase-like 1
6	Rn30004185	—	2.3045	Antisense RNA overlapping MCH protein.
7	Rn30021148	Ifit3	2.2743	Tetratricopeptide repeat 3
8	Rn30015485	Gbp4	2.2279	Similar to guanylate nucleotide-binding protein 4
9	Rn30001055	Oasl1	2.2037	2'-5'-Oligoadenylate synthetase-like 1
10	Rn30020145	—	2.1558	
11	R000595_01	Cx3cl1	2.0349	Chemokine (C-X3-C motif) ligand 1
12	Rn30020616	Cxcl11	1.9534	Chemokine (C-X-C motif) ligand 11
13	R001188_01	Il1b	1.9086	Interleukin-1 beta precursor (IL-1 beta).
14	Rn30005625	Mdm2	1.8729	Similar to mdm2 gene product
15	Rn30024865	isg12(b)	1.8722	Putative ISG12(B) protein.
16	Rn30016690	Isg20	1.8707	Interferon-stimulated protein (predicted)
17	Rn30021722	Apol9a	1.8058	
18	Rn30005624	Mdm2	1.767	Similar to mdm2 gene product
19	R003548_01	Kcnt1	1.7082	Potassium channel subfamily T member 1
20	Rn30015487	Gbp5	1.6855	Similar to guanylate-binding protein 5
21	R003549_01	Csf2	1.6817	Colony-stimulating factor
22	R001026_01	Atf3	1.665	Cyclic AMP-dependent transcription factor ATF-3
23	Rn30004011	Scin	1.6441	Scinderin
24	R001191_01	Il6	1.6382	Interleukin-6
25	Rn30015486	Gbp5	1.6332	Similar to guanylate-binding protein 5
26	Rn30008444	Ifi271	1.6328	Putative ISG12(a) protein
27	Rn30008951	Elovl4	1.6118	Similar to Elovl4
28	Rn30016019	Irf7	1.5875	Interferon regulatory factor 7
29	R002891_01	Cyp2d4v1	1.573	Cytochrome P450 2D18
30	Rn30001796	Mx2	1.5626	Interferon-induced GTP-binding protein Mx3.
31	R000644_01	Ccl2	1.5596	Chemokine (C-C motif) ligand 2
32	Rn30023693	—	1.5285	—
33	Rn30026248	—	1.528	Macrophage inflammatory protein 2-beta precursor
34	Rn30016526	Ccdc37	1.5222	Similar to C230069K22Rik protein
35	Rn30012826	Aadac	1.5185	Arylacetamide deacetylase
36	Rn30001056	Oasl2	1.5141	2'-5'-Oligoadenylate synthetase-like 2
37	Rn30008269	Phlda3	1.502	Pleckstrin homology-like domain family A member 3
38	R003539_01	Mapk8ip1	0.6656	C-Jun-amino-terminal kinase-interacting protein 1
39	Rn30026086	—	0.6545	Ankyrin repeat domain 11
40	Rn30018744	Ptov1	0.6483	Prostate tumor overexpressed gene 1
41	Rn30003851	Sh3kbp1	0.6376	SH3-domain kinase binding protein 1
42	Rn30001871	—	0.6282	Steroid-sensitive protein 1
43	R002280_01	Thra_v2	0.6144	Thyroid hormone receptor alpha
44	R002312_01	Pla2g2a	0.512	Phospholipase A2, membrane-associated precursor
45	Rn30001610		0.4945	Leucine-rich repeats and calponin homology (CH) domain containing 3

TABLE 3: Differential gene expression profiles between COX-2-shRNA-1 and sh-NC at 72 hours posttransfection.

	Oligo_id	Name	Ratio	Description
1	Rn30022454	—	6.1452	Tripartite motif-containing 65
2	Rn30006362	Purb	3.4474	
3	R001746_01	Cxcl10	2.8634	Small inducible cytokine B10 precursor
4	Rn30020145	—	2.652	
5	Rn30001057	Oasl1	2.6028	2'-5'-Oligoadenylate synthetase-like 1
6	Rn30005291	Cdc27	2.4796	Cell division cycle 27 homolog
7	Rn30006471	—	2.3552	PFTAIRE protein kinase 1
8	Rn30015483	Gbp4	2.3519	Similar to guanylate nucleotide-binding protein 4
9	Rn30021673	—	2.3392	"C
10	R000595_01	Cx3cl1	2.3344	Fractalkine precursor
11	Rn30001055	Oasl1	2.3048	2'-5'-Oligoadenylate synthetase-like 1
12	Rn30002812	Enah	2.2328	Enabled homolog
13	Rn30018127	—	2.2025	Diaphanous homolog 1
14	Rn30024865	isg12(b)	2.2007	Putative ISG12(B) protein
15	Rn30025470	—	2.1365	RNA-binding motif protein 27
16	Rn30025880	—	2.0773	Topoisomerase (DNA) II beta-binding protein
17	Rn30021148	Ifit3	2.0594	Human interferon-inducible proteins
18	Rn30002530	Rfc1	2.035	VIP-receptor-gene repressor protein
19	Rn30006955	—	1.9683	Peptidyl-prolyl <i>cis-trans</i> isomerase G
20	Rn30024339	Gbf1	1.9515	Golgi-specific brefeldin A-resistance factor 1
21	Rn30015485	Gbp4	1.9459	Similar to guanylate nucleotide-binding protein 4
22	Rn30009903	Trp53	1.8363	Cellular tumor antigen p53
23	Rn30010375	—		
24	Rn30006914	Loxl3	1.7087	Similar to lysyl oxidase-like 3
25	Rn30018257	Hsp90ab1	1.7047	Heat shock protein HSP 90-beta (HSP 84).
26	Rn30016830	Chd4	1.6964	Mi-2 autoantigen
27	Rn30021722	Apol9a	1.6932	"C
28	Rn30015487	Gbp5	1.6803	Similar to guanylate-binding protein 5
29	Rn30009073	—	1.6721	"C
30	Rn30008992	—	1.6509	WAS protein family, member 2
31	Rn30017948	Psmb10	1.6478	Proteasome subunit, beta type 10
32	Rn30005283	—	1.644	Rap1 interacting factor 1 homolog (yeast)
33	Rn30019336	Pogz	1.6139	Pogo transposable element with ZNF domain
34	Rn30023995	NIPBL	1.6115	PREDICTED: similar to delangin
35	Rn30021836	Olr1214	1.5675	Olfactory receptor Olr1214
36	R003169_01	Acs16	1.5651	Long-chain-fatty-acid-CoA ligase 6
37	R004194_01	Zfp709	1.565	Zinc finger protein 14
38	Rn30004337	RGD1565597	1.5561	Similar to RIKEN cDNA 2210421G13
39	Rn30007454	—	1.5487	Complement receptor 2
40	Rn30003220	—	1.545	13-day embryo male testis cDNA
41	Rn30015924	Trub1	1.5415	TruB pseudouridine (psi) synthase homolog 1
42	Rn30019481	Gabpb2	1.5354	Transcription factor GABP beta 2-1 chain
43	Rn30009011	RGD1305500	1.5248	DNA segment, Chr 19, ERATO Doi 737
44	Rn30015486	Gbp5	1.5204	Guanylate-binding protein 5
45	Rn30015488	—	1.5197	Guanylate nucleotide-binding protein 2
46	R002891_01	Cyp2d4v1	1.5187	Cytochrome P450 2D18
47	Rn30016434	—	1.5155	RGD1310888-predicted protein

TABLE 3: Continued.

	Oligo_id	Name	Ratio	Description
48	Rn30001002	—	1.5108	
49	R001164_01	—	1.5085	Granzyme M precursor
50	Rn30023011	—	1.5073	
51	Rn30000745	Hspa1b	0.6661	Heat shock 70 kDa protein 1A/1B
52	Rn30010844	—	0.6653	
53	R002646_01	—	0.665	
54	Rn30013433	Med31	0.6623	RNA polymerase II transcription subunit 31
55	Rn30003203	Dynlt3	0.6605	T-complex-associated testis-expressed 1-like
56	Rn30022044	—	0.6596	—
57	Rn30003825	Nt5c1b	0.6581	Retinol dehydrogenase 14
58	Rn30025065	P4ha2	0.6576	Procollagen-proline, 2-oxoglutarate-4-dioxygenase, alpha II polypeptide
59	Rn30000745	Hspa1b	0.6555	Heat shock 70 kDa protein 1A/1B
60	Rn30019963	—	0.6548	—
61	Rn30005770	Ero1l	0.6482	ERO1-like protein alpha precursor
62	Rn30024141	—	0.6465	~C
63	Rn30019903	Rassf1	0.6464	Ras association domain family 1 isoform 2
64	Rn30003533	Dusp1	0.6461	Dual specificity protein phosphatase 1
65	Rn30020658	RGD1563649	0.6446	Similar to ORF4
66	Rn30006166	Pea15a	0.6435	Phosphoprotein enriched in astrocytes 15
67	Rn30012217	Arg1	0.6427	Arginase-1 (EC 3.5.3.1)
68	Rn30023334	—	0.6392	
69	Rn30023867	Rnase1l2	0.6379	
70	Rn30002222	Pgk1	0.6341	Phosphoglycerate kinase 1
71	R002211_01	Aldoc	0.6301	Fructose-bisphosphate aldolase C
72	Rn30002683	RGD1560553	0.6288	PREDICTED: similar to hypoxia-induced gene 1
73	Rn30018943	—	0.6233	DNA-3-methyladenine glycosylase
74	Rn30006243	—	0.619	
75	Rn30025357	—	0.6154	
76	Rn30000745	Hspa1b	0.6086	Heat shock 70 kDa protein 1A/1B
77	Rn30003851	Sh3kbp1	0.6013	SH3-domain kinase binding protein 1
78	Rn30000745	Hspa1b	0.5944	Heat shock 70 kDa protein 1A/1B
79	Rn30005084	LOC499742	0.5851	LRRGT00150.
80	Rn30000745	Hspa1b	0.5848	Heat shock 70 kDa protein 1A/1B
81	Rn30015841	Bnip3	0.581	BCL2/adenovirus E1B-interacting protein 3
82	Rn30014271	RGD1559815	0.5793	Da1-12
83	Rn30000745	Hspa1b	0.5732	Heat shock 70 kDa protein 1A/1B
84	Rn30000745	Hspa1b	0.5731	Heat shock 70 kDa protein 1A/1B
85	Rn30000745	Hspa1b	0.5706	Heat shock 70 kDa protein 1A/1B
86	Rn30000745	Hspa1b	0.5699	Heat shock 70 kDa protein 1A/1B
87	Rn30026196	Hist1h4m	0.5511	Histone H4.
89	Rn30000745	Hspa1b	0.545	Heat shock 70 kDa protein 1A/1B
90	Rn30024135	LOC499698	0.5425	C-reactive protein precursor.
91	Rn30000745	Hspa1b	0.5364	Heat shock 70 kDa protein 1A/1B
92	Rn30015672	Car9	0.51	Similar to carbonic anhydrase 9
93	Rn30004468	Upp1	0.4769	Uridine phosphorylase 1
94	Rn30014956	Plcd4	0.4744	Phospholipase C, delta 4
95	R001854_01	Ccl20	0.4709	Small inducible cytokine A20 precursor
96	Rn30022206	Dusp6	0.4411	Dual specificity protein phosphatase 6

FIGURE 4: Continued.

(c)

FIGURE 4: Differential gene expression profiles in COX-2 knockdown cells. (a) Expression of CDC27 and Sh3kbp1 were detected by RT-PCR in cells. (b) Expression of Phlcd4 and Dusp1 was detected by RT-PCR. (c) The expressions of Serpinb2, cxcl10, Dusp6, and TP53 genes were measured by RT-PCR. * $p < 0.05$ and ** $p < 0.01$, compared with the sh-NC group.

FIGURE 5: Continued.

FIGURE 5: Effects of shRNA-mediated COX-2 knockdown on HSCs related with the changed expression of CDC27. (a) Western blotting for CDC27 protein expression in all groups. (b) Cell proliferation was detected in cotransfection of COX-2 and CDC27 shRNA cells, COX-2-shRNA-1 cells, and sh-NC cells. (c) HSC-T6 cells were transfected with COX-2-shRNA-1, or sh-NC, or cotransfected with COX-2-shRNA-1 and sh-CDC27. Cell cycle analysis was examined by flow cytometry. (d) Apoptosis of HSC-T6 cells transfected with COX-2-shRNA and CDC27-shRNA, COX-2 nanomedicine-shRNA-1, and sh-NC was examined by flow cytometry. * $p < 0.05$ and *** $p < 0.001$, compared with the sh-NC group and COX-2-shRNA-1 group.

proproliferation effect of CDC27 (Figure 5(b)). In the previous results, knockdown of COX-2 induced more cells to arrest in G0/G1 phase, but on this basis, knockdown of CDC27 showed a weakened effect (Figure 5(c)). Cotransfection of CDC27-shRNA and COX-2-shRNA-1 decreased the cell apoptotic rate when compared with the COX-2-shRNA-1 group, suggesting that knockdown of CDC27 attenuated the proapoptotic effect of COX-2-shRNA-1 in HSCs (Figure 5(d)). What emerges from the results reported in the above is that COX-2 knockdown suppressed proliferation and accelerated apoptosis via activating CDC27 expression in HSCs.

4. Discussion

The primary effector cell in hepatic fibrosis is the HSCs [9]. HSCs are activated and transformed from quiescent to acti-

vated when the liver is damaged by mechanical stimulation or the inflammation. Activated HSCs have a strong ability to synthesize extracellular matrix, then leading to the formation of liver fibrosis or cirrhosis. Therefore, inhibiting the proliferation of HSCs and promoting its apoptosis have become the main strategy to treat liver fibrosis [10].

Previous studies have shown that COX-2 may be involved in the development of hepatic fibrosis by regulating the proliferation and apoptosis of HSCs [4]. SC-236 was used to prevent the occurrence of carbon tetrachloride-induced hepatic fibrosis in rats, which is mainly related to its regulation on the apoptosis and proliferation of HSCs [4]. However, the side effect of COX-2 inhibitors such as cardiovascular events or gastrointestinal bleeding limits its wide application [11]. Therefore, the nucleotide-based inhibitor for gene expression has been raised as a novel treatment approach. RNA interference (RNAi), a phenomenon of posttranscriptional gene silencing (PTGS),

provides new insights for treating liver fibrosis [12]. Previous experiments showed that COX-2-shRNA-1 can effectively treat experimental liver fibrosis [3].

In this study, we found that knockdown of COX-2 effectively suppressed the proliferation of HSCs in the liver of rats with hepatic fibrosis, which has been proved by the decrease in α -SMA and the number of α -SMA immune-positive cells. More clearly, knockdown of COX-2 suppressed proliferation and facilitated apoptosis of HSCs. Our results were consistent with most literature reports [1, 6]. We argue that the regulation of COX-2 on HSC cell behavior might be the primary mechanism of it in hepatic fibrosis pathogenesis.

For the further evidences of COX-2 in regulating hepatic fibrosis, we employed mRNA microarray assay to examine the expression of a series of proliferative and apoptotic relative genes. Knockdown of COX-2 in HSCs leads to some cell proliferation genes to change; some of them upregulated, such as *Il6*, *Csf2*, *Atf3*, *Cdc27*, *Tp53*, and *Cxcl10*; and some of them downregulated including *Dusp1*, *Serpinb2*, and *Plcd4*. As for the apoptotic related genes, upregulation of *Il1b*, *Il6*, *Ccl2*, *Phlda3*, and *Tp53* and downregulation of *Sh3kbp1*, *Peal5*, and *Plcd4* genes were demonstrated. *Sh3kbp1* gene may regulate various cell behaviors [13], and *Plcd4* gene expression was associated with breast cancer cell proliferation [14]; *CXCL10* induced cell proliferation and apoptosis [15, 16]. After silencing the COX-2 gene in HSCs, the expression of *CDC27* is enhanced, which is the only gene with consistent results in DNA chips and RT-PCR. *CDC27* was previously reported to facilitate proliferation [7]. It has potent protein binding affinity [8, 17] and participates in various biological processes including ubiquitination mediated proteolysis [18], cell cycle regulation [19], cell division [20], and transformation at metaphase of mitosis [21, 22]. In this study, we found that *CDC27* knockdown could accelerate HSC proliferation and inhibit cell apoptosis, indicating that RNAi silencing of COX-2 could modulate HSCs growth, inhibit cell proliferation, and induce cell apoptosis via *CDC27* upregulation. Moreover, *CDC27* has been postulated to be one major target for induced cell apoptosis and cell cycle arrest in cancer cells [23]. The expression of COX-2 and *CDC27* has been associated during immune response regarding macrophage proliferation [24]. This study, on the other hand, revealed the regulation of *CDC27* gene expression by COX-2 knockdown, suggesting that *CDC27* exerted its role at the downstream of COX-2. Our data thus provide the first-hand information on how COX-2 regulates HSC proliferation, cell cycle, and cell apoptosis by regulating *CDC27* gene expression, revealing an unknown pathway of HSC behavior. However, the detailed molecular mechanism for COX-2 regulation on *CDC27* gene expression is still unclear yet and thus requires further mechanistic studies to fulfill the weakness of the current study.

In summary, the current study described the regulation of HSC proliferation and apoptosis by COX-2/*CDC27* axis and found that such regulatory pathway played important roles in mediating severity of hepatic fibrosis. Our overall findings provide a promising treatment target for early intervention of liver fibrosis.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of University of South China (2011002A), Hengyang, China.

Conflicts of Interest

The authors declare no conflict of interest, financial, or otherwise.

Authors' Contributions

Yang Hu designed the experiments. Yang Hu, Li Xian Chen, and Nian Fu performed the experiments and analyzed the data. Yang Hu wrote the manuscript. Jian Hua Xiao and Xue Feng Yang have modified the language expression of the article. All authors have read and approved the manuscript.

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References

- [1] Y. H. Paik, J. K. Kim, J. I. Lee et al., "Celecoxib induces hepatic stellate cell apoptosis through inhibition of Akt activation and suppresses hepatic fibrosis in rats," *Gut*, vol. 58, no. 11, pp. 1517–1527, 2009.
- [2] H. Hu, X. Jing, X. Zou, and J. Wu, "Role of cyclooxygenase 2 and its inhibitor valdecoxib in liver fibrosis," *Zhonghua Yi Xue Za Zhi*, vol. 94, no. 10, pp. 784–787, 2014.
- [3] X. Ni, H. W. Liao, W. S. Ou et al., "Construction of COX-2 short hairpin RNA expression vector and its inhibitory effect on hepatic fibrosis," *Biotechnology & Biotechnological Equipment*, vol. 32, no. 3, pp. 1–10, 2018.
- [4] L. Chang, Z. Zhang, J. Yang, S. H. McLaughlin, and D. Barford, "Atomic structure of the APC/C and its mechanism of protein ubiquitination," *Nature*, vol. 522, no. 7557, pp. 450–454, 2015.
- [5] J. Zhu, Z. Luo, Y. Pan et al., "H19/miR-148a/USP4 axis facilitates liver fibrosis by enhancing TGF- β signaling in both hepatic stellate cells and hepatocytes," *Journal of Cellular Physiology*, vol. 234, no. 6, pp. 9698–9710, 2019.
- [6] A. Planagumà, J. Clària, R. Miquel et al., "The selective cyclooxygenase-2 inhibitor SC-236 reduces liver fibrosis by mechanisms involving non-parenchymal cell apoptosis and PPARgamma activation," *The FASEB Journal*, vol. 19, no. 9, pp. 1120–1122, 2008.
- [7] L. Qiu, J. Wu, C. Pan et al., "Downregulation of *CDC27* inhibits the proliferation of colorectal cancer cells via the accumulation

- of p21Cip1/Waf1," *Cell death & disease*, vol. 7, no. 1, p. e2074, 2016.
- [8] V. P. Bermudez, S. A. MacNeill, I. Tappin, and J. Hurwitz, "The influence of the Cdc27 subunit on the properties of the *Schizosaccharomyces pombe* DNA polymerase delta," *Journal of Biological Chemistry*, vol. 277, no. 39, pp. 36853–36862, 2002.
 - [9] S. Karvar, E. A. Ansa-Addo, J. Suda et al., "Moesin, an ERM family member, regulates hepatic fibrosis," *Hepatology*, vol. 72, no. 3, pp. 1073–1084, 2019.
 - [10] S. Bi, F. Chu, M. Wang et al., "Ligustrazine-oleanolic acid glycine derivative, G-TOA, selectively inhibited the proliferation and induced apoptosis of activated HSC-T6 cells," *Molecules*, vol. 21, no. 11, p. 1599, 2016.
 - [11] C. C. Szeto, K. Sugano, J. G. Wang et al., "Non-steroidal anti-inflammatory drug (NSAID) therapy in patients with hypertension, cardiovascular, renal or gastrointestinal comorbidities: joint APAGE/APLAR/APSDE/APSH/APSN/PoA recommendations," *Gut*, vol. 69, no. 4, pp. 617–629, 2020.
 - [12] J. M. Bangen, L. Hammerich, R. Sonntag et al., "Targeting CCL4 -induced liver fibrosis by RNA interference-mediated inhibition of cyclin E1 in mice," *Hepatology*, vol. 66, no. 4, pp. 1242–1257, 2017.
 - [13] R. Dejournett, R. Kobayashi, S. H. Pan et al., "Phosphorylation of the proline-rich domain of Xp95 modulates Xp95 interaction with partner proteins," *The Biochemical Journal*, vol. 401, no. 2, pp. 521–531, 2007.
 - [14] D. W. Leung, C. Tompkins, J. Brewer et al., "Phospholipase C delta-4 overexpression upregulates ErbB1/2 expression, Erk signaling pathway, and proliferation in MCF-7 cells," *Molecular cancer*, vol. 3, no. 1, p. 15, 2004.
 - [15] Q. Jiang, F. Wang, L. L. Shi et al., "C-X-C motif chemokine ligand 10 produced by mouse Sertoli cells in response to mumps virus infection induces male germ cell apoptosis," *Cell death & disease*, vol. 8, no. 10, p. e3146, 2017.
 - [16] M. Wornle, H. Schmid, M. Merkle, and B. Banas, "Effects of chemokines on proliferation and apoptosis of human mesangial cells," *BMC Nephrology*, vol. 5, no. 1, 2004.
 - [17] A. Schreiber, F. Stengel, Z. Zhang et al., "Structural basis for the subunit assembly of the anaphase-promoting complex," *Nature*, vol. 470, no. 7333, pp. 227–232, 2011.
 - [18] L. A. MacFarlane and P. R. Murphy, "Regulation of FGF-2 by an endogenous antisense RNA: effects on cell adhesion and cell-cycle progression," *Molecular Carcinogenesis*, vol. 49, no. 12, pp. 1031–1044, 2010.
 - [19] J. M. Pérez-Pérez, O. Serralbo, M. Vanstraelen et al., "Specialization of CDC27 function in the *Arabidopsis thaliana* anaphase-promoting complex (APC/C)," *The Plant Journal*, vol. 53, no. 1, pp. 78–89, 2008.
 - [20] D. Li, G. Morley, M. Whitaker, and J. Y. Huang, "Recruitment of Cdc20 to the kinetochore requires BubR1 but not Mad2 in *Drosophila melanogaster*," *Molecular and Cellular Biology*, vol. 30, no. 13, pp. 3384–3395, 2010.
 - [21] L. Zhang, T. Fujita, G. Wu, X. Xiao, and Y. Wan, "Phosphorylation of the anaphase-promoting complex/Cdc27 is involved in TGF-beta signaling," *The Journal of Biological Chemistry*, vol. 286, no. 12, pp. 10041–10050, 2011.
 - [22] T. Fujita, M. W. Epperly, H. Zou, J. S. Greenberger, and Y. Wan, "Regulation of the anaphase-promoting complex-separase cascade by transforming growth factor-beta modulates mitotic progression in bone marrow stromal cells," *Molecular Biology of the Cell*, vol. 19, no. 12, pp. 5446–5455, 2008.
 - [23] S. J. Lee and S. A. Langhans, "Anaphase-promoting complex/cyclosome protein Cdc27 is a target for curcumin-induced cell cycle arrest and apoptosis," *BMC Cancer*, vol. 12, no. 1, 2012.
 - [24] W. C. Lim and V. T. Chow, "Gene expression profiles of U937 human macrophages exposed to *Chlamydomonas pneumoniae* and/or low density lipoprotein in five study models using differential display and real-time RT-PCR," *Biochimie*, vol. 88, no. 3-4, pp. 367–377, 2006.