The liver is the only major organ in the adult body that has the ability to regenerate after injury. After the loss or injury of liver tissue caused by surgery, infection, poisoning or trauma, the remaining liver tissue can be restored to its original size and function in rats (15). Liver regeneration is an extremely complicated process, involving many cells, many factors, and many signal pathways. In the process of liver regeneration, energy metabolism is essential. As an important component of the body, adipose tissue is the main site for energy storage and oxidation, and it also has important significance for cell recognition and information transmission (10). Liver regeneration is often accompanied by fat accumulation (6-9).

C-C chemokine ligand 2 (CCL2) was the first chemokine discovered by Valente in 1988 (23). CCL2, also named monocyte (Mn) chemoattractant protein-1 (MCP-1), is secreted by injured Kupffer cells, stellate cells, hepatocytes due to massive accumulation of Mn; it plays important roles in inflammatory responses, angiogenesis, injury repair, and tumorigenesis (11, 18). CCL2 has also been found to play important roles in the occurrence and development of many liver diseases, including hepatitis (3, 19), liver fibrosis (1) and liver cancer (13); nevertheless, its role in liver regeneration is not clear.

In the present study we constructed an interference plasmid of CCL2 and transferred it into a partial hepatectomy rat liver model using hydraulic transgenic technology, so as to preliminarily study the role of this protein in liver regeneration and to begin to clarify the mechanism of liver regeneration.

Material and methods

Experimental animals. Sprague Dawley (SD) rats, 200 ± 20 g, male, 40 days, were raised at a constant temperature (21-22°C) in an animal house. The rats were fed and watered ad libitum during the experiment. This study was conducted in accordance with the international guidelines for animal studies and obtained the approval of from the ethics committee of Xinxiang Medical University.

Construction of a CCL2 shRNA expression plasmid. Two single-stranded oligonucleotides were designed and synthesized from the cDNA sequence of CCL2 in GenBank (sequence number nm_031530.1). We used BamH I and Hind III to create the expression vector pGenesil-1.0-
plasmid (AXYBIO, China). After annealing, two single-stranded oligonucleotides were connected to pgenesil-1.0 for 18 h, and then JM109 competent cells were transformed. Using double enzyme digestion identification and sequencing, the positive clone was named pGenesil-1.0-ccl2. The ShRNA fragment sequence was as follows:

ccl2 Sense:
5'-GATCCAAAAAGAAGCTGAGATTTTTCAAGAC-GAAATACTACAGCTTTTGGTTTTA-3'
ccl2 Anti-sense:
5'-AGCTTAAAAAACAAAGAAGCTGTAGTATTTC-GTCTGAAAAATACTACAGCTTTTGG-3'

Establishment of the partial hepatectomy model in rats. After the rats were anesthetized with pentobarbital sodium (40 mg/kg), the hair on the abdomen and chest was removed. The rats were sterilized with ethanol and fixed on the operating table in the supine position. The skin was cut along the midline from 3 cm below the sternoxiphoid process to expose the abdominal cavity. The left lobe and middle lobe of liver (accounting for about 70% of total liver mass) was extruded. After ligation of the liver leaf root with silk thread, we excised the liver leaf, sutured the wound, and applied tetracycline powder to prevent infection.

Transfection efficiency observation. After partial hepatectomy in rats, pGenesil-1.0-ccl2 plasmid was transferred using the tail vein hydraulic transgenic method. At 6 h after partial hepatectomy, the rats were sacrificed using cervical dislocation. The right lobe of the liver was removed for frozen section, and the expression of green fluorescent protein was measured under fluorescence microscopy.

Liver function test. At 144 h after partial hepatectomy, 5 rats in each of the pGenesil-1.0-ccl2 group and partial hepatectomy group were anesthetized using a pentobarbital sodium intraperitoneal injection, fixed in supine position, the abdominal cavity was opened, and 1 mL blood was collected from the inferior vena cava. The blood was collected at room temperature for 30 min, centrifuged at 4000 r/min for 5 min, and 300 µl serum was collected. The content of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum was measured using a biochemical analyzer (Hitachi, Japan).

Liver index calculation. The weights of the rats and those of the regenerated livers were weighed at 48, 96, 144, 192, 240 and 288 h after partial hepatectomy. The liver index was calculated according to the weight of the regenerated liver/body weight × 100%.

Proliferating cell nuclear antigen (PCNA) positive cell rate test. After 48 hours of partial liver resection and recovery, the liver tissues were prepared into paraffin sections, dewaxed to water, treated with 3% H2O2 at room temperature for 15 min, modified with microwave antigen in citric acid buffer for 3 min, sealed with 10% serum at 37°C for 30 min, and then the liquid was aspirated. PCNA (Sigma, American, 1 : 100) was added and incubated overnight in a refrigerator at 4°C. Secondary antibody (Sigma, American) was incubated at 37°C for 20 min. SABC was incubated at 37°C for 20 min, DAB-H2O2 was developed, and neutral gum was used as a sealant. Ten fields were randomly selected and counted under microscopy, and the PCNA-positive cell rate was calculated according as PCNA-positive cell numbers in the field/total cell number in the cross section × 100%.

The expression of fat-related proteins. After partial liver resection and recovery for 48, 96, 144, 192, 240 and 288 h, the regenerated liver homogenate was centrifuged and collected for supernatant at 4°C, SDS-PAGE was used to separate the same amount of protein. The proteins were then transferred to the PVDF membrane. 5% skim milk powder sealant was sealed for 1 hour, adipogenic differentiation-related protein (ADRP) (Abcam, England) was added, overnight at 4°C, and β-actin was the internal reference protein. Horseradish peroxidase-labeled secondary antibodies (Abcam, England) were added and combined at room temperature for 1 h. Beyo ECL Plus (Beyotime, China) chemiluminescence kit was used to develop bands.

Statistical analysis. SPSS 13.0 statistical software was used to conduct t-tests on the data of the experimental and control groups.

Results and discussion

Fat is one of the major energy sources in mammals. It plays important roles in normal cell growth and metabolism. Fatty acids can be broken down into carbon dioxide and water in the body, after which substantial amounts of energy are released in the form of ATP to be used by organisms. Fat accumulation occurs in liver tissue during liver regeneration (6, 17). In recent years, studies have found that lowering the accumulation of fat in liver regeneration through drug interventions or genetic changes can inhibit liver regeneration (20, 21, 24). However, the relationship between fat accumulation and liver regeneration, as well as the related signaling pathways, is unclear.

Adipogenic differentiation related protein (ADRP) is also known as perilipin-2 (PLIN2). It is a lipid droplet surface protein with a relative molecular weight of 48 kD, found in primate fat cells. ADRP is a surface protein of lipid droplets. It is widely expressed in almost all mammalian cultured cells, expressed in adipose tissue, liver, muscle, breast, pancreas, and small intestinal mucosa. ADRP is a major intracellular lipid droplet protein in the liver (16), and is expressed in both normal hepatocytes and astrocytes (22).

The content of ADRP positively correlated with the content of lipid in hepatocytes. PLIN2 knockout mice were resistant to obesity induced by a high-fat diet, inhibiting the formation of inflammatory lesions and steatosis in the white adipose tissue of the liver, and reducing energy intake (14). PLIN2 knockout also down-regulated the expression of genes related to adipogenesis and triglyceride synthesis, and inhibited alcohol-induced liver steatosis in mice (4). For these reasons, ADRP is considered a marker of liver lipid accumulation.

Kohjima et al. performed partial hepatectomy on ADRP knockout mice and found that the content of triglycerides in the liver decreased, cell cycle slowed down, beta-oxidation and fatty acids in the cytoplasm
decreased, and liver regeneration slowed down. They also found reduced accumulation of liver fat and decreased cell proliferation in CCL4-induced ADRP knockout mice (12). They concluded that the accumulation and utilization of fat in the liver is beneficial to liver regeneration caused by partial hepatectomy and liver injury, and ADRP plays an important role in this process.

Clement et al. found that CCL2 can directly act on liver parenchymal cells and cause liver parenchymal cells to express phosphoenolpyruvate carboxykinase, promoting fatty acid esterification, and reducing the secretion of fat, resulting in cell fat accumulation (5). Ambade et al. showed that CCL2 caused the increase of ADRP in liver cells (2).

In this study, to investigate the role of CCL2 and ADRP in fat metabolism during liver regeneration, we constructed a plasmid pGenesil-1.0-ccl2. After partial hepatectomy in rats, the pGenesil-1.0-ccl2 plasmid was transferred using the tail vein hydraulic transgenic method. The expression of green fluorescent protein at 6 h after partial hepatectomy reached 35% (Fig. 1). It showed that CCL2 shRNA was highly expressed in the liver.

Venous blood was taken from the rats 144 h after partial hepatectomy to measure liver functioning. ALT and AST levels in the pGenesil-1.0-ccl2 group were not significantly different from those in the partial hepatectomy group (Tab. 1). After partial hepatectomy, the liver indexes of rats treated with the pGenesil-1.0-ccl2 plasmid at 48, 96, 144, 192, 240 and 288 h were lower than those of the control group only undergoing partial hepatectomy, with a significant difference at 144 h (Fig. 2). The immunohistochemical results of 48 h after partial hepatectomy showed that the PCNA positive cell rate was 14% in the partial hepatectomy group and 8% in the pGenesil-1.0-ccl2 group, slightly lower than that of the control group (Fig. 3). These experiments showed that decreased CCL2 expression did not cause liver damage, but affected the rate of liver regeneration.

Western blotting results showed that, 48, 96, 144, 192, 240 and 288 h after partial hepatectomy, the

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
</tr>
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<tr>
<td>PH</td>
<td>83.42 ± 5.13</td>
<td>112.53 ± 7.42</td>
</tr>
<tr>
<td>pGenesil-1.0-ccl2</td>
<td>76.92 ± 8.46</td>
<td>109.68 ± 9.38</td>
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Fig. 1. Expression of pGenesil-1.0-ccl2 plasmid

Fig. 2. Effect of CCL2 on liver index in rats

Fig. 3. Immunohistochemical results of PCNA
amounts of ADRP in the pGenesil-1.0-ccl2 group was lower than that of the group with partial hepatectomy only (Fig. 4). It indicated that CCL2 interference vector caused decreased ADRP expression, consistent with previous research results.

In conclusion, CCL2 is a liver regeneration related protein that regulates the formation of fat and promotes liver regeneration by regulating the expression of its downstream protein ADRP. Nevertheless, the specific role of fat droplets in liver regeneration and the related molecular mechanisms need to be further studied.

References