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***Basic Study***

**Recombinant adeno-associated virus 8-mediated inhibition of microRNA let-7a ameliorates sclerosing cholangitis in a clinically relevant mouse model**

Hua H *et al.* Inhibition of let-7a-5p ameliorates sclerosing cholangitis

Hui Hua, Qian-Qian Zhao, Miriam Nkesichi Kalagbor, Guo-Zhi Yu, Man Liu, Zheng-Rui Bian, Bei-Bei Zhang, Qian Yu, Yin-Hai Xu, Ren-Xian Tang, Kui-Yang Zheng, Chao Yan

**Hui Hua, Qian-Qian Zhao, Miriam Nkesichi Kalagbor, Guo-Zhi Yu, Man Liu, Zheng-Rui Bian, Bei-Bei Zhang, Qian Yu, Ren-Xian Tang, Kui-Yang Zheng, Chao Yan,** Jiangsu Key Laboratory of Immunity and Metabolism, Department of Pathogenic Biology and Immunology, National Demonstration Center for Experimental Basic Medical Science Education, Laboratory of Infection and Immunity, Xuzhou Medical University, Xuzhou 221004, Jiangsu Province, China

**Yin-Hai Xu,** Department of Laboratory Medicine, The Affiliated Hospital of Xuzhou Medical University, Xuzhou 221002, Jiangsu Province, China

**Co-corresponding authors:** Kui-Yang Zheng and Chao Yan.

**Author contributions:** Hua H and Zhao QQ performed the majority of experiments; Kalagbor MN, Yu GZ, Liu M, Bian ZR, Zhang BB, Yu Q, Xu YH, and Tang RX contributed to the acquisition of data and improving the manuscript. Zheng KY and Yan C contributed equally to this work as co-corresponding authors; Zheng KYsupervised the experiments, corrected the data, and revised the manuscript; Yan C conceived and designed the main content of this study and was responsible for analyzing the research results.

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**Corresponding author: Chao Yan, MD, PhD, Academic Research, Professor,** JiangsuKey Laboratory of Immunity and Metabolism, Department of Pathogenic Biology and Immunology, National Demonstration Center for Experimental Basic Medical Science Education, Department of Pathogenic Biology and Immunology, Xuzhou Medical University, No. 209 Tongshan Road, Xuzhou 221004, Jiangsu Province, China. yanchao6957@xzhmu.edu.cn

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**Abstract**

BACKGROUND

Primary sclerosing cholangitis (PSC) is characterized by chronic inflammation and it predisposes to cholangiocarcinoma due to lack of effective treatment options. Recombinant adeno-associated virus (rAAV) provides a promising platform for gene therapy on such kinds of diseases. A microRNA (miRNA) let-7a has been reported to be associated with the progress of PSC but the potential therapeutic implication of inhibition of let-7a on PSC has not been evaluated.

AIM

To investigate the therapeutic effects of inhibition of a miRNA let-7a transferred by recombinant adeno-associated virus 8 (rAAV8) on a xenobiotic-induced mouse model of sclerosing cholangitis.

METHODS

A xenobiotic-induced mouse model of sclerosing cholangitis was induced by 0.1% 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine (DDC) feeding for 2 wk or 6 wk. A single dose of rAAV8-mediated anti-let-7a-5p sponges or scramble control was injected *in vivo* into mice onset of DDC feeding. Upon sacrifice, the liver and the serum were collected from each mouse. The hepatobiliary injuries, hepatic inflammation and fibrosis were evaluated. The targets of let-7a-5p and downstream molecule NF-κB were detected using Western blot.

RESULTS

rAAV8-mediated anti-let-7a-5p sponges can depress the expression of let-7a-5p in mice after DDC feeding for 2 wk or 6 wk. The reduced expression of let-7a-5p can alleviate hepato-biliary injuries indicated by serum markers, and prevent the proliferation of cholangiocytes and biliary fibrosis. Furthermore, inhibition of let-7a mediated by rAAV8 can increase the expression of potential target molecules such as suppressor of cytokine signaling 1 and Dectin1, which consequently inhibit of NF-κB-mediated hepatic inflammation.

CONCLUSION

Our study demonstrates that a rAAV8 vector designed for liver-specific inhibition of let-7a-5p can potently ameliorate symptoms in a xenobiotic-induced mouse model of sclerosing cholangitis, which provides a possible clinical translation of PSC of human.

**Key Words:** Primary sclerosing cholangitis; Recombinant adeno-associated virus 8; Let-7a-5p; Therapeutic effects; Inflammation

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**Core Tip:** Primary sclerosing cholangitis (PSC) have a high risk of cholangiocarcinoma with a lack of effective treatment options. Then the present study aimed to investigate the therapeutic effects of inhibition of a microRNA let-7a transferred by recombinant adeno-associated virus 8 (rAAV8) on a 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine-induced mouse model of sclerosing cholangitis. And the results of our study demonstrates that a rAAV8 vector designed for liver-specific inhibition of let-7a-5p can potently ameliorate symptoms in a xenobiotic-induced mouse model of sclerosing cholangitis, which provides a possible clinical human clinical translation of PSC.

**INTRODUCTION**

Primary sclerosing cholangitis (PSC) is an idiopathic cholestatic disease and it affects intrahepatic and/or extrahepatic bile ducts characterized by chronic inflammation, ductal stricture, cholestasis, and fibrosis[1,2].PSC patients can progress to liver cirrhosis and have a great risk of cholangiocarcinoma with liver failure at the end stage. PSC has been considered an orphan disease since the prevalence of PSC is up to 16.2 per 100000 population with variable distribution due to different regions[3-5]. Although the occurrence of PSC is rare, it showed significant morbidity and mortality in PSC patients as there is a very limited medical option to interfere with the course of PSC, and liver transplant is the exclusively therapeutic option at the end-stage of the disease[1,6].MicroRNA (miRNA) represents a kind of small non-coding RNA with 18-23 nucleotide length. The miRNAs widely participate in various processes in physiological and disease conditions by regulating of expression mRNAs at the post-transcriptional level. Although the etiology and pathogenesis of PSC are complex and largely unknown, increasing data demonstrate that the dysregulation of miRNA can also contribute to the pathogenesis of PSC[7-9].These aberrant miRNAs have been reported to be involved in almost all aspects of diseases including inflammation, hyperplasia, fibrosis, and malignancy of cholangiocytes in patients or mouse models of PSC[10,11]. Of these miRNAs, the let-7a family is one of the latest identified miRNAs that are dysregulated in cholestasis[12-16], suggesting possible medical targets of let-7a on cholestatic diseases. However, the therapeutic effect of targeting let-7a in cholestasis is yet to be determined.

Recombinant adeno-associated virus (rAAVs) provide a promising vehicle for gene therapy as they can efficiently transfer genes to the target tissue with low immunogenicity, low toxicity, and long persistence[17-23]. rAAV8 is one serotype that can transduce hepatocytes and cholangiocytes with high efficiency and specificity due to its liver tropism and has been implicated in many liver diseases including cholangiopathies[19,24-28]. Increasing data showed that rAAVs also can deliver miRNAs (mimics, precursor, or its antisense) to play the significant therapeutic effects of miRNAs on many other liver diseases[29-32], but there are rare therapeutic data about miRNAs delivered by rAAVs on cholangiopathies. Given the aberrance and importance of let-7a in cholangiopathies especially in PSC, we hypothesize that inhibition of let-7a delivered by rAAV8 has potential therapeutic effects on PSC. To support our hypothesis, we used a well-established mouse model of PSC- a mouse model of sclerosing cholangitis *via* 0.1% 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine (DDC) feeding, following treatment with a single dose injection of rAAV8-delivered inhibitors (anti-senses of let-7a sponges) of let-7a for 2 wk or 6 wk. Our data showed that we have successfully developed a possible therapeutic strategy for PSC based on the administration of an AAV8 vector designed for liver-specific inhibition of let-7a. This strategy could markedly ameliorate biliary injuries by the decreased proliferation of cholangiocytes, biliary inflammation, and fibrosis in the mice induced by 0.1% DDC feeding for 2 wk or 6 wk. Furthermore, the therapeutic effects may be associated with the inhibition of NF-κB-mediated hepatic inflammation. The present study demonstrates that the rAAV-mediated miRNAs strategy provides a promising therapeutic opportunity for this debilitating and life-threatening disease.

**MATERIALS AND METHODS**

***Experimental animals and ethics***

Female C57BL/6J mice without specific pathogen, 6-wk old, 18-25 g in weight, were raised in a clean-grade animal house, free to eat and drink, and the animal lab environment as the following specifications: temperature range from 20 °C to 26 °C, humidity range from 40% to 70%, and 12 h/12 h light/dark. Tail vein injection and other operations were conducted in the animal barrier experimental operation room of the Experimental Animal Center of Xuzhou Medical University [SYXK (Su) 2016-0028]. Animal care and all experiments in this study were carried out following the guidelines of the National Laboratory Animal Center and were given humanitarian care according to the 3R principle of laboratory animals. The main procedures and protocols were approved by the Animal Care and Use Committee of Xuzhou Medical University (license IACUC: 201801w003).

***Mouse model of DDC-feeding induced sclerosing cholangitis and the adeno- associated virus infection***

A sclerosing cholangitis mouse model was induced by 0.1% DDC feeding for 2 wk or 6 wk. Specifically, 24 mice were fed DDC food while the other 12 mice were fed chow food as a control. The adeno-associated virus (AAV) labeled with EGFP for targeting knocking down the expression of let-7a-5p in the liver was purchased from Genechem (Shanghai, China). The mice in the two DDC-feeding groups were intravenously injected with 100 μL 4 × 1012 vector genomes (VG)/mL AAV that overexpressed mmu-let-7a-5P-sponges or empty vehicles (4 × 1011 vg per mice). And the 12 normal control mice were injected with the same volume of PBS (LPS-free) in which the AAV original liquid was diluted. Following, half of the mice from each group were sacrificed by euthanasia in 2 wk, while the half of rest were sacrificed in 6 wk. The livers and sera from each mouse were harvested for further experiments. The inhibitor sponges of mmu-let-7a-5P are ACCACACAAgacCTACCTCCcttcACCACACAAgacCTACCTCCcttcACCACACAAgacCTACCTCCatccgtaACCACACAAgacCTACCTCCcttcACCACACAAgacCTACCTCC, and the specific molding method is shown as Figure 1.

***RNA extraction and qPCR analysis***

Total RNAs from liver tissues were extracted using the TRIZOL reagent (Invitrogen, Carlsbad, CA, United States), following the manufacturer’s instructions, and then reverse transcribed into cDNA using the miRcute Plus miRNA First-Strand cDNA Kit (TIANGEN Biotech, Beijing, China) or FastKing RT Kit (With gDNase) (TIANGEN Biotech, Beijing, China). Then, qPCR was performed using the SYBR Green Master Mix (TransGen Biotech, Beijing, China) and run on a StepOne Plus Real-Time PCR System (Roche Applied Science, Mannheim, Germany). The relative expression levels of miRNAs or mRNAs were normalized to *U6* small nuclear RNA (snRNA) or β-actin following the 2-ΔΔCt comparative method. The sequences of the primers used in this study were optimized as Table 1.

***Activities of serum enzyme detection***

The mouse serum was collected and transported to the Laboratory Department of the Affiliated Hospital of Xuzhou Medical University at low temperature to detect the activities of bile duct injury-related enzymes [alanine aminotransferase (ALT), total bilirubin (BILT), and total bile acid (TBA)].

***Hematoxylin and eosin and Sirius red staining***

For histological analysis, liver tissues were excised and fixed with 4% paraformaldehyde for 24 h at least. Thereafter, the fixed tissues were embedded in paraffin, sliced to a thickness of 4 μm, and routinely stained with hematoxylin and eosin (H&E) and Sirius red staining according to the recommendation of the manufacturer (Shanghai Xinfan Biotechnology, Shanghai, China), after sealing the slides with neutral adhesive, the pathological changes of stained histological sections were observed by a microscope (Olympus, Japan). The staining pictures were quantitatively analyzed with Image J, 6 pictures zoomed in 100 × were selected for each mouse for quantitative analysis, and the average value was taken as the quantitative value of that mouse, *n* = 6.

***Immunohistochemistry staining***

The liver tissue was deparaffinized, hydrated, and heated in citric acid buffer at 95 °C for 15 min and blocked with 5% BSA for 30 min, and then incubated overnight with primary anti-CK19 (1:500, ab52625, Abcam, Cambridge, United States) and then incubated with secondary antibodies for 30 min at room temperature. After washing with PBS, DAB (1:20, ZSGB-BIO, Beijing, China) as an enzyme substrate was added. Six high-power fields (× 100 magnifications, Olympus, Japan) were randomly selected from each mouse staining section and the percentage of CK19 positive area was calculated by ImageJ software (NIH, United States).

***Immunofluorescence*** ***staining***

The slides with liver tissues were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then blocked with 5% BSA. Slides were incubated with the indicated primary antibodies anti-GFP (1:100; Abcam, Cambridge, MA, United Kingdom) overnight at 4 °C and then incubated with fluorescence-labeled secondary antibodies for 1 h at room temperature. Nuclei were stained using DAPI for 10 min. Six images zoomed in 100 × per mouse were captured under a fluorescence microscope (AX70, Olympus, Japan), and the average signal value from the 6 images was used to represent the value from one mouse liver. Ten fields from each slide were randomly selected, and the positive color area was measured quantitatively using Image-Pro Plus 6.0 software.

***Detection of hepatic hydroxyproline contents***

Hepatic hydroxyproline (Hyp) content was determined using a commercially available kit according to the manufacturer’s recommendations (Jiancheng Institute of Biotechnology, Nanjing, Jiangsu Province, China).

***Western blot analysis***

Liver homogenates were harvested and washed in cold PBS twice and then were treated with the lysis buffer (Beyotime, Shanghai, China) on ice for 30 min. The lysate was collected into microtubes and centrifuged for 15 min at 12000 rpm at 4 °C. Protein samples (20 mg) were denatured with the 5 × SDS loading buffer at 100 °C for 5 min then were segregated on a 10% SDS polyacrylamide gel electrophoresis and transferred onto 0.2 um nitrocellulose membranes. After 60 min of blocking with 5% fat-free milk, membranes were incubated with suppressor of cytokine signaling 1 (SOCS1) (1:1000; Cell Signaling Technology, United States), Dectin-1 (1:1000; Abclonal, Wuhan, Hubei Province, China), P-p65 (1:1000; Cell Signaling Technology, United States) and GAPDH antibody (1:2000; Abmart, Shanghai, China) overnight at 4 °C. After washing with TBST 3 times, blots were incubated with the anti-rabbit secondary antibody (1:5000; Abclonal, Wuhan, China) for 1 h. After washing, immunoreactive protein bands were detected by using enhanced chemiluminescence reagents (Bio-Rad, California, United States). Band intensities were normalized to GAPDH and analyzed using ImageLab software.

***Statistical analysis***

All data are presented as means ± SEM. The statistical analysis was performed with the use of the software package SPSS version 19.0 (SPSS Inc, Chicago, United States). One-way ANOVA analysis was used for the comparison of differences among more than two groups, which was followed by the Least Significant Difference test unless otherwise stated. In the case of the comparison of two groups, the differences were evaluated using a two-tailed Student’s *t*-test, a value of *P* < 0.05 was considered significant.

**RESULTS**

***Anti-let-7a-5p sponges delivered by rAAV8 depressed the expression of let-7a-5p in the liver of mice with DDC feeding***

Since the expression of let-7a-5p is increased in both experimental obstructive cholestasis and patients[12], we investigated the potential therapeutic effects of an inhibitor of let-7a-5p delivered by highly hepatotropic rAAV8 on a mouse model of PSC at 2 wk and 6 wk. To address this, we generated rAAV8 expression plasmids containing either the scramble control (anti-SCR) or anti-let-7a-5p sponges under the control of a liver-specific thyroxine-binding globulin (TBG) promoter (Figure 1A) and injected rAAV8-anti-let-7a-5p (anti-let-7a-5p, 4 × 1011 vg/mouse) or rAAV8-scramble (anti-SCR, 4 × 1011 vg/mouse) with a single dose at the beginning of DDC feeding for 2 wk and 6 wk, respectively (*n* = 6 per group, Figure 1B).

After 2 or 6 wk for DDC feeding, we found that the GFP-flagged rAAVs were extensively observed in the livers of mice injected with rAAVs anti-SCR control or anti-let-7a-5p sponges (Figure 2A-C), and the relative expression of GFP indicating the amounts of virus in the liver were significantly increased both in anti-SCR and inhibitor mice at 2 wk or 6 wk, although it seems that the titer of recombinant adenovirus has dropped from 2 wk to 6 wk. We further evaluated the inhibition efficiency of let-7a-5p using anti-let-7a-5p sponges, it was found that anti-let-7a-5p sponges can significantly depress approximately 50% (at 2 wk) and approximately 60% (at 6 wk) of let-7a-5p in the liver of rAAV8-anti-let-7a-5p-injected mice, compared with anti-SCR mice (Figure 2D and E).

***Inhibition of let-7a-5p delivered by rAAV8 alleviates hepato-biliary injuries caused by DDC***

Next, we evaluated the amelioratory effects of let-7a-5p on hepato-biliary injuries caused by DDC (Figure 3). We found that DDC feeding for 2 wk or 6 wk both can significantly increase the ratio of liver weight to body weight which may indicate hepatomegaly, but let-7a-5p inhibitor depressed the ratio, compared with anti-SCR control at 2 wk or 6 wk (*P* < 0.05; Figure 3A). In addition, let-7a-5p inhibitor delivered by rAAVs can also reduce the levels of aspartate aminotransferase and BILT that were increased due to hepatic-biliary damages induced by DDC feeding both at 2 wk and 6 wk (*P* < 0.05, Figure 3B and C). At 2 wk, although there was no statistical significance of ALT and alkaline phosphatase (ALP) between anti-let-7a-5p mice and anti-SCR control mice, there are decreasing trends of ALT and ALP activities in anti-let-7a-5p mice than those anti-SCR control mice [*P* > 0.05, Figure 3D and E (upper panel)]. However, after 6 wk, there are significant decreases of ALT and ALP in the sera of anti-let-7a-5p mice, compared with anti-SCR control [*P* < 0.05, Figure 3D and E (nether panner)]. Taken together, let-7a-5p inhibitor delivered by rAAV8 ameliorates the hepato-biliary injuries caused by DDC feeding.

To assess the potential side effects of rAAV delivering anti-let-7a-5p sponges or anti-SCR on kidney function, we also detected serum urea, creatinine, and uric acid in those administrated mice both at 2 wk and 6 wk. Compared with normal control mice, we didn’t find any increases in these indexes after injection of rAAV8, suggesting that rAAV8 injection can’t induce kidney injuries (Supplementary Figure 1).

***let-7a-5p inhibition mediated by rAAV8 ameliorates histologic changes and proliferation of cholangiocytes***

H&E staining showed DDC feeding causes a moderated ductular reaction at 2 wk, and DRs became severe with the extended time of DDC feeding at 6 wk (Figure 4A), but rAAV8-let-7a-5p inhibitor injected mice showed a significant amelioration of the ductular reaction both at 2 wk and 6 wk, compared with anti-SCR control mice (Figure 4A). We also stained the CK19-specific marker for cholangiocytes in the liver, we found that the percentage of positive CK19 cells was dramatically increased after DDC feeding, compared with normal control mice, but anti-let-7a-5p mediated by rAAV8 significantly decreased the percent of positive CK19 cholangiocytes both at 2 wk and 6 wk, compared with anti-SCR control mice (*P* < 0.05, Figure 4B and C).

***rAAV8-delivered let-7a-5p inhibition attenuates liver fibrosis***

Sirus-red staining showed there are massive “strip shape” collagen fibers deposition around bile ducts after DDC feeding for 2 wk and 6 wk, however, after treatment with rAAV8 mediated anti-let-7a-5p for 2 or 6 wk, the deposition of these collagen fibers was significantly decreased (Figure 5A). Furthermore, we detected other fibrotic biomarkers such as *Acta2* (encoding α-SMA) and *Tgfb* using qPCR, after 2 or 6 wk of the feeding of DDC, we found that the relative expression of *Acta2* and *Tgfb* was significantly higher in the DDC feeding mice than those in normal control mice without DDC feeding, but the mice injected with let-7a-5p inhibitor delivered by rAAV8 showed a noteworthy drop (almost five times drop) in *Acta2* and *Tgfb* expression, compared with anti-SCR control mice when they were fed with DDC for 2 or 6 wk (*P* < 0.001, Figure 5B and C). We also detected Hyp-another liver fibrosis marker, it was shown that there was no significant difference in anti-SCR control mice and anti-let-7a-5p mice at 2 wk for DDC feeding, but after 6 wk of treatment, the content of Hyp was significantly decreased in anti-let-7a-5p mice, compared with anti-SCR control mice (*P* < 0.05, Figure 5D). Taken together, rAAV8 delivered let-7a-5p inhibition abates liver fibrosis in experimental sclerosing cholangitis.

***Let-7a-5p inhibition mediated by rAAV8 increased the expression of SOCS1 and Dectin-1*, *thus depression of NF-κB-mediated proinflammatory cytokines***

Our previous and other studies demonstrated that let-7a-5p can potently regulate immune responses to depression NF-κB-mediated pro-inflammation by targeting several molecules such as SOCS1 and Dectin-1[33]. Therefore, we detected hepatic expression of SOCS1, Dectin-1, and P-p65 in the DDC feeding mice that were injected with rAAV8 mediated anti-SCR control or anti-let-7a-5p. Regarding to 2 wk’ DDC feeding, there was no significant differences in the expression of SOCS1 between anti-SCR and anti-let-7a-5p group (*P* > 0.05, Figure 6A and B), but compared with rAAV8 mediated anti-SCR control group, the expression of Dectin-1 in rAAV8 mediated anti-let-7a-5p mice were significantly increased (*P* < 0.05, Figure 6A and C), and the level of downstream inflammatory transfactor P-p65 of NF-κB were remarkably decreased (*P* < 0.05, Figure 6A and D); For 6 wk DDC feeding, anti-let-7a-5p can both depressed the expression of SOCS1 (*P* < 0.05, Figure 6E and F) and Dectin-1 (*P* < 0.05, Figure 6E and G), which induced the lower level of P-p65 of NF-κB (*P* < 0.05, Figure 6E and H).

Sequentially, we further detected the pro-inflammatory cytokines such as IL-6, TNF-α, and CCl2. At 2 wk, DDC feeding induced high levels of *Il6*, *Tnfa*, and *Ccl2* mRNA production, but rAAV8-mediated let-7a-5p inhibitor depressed the production of these cytokines, compared with an anti-SCR group (*P* < 0.01, Figure 7A-C, upper panel). The production of these cytokines in the mice at 6 wk after DDC feeding seems to be declined compared with those in the mice with DDC feeding for 2 wk, but they were still higher than those in the normal control mice (at least more than 3 times; *P* < 0.001, Figure 7A-C, nether panel); furthermore, the production of these cytokines were also significantly decreased in rAAV8 mediated anti-let-7a-5p mice, compared with anti-SCR group mice when they were feeding DDC for 6 wk (*P* < 0.01, Figure 7D-F, D for *II6*, E for *Tnfa*, and F for *Ccl2* nether panel). Taken together, our data demonstrated that anti-let-7a-5p delivered by rAAV8 ameliorate experimental sclerosing cholangitis by targeting at Dectin-1 or SOCS1 -negatively regulated NF-κB.

**DISCUSSION**

PSC is a rare but life-threatening chronic disease and there is no effective medical option to cure it except liver transplantation. DDC-fed (0.1%) mice are a xenobiotic- induced mouse model of cholangiopathy. It can induce reactive cholangiocytes, biliary inflammation, pericholangitis, periductal fibrosis, and ultimately portal-portal bridging affecting large bile ducts, which more closely resembles human sclerosing cholangitis and biliary fibrosis[34,35]. In the present study, using this experimental model of mice, we found that we demonstrate that a single dose of rAAV8-mediated inhibition of let-7a can protect mice from hepatic (biliary) injuries, biliary inflammation, proliferation, and fibrosis in experimental sclerosing cholangitis for 2 wk and 6 wk, suggesting that inhibition let-7a delivered by rAAV8 provides a potential therapeutic strategy for sclerosing cholangitis (Figure 8).

rAAV-based therapeutic has proved as the most effective strategy for gene therapy because rAAV8 can provide an effective, safe, and long-lasting vehicle to deliver genes (including miRNAs) to the targeted organs[36,37]. Currently, rAAV has been implicated in clinical trials in some human diseases such as cystic fibrosis, and Parkinson’s disease, and Glybera-the commercial drug based on rAAV has been applied in the treatment of lipoprotein lipase deficiency[38-43]. rAAV8 with TBG promoters is a liver-tropism serotype that has been widely used to deliver genes targeting hepatocytes with high transduction efficiency, low immunogenicity, and toxicity to the cell. In our present study, we also found that systematic injection of rAAV8 has no side effects on other organs such as the kidney (Supplementary Figure 1), suggesting that rAAV8 is relatively low side effects on other organs[44,45]. In our present study, we attempted to use rAAV8 as a tool to deliver inhibitors of let-7a (anti-sense of let-7a-5p sponge). After injection, rAAV8 can not only invades hepatocytes but also can transfect cholangiocytes, a study showed that 6.8% and 30.9% of cholangiocytes were rAAV8 positive at 10 d and 56 d of transfection, respectively[24]. These data demonstrated that rAAV8 may act as a useful tool for the intervention of cholangiopathies.

Increasing evidence demonstrates that miRNAs play critical roles in cholangiopathies[6]. Let-7 family is the first identified miRNA and increasing data suggested that let-7a plays pro-inflammatory or anti-inflammatory roles by inhibition of several targets[46-50]. Of these signaling pathways that let-7 is involved in, the TLR/NF-κB signaling pathway that is critical to the induction of pro-inflammatory cytokines can be also well-regulated by let-7. For example, let-7a targeted the SOCS1-the feedback inhibitor of NF-κB to facilitate the transcriptional activity of NF-κB and subsequent production of pro-inflammatory cytokines[51]. Similarly, let-7a has been reported to target A20-another inhibitor of the NF-κB pathway, leading to the increased TNF, IL-1β, and nitrite during Mycobacterium tuberculosis infection[52]. In agreement with these observations, we found that the pro-inflammatory cytokines such as TNF, IL-6, and CCl2 were decreased after the treatment with anti-let-7a delivered by rAAV8, which may be associated with the increased SOCS1 expression and the increased activities of NF-κB due to the loss of inhibitor effects of let-7a.

Many miRNAs have been demonstrated to participate in cholestasis[53-55]. Of these miRNAs, let-7a-5p is a recently identified miRNA that is dysregulated in animal models of cholangiopathies (such as BDL and *Mdr2* KO mice) and patients with BA or PSC. Intriguing, it seems that let-7a-5p showed multiple contradictory functions in the different contexts of cholangiopathies due to targeting different genes. For example, let-7a decreased cholangiocytes in BDL mice and inhibits the secretin produced by cholangiocytes and S cells by targeting *NGF*, leading to a repression of the proliferation of cholangiocytes[13]. Similarly, in another study, the expression of let-7a was decreased in the liver of *Mdr2* KO mice or human PSC, which probably increase downstream targets of let-7a (such as the NF-κB, IL-13, and NR1H4) and accelerate the severity of disease[14]. However, in another study, an increased let-7a-5p inhibited BCC2/Abcc2 (also known as MRP2) expression in obstructive cholestasis; the latter is critical for biliary excretion of conjugated bilirubin and the deficiency of BCC2 lead to obstructive cholestasis[12]. In our present study, we found that the inhibition of let-7a delivered by rAAV8 showed therapeutic effects on sclerosing cholangitis induced by DDC feeding at early (2 wk) or chronic stage (6 wk). The expression pattern of let-7a is discrepant in different cell types and the role of let-7a seems cell type-dependent in the different contexts of cholangiopathies. Glaser *et al*[13] found that the more than 15-fold increased expression of let-7a in hepatocytes, but deceased in cholangiocytes in the context of BDL mice. This may partly account for the diverse roles of let-7a in different cholangiopathies.

**CONCLUSION**

In summary, rAAV8 mediated let-7a-5p inhibitor provides powerful therapeutical effects on the DDC-induced sclerosing cholangitis, which can prevent hepato-biliary injuries, ductal reaction, and fibrosis by interfering with pro-inflammatory cytokines production. Our present study provides a possible clinical human clinical translation of PSC using rAAV8 systems to manipulate the expression of let-7a-5p. Other mechanisms by which therapeutic implication of inhibition of let-7a in DDC-induced sclerosing cholangitis are not excluded and further investigations are warranted.

**ARTICLE HIGHLIGHTS**

***Research background***

Primary sclerosing cholangitis (PSC) which may progress to cholangiocarcinoma is an idiopathic cholestatic disease and there is a very limited medical option to interfere with the course of PSC. Recombinant adeno-associated virus (rAAV) provides a prospective platform for gene therapy on such kinds of diseases. A microRNA (miRNA) let-7a has been reported to be associated with the progress of PSC but the potential therapeutic implication of inhibition of let-7a on PSC has not been evaluated.

***Research motivation***

To investigate the potential function and mechanisms of miRNA let-7a-5p transferred by rAAV8 in animal model of PSC.

***Research objectives***

To study the therapeutic effects of inhibition of let-7a-5p transferred by rAAV8 on a 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine (DDC)-induced mouse model of sclerosing cholangitis.

***Research methods***

A mouse model of sclerosing cholangitis was induced by 0.1% DDC feeding for 2 wk or 6 wk, and rAAV8-mediated anti-let-7a-5p sponges or scramble control was injected *in vivo* onset of DDC feeding. After sacrifice of the mice, the liver and the serum were collected from each mouse. The hepatobiliary injuries, hepatic inflammation was evaluated. The targets of let-7a-5p and downstream molecule NF-κB were detected using Western blot.

***Research results***

The reduced expression of let-7a-5p can alleviate hepato-biliary injuries indicated by serum markers, and prevent the proliferation of cholangiocytes and biliary fibrosis. Furthermore, inhibition of let-7a mediated by rAAV8 can increase the expression of potential target molecules such as SOCS1 and Dectin1, which consequently inhibition of NF-κB-mediated hepatic inflammation.

***Research conclusions***

Our findings suggested that a rAAV8 vector designed for liver-specific inhibition of let-7a-5p can potently ameliorate symptoms in a xenobiotic-induced mouse model of sclerosing cholangitis, which provides a possible therapeutic strategy for PSC.

***Research perspectives***

The present study demonstrates that the rAAV-mediated miRNAs strategy may provide a promising therapeutic opportunity for this debilitating and life-threatening disease.

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**Footnotes**

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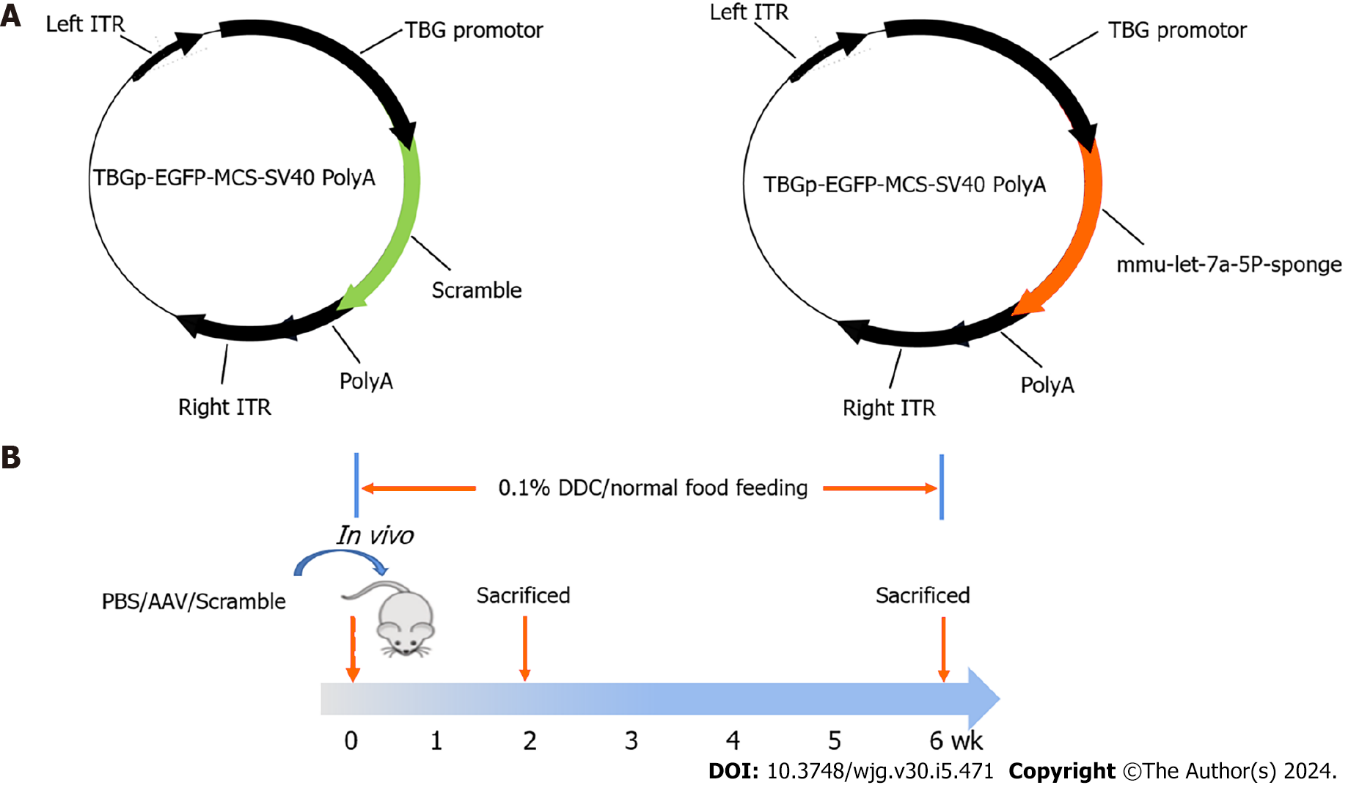
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Grade D (Fair): 0

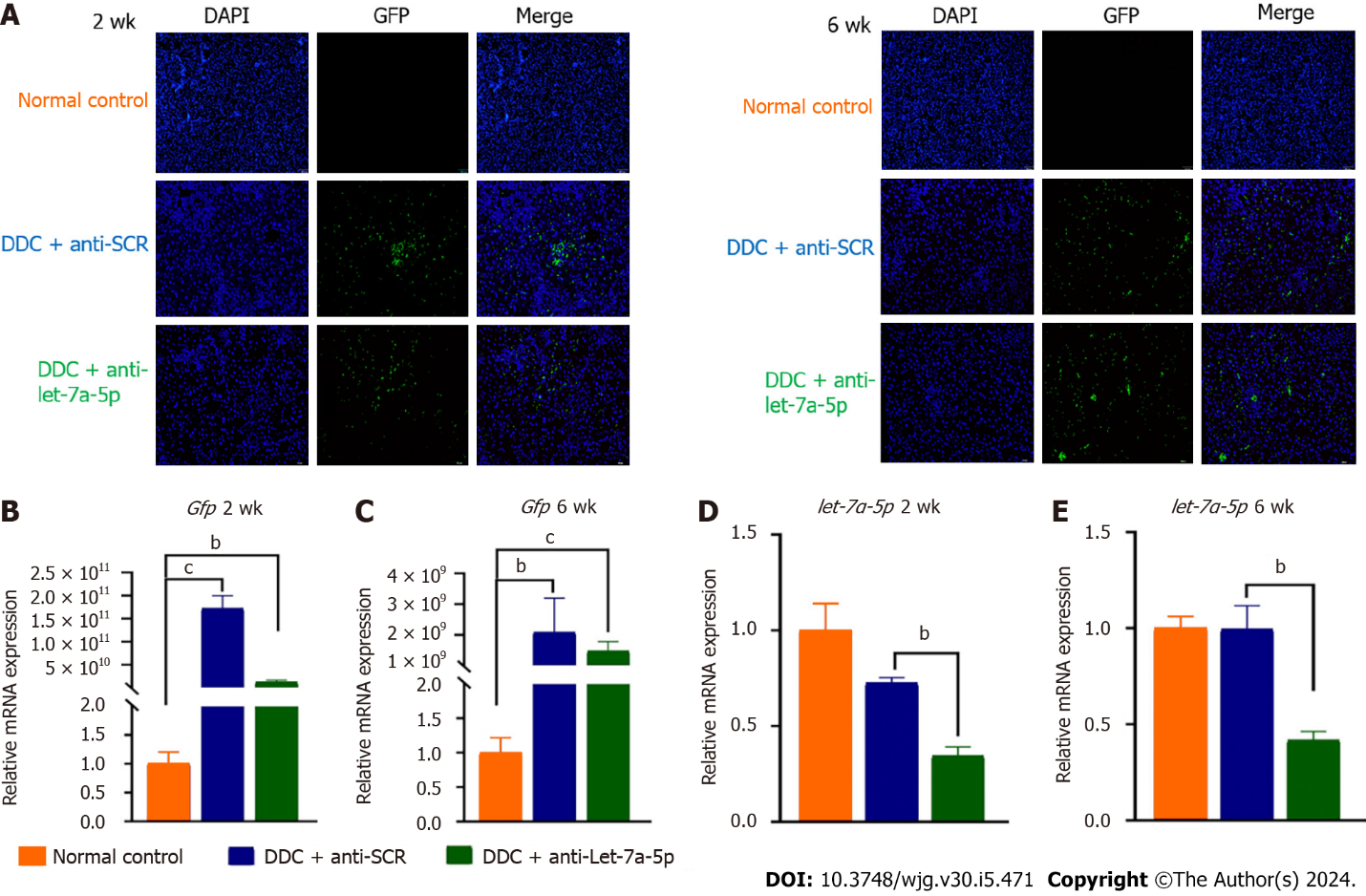
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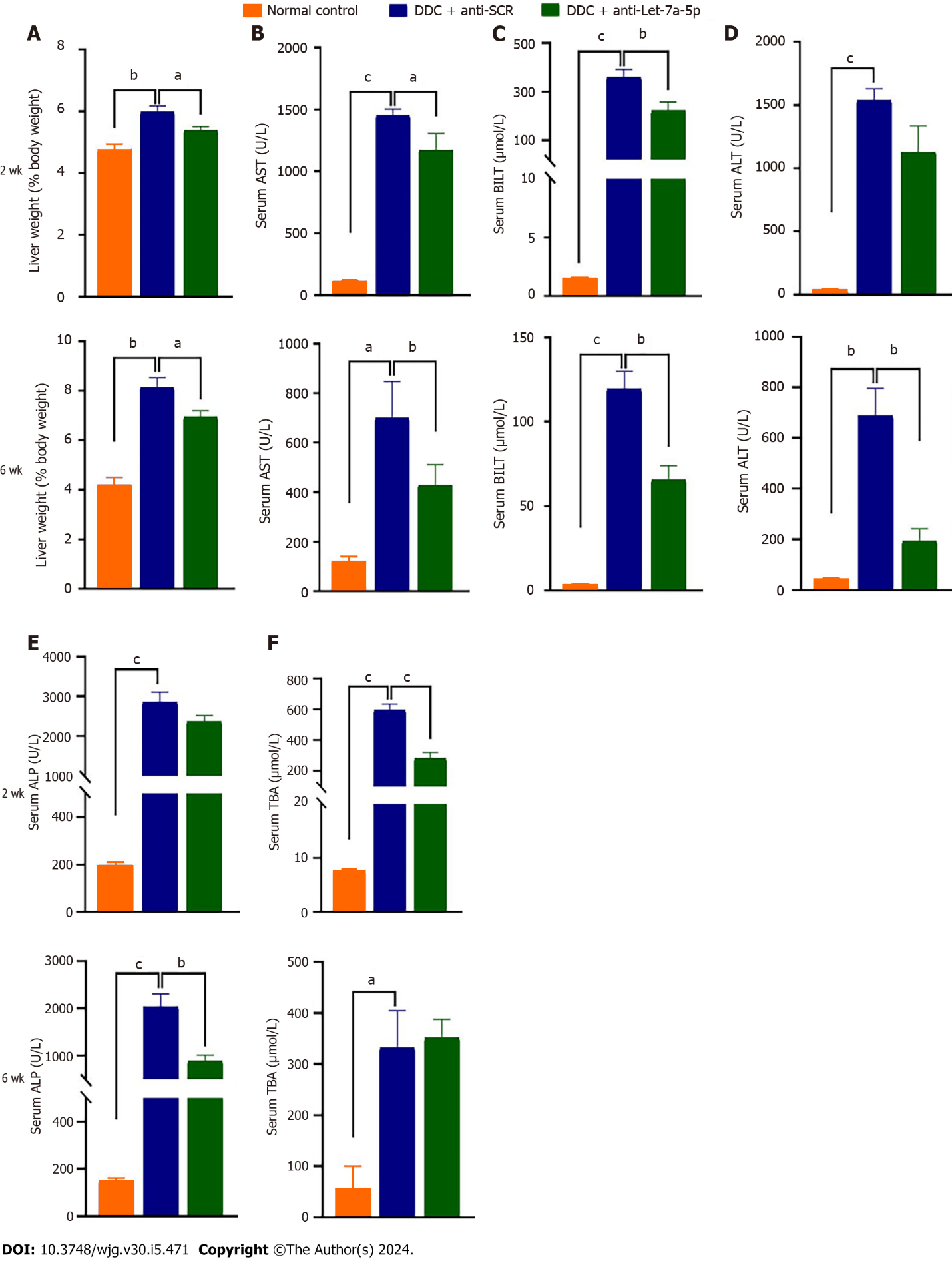
**Figure Legends**



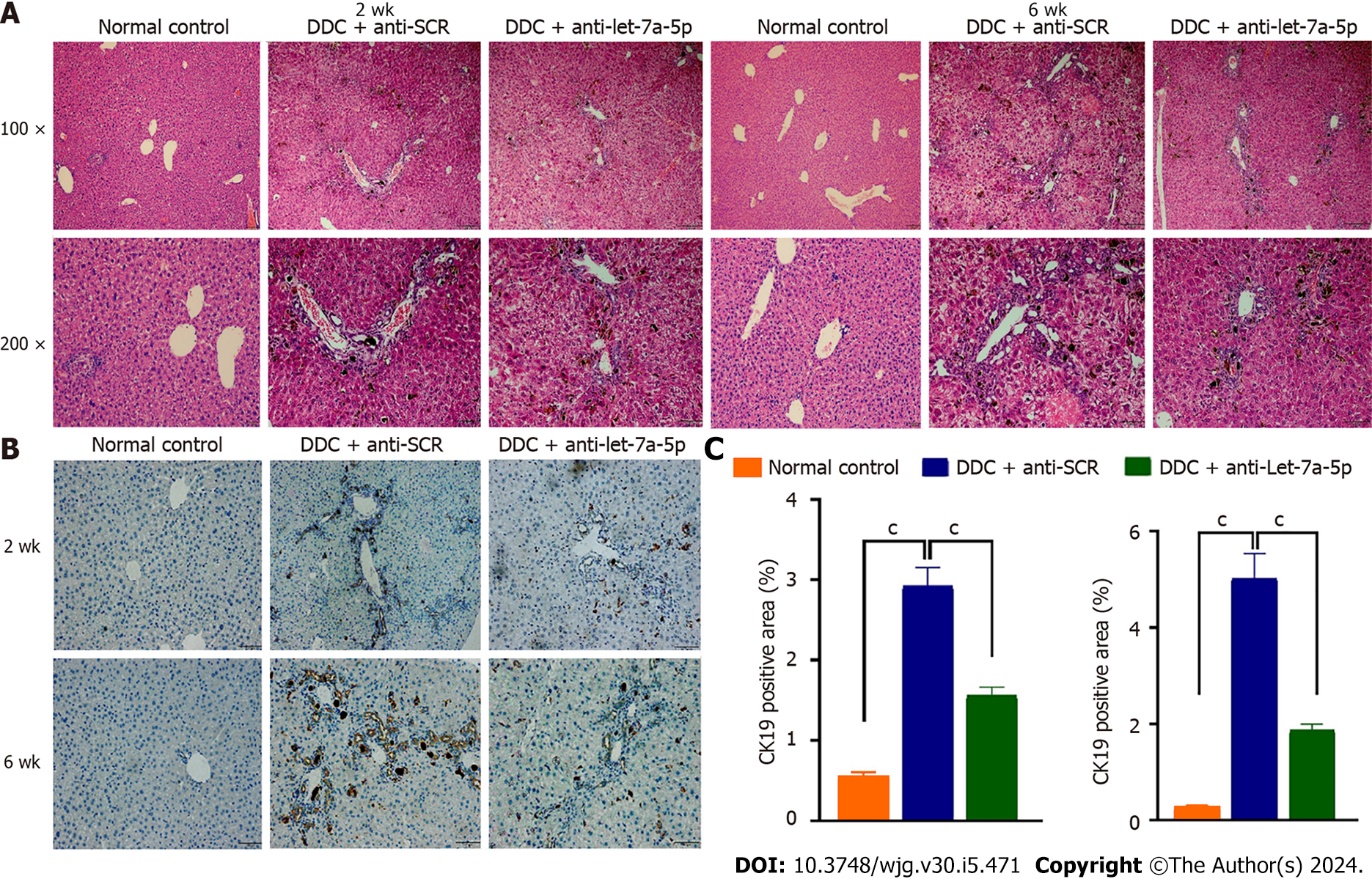
**Figure 1 Schematic representation of recombinant adeno-associated virus 8 delivered let-7a-5p inhibitor and the design of experiment.** A: Diagrams of anti-let-7a-5p sponges’ vector and anti-scramble control (SCR) control vector; B:The design of the experiment. The mice were divided 3 groups (*n* = 6 for each group): (1) Normal control mice with feeding chow; (2) 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine (DDC) feeding mice who were iv injected with adeno-associated virus 8-anti-SCR at the dose of 4 × 1011 vg per mouse; and (3) DDC feeding mice were iv injected with anti-let-7a-5p sponges (anti-let-7a-5p) at the dose of 4 × 1011 vg per mice. The mice in each group were sacrificed at 2 wk and 6 wk after DDC feeding to evaluate the expression of let-7a-5p and the therapeutic effects of let-7a-5p inhibitor. ITR: Invert terminal repeat; TBG: Thyroxine-binding globulin; DDC: 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine; AAV: Adeno-associated virus.



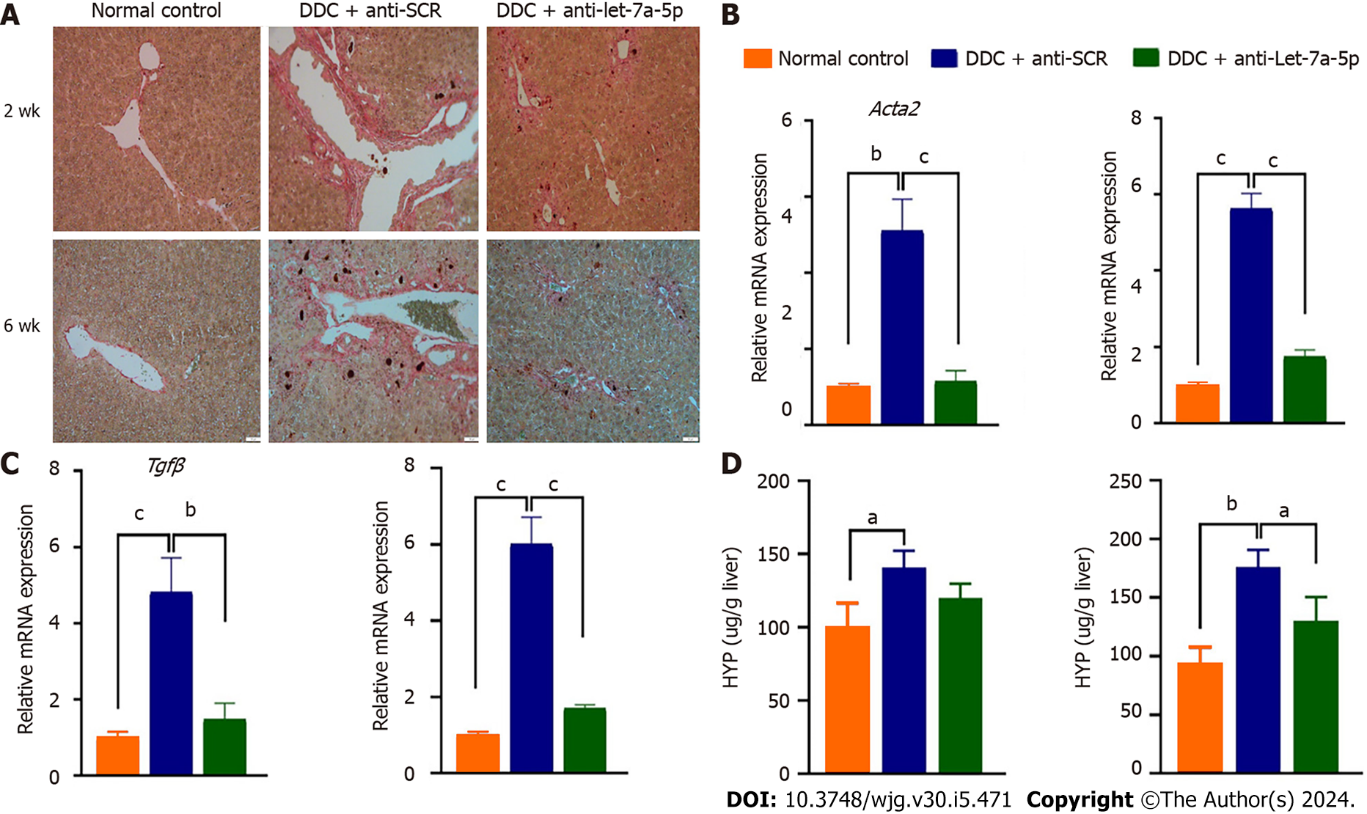
**Figure 2 The expression of let-7a-5p is depressed using anti-let-7a-5p sponges delivered by recombinant adeno-associated virus 8.** A: The recombinant adeno-associated virus 8 was extensively distributed in the liver of mice after 2 or 6 wk of DDC feeding; B and C: The relative expression of GFP indicates the relative amounts of virus; D and E: The relative expression of let-7a-5p in the liver of mice injected by adeno-associated virus 8-mediated scramble control or anti-let-7a-5p sponges after 2 wk or 6 wk of DDC feeding. DDC: 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine; SCR: Scramble control. b*P* < 0.01, c*P* < 0.001.



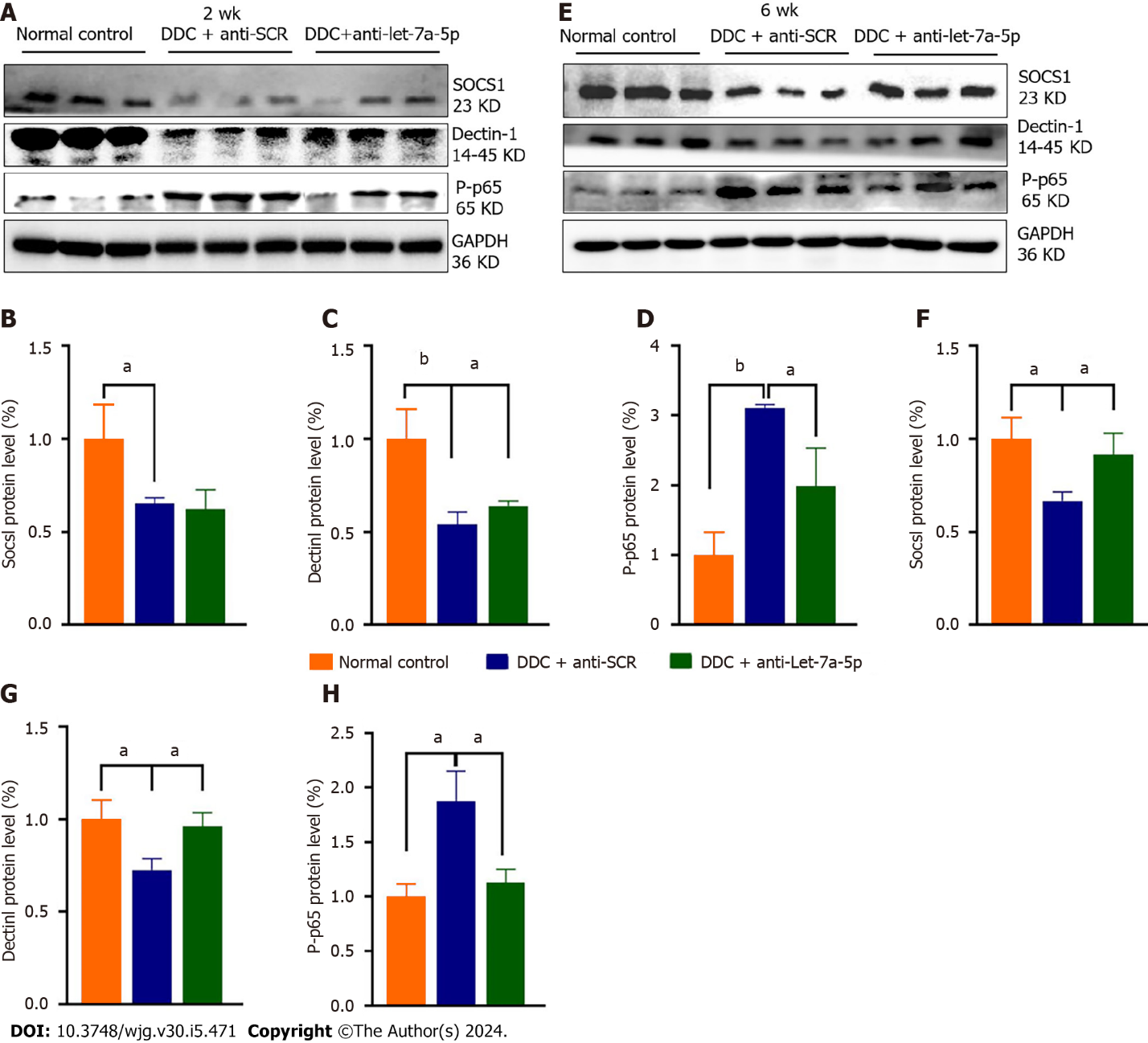
**Figure 3 Recombinant adeno-associated virus 8 mediated anti-let-7a-5p protects from 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine-induced hepato-biliary damages.** A:The ratio of Liver weight to body weight; B-E:Serum levels of aspartate aminotransferase, total bilirubin, alanine aminotransferase, and alkaline phosphatase in the normal control, scramble control, and anti-let-7a sponge (anti-let-7a-5p) mice for 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine feeding at the indicated time (2 wk and 6 wk). DDC: 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine; SCR: Scramble control; AST: Aspartate aminotransferase; BILT: Total bilirubin; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; TBA: Total bile acid. a*P* < 0.05, b*P* < 0.01, c*P* < 0.001.



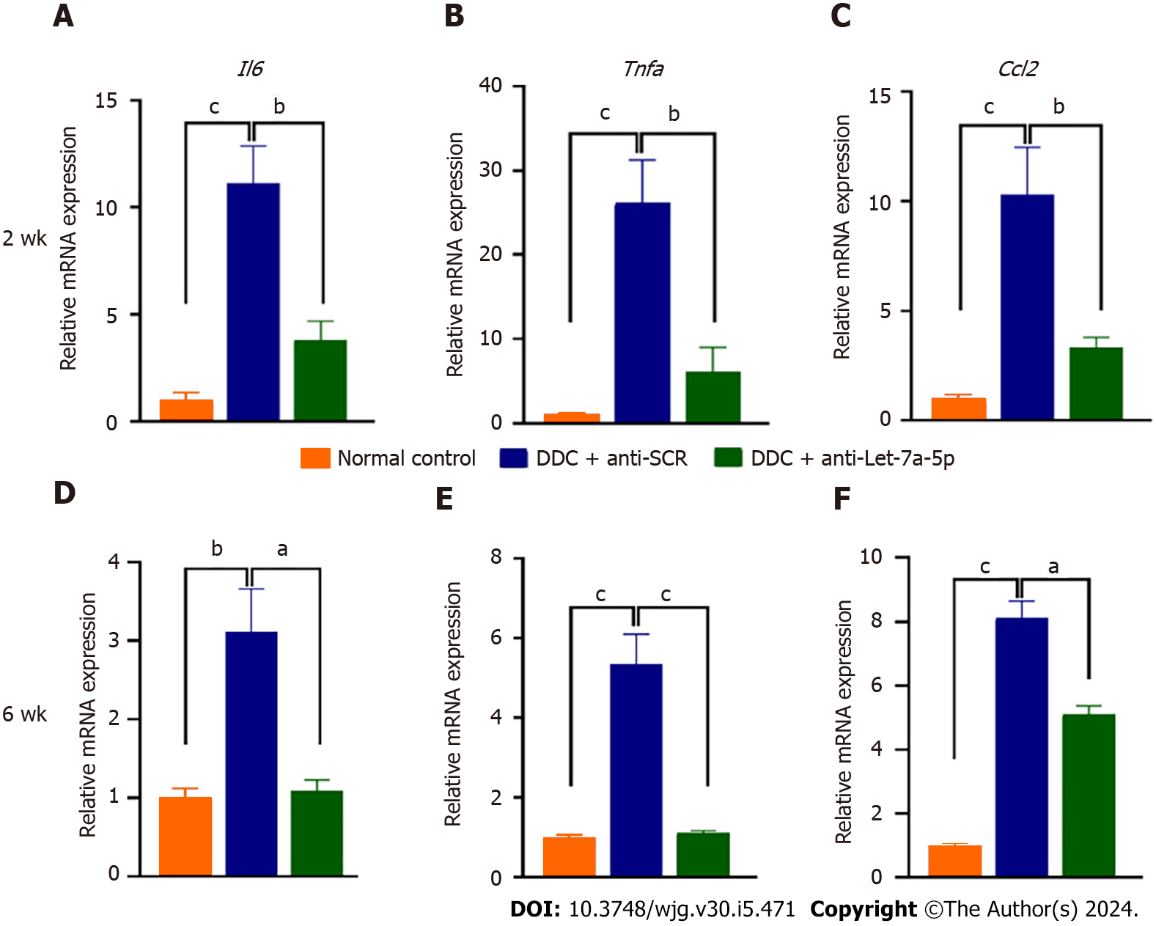
**Figure 4** **Let-7a-5p inhibition delivered by recombinant adeno-associated virus 8 prevents liver pathology and the proliferation of cholangiocytes.** A: Histological changes at 2 and 6 wk was evaluated using hematoxylin and eosin staining. The upper panel was shown in 100 times amplification; the nether panel was shown in 200 times amplification; B: CK19 was stained for indicating the proliferation of cholangiocytes using Immunohistochemistry staining; C: and the percent of CK19 positive cells in the liver was calculated at 2 and 6 wk. DDC: 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine; SCR: Scramble control. c*P* < 0.001.



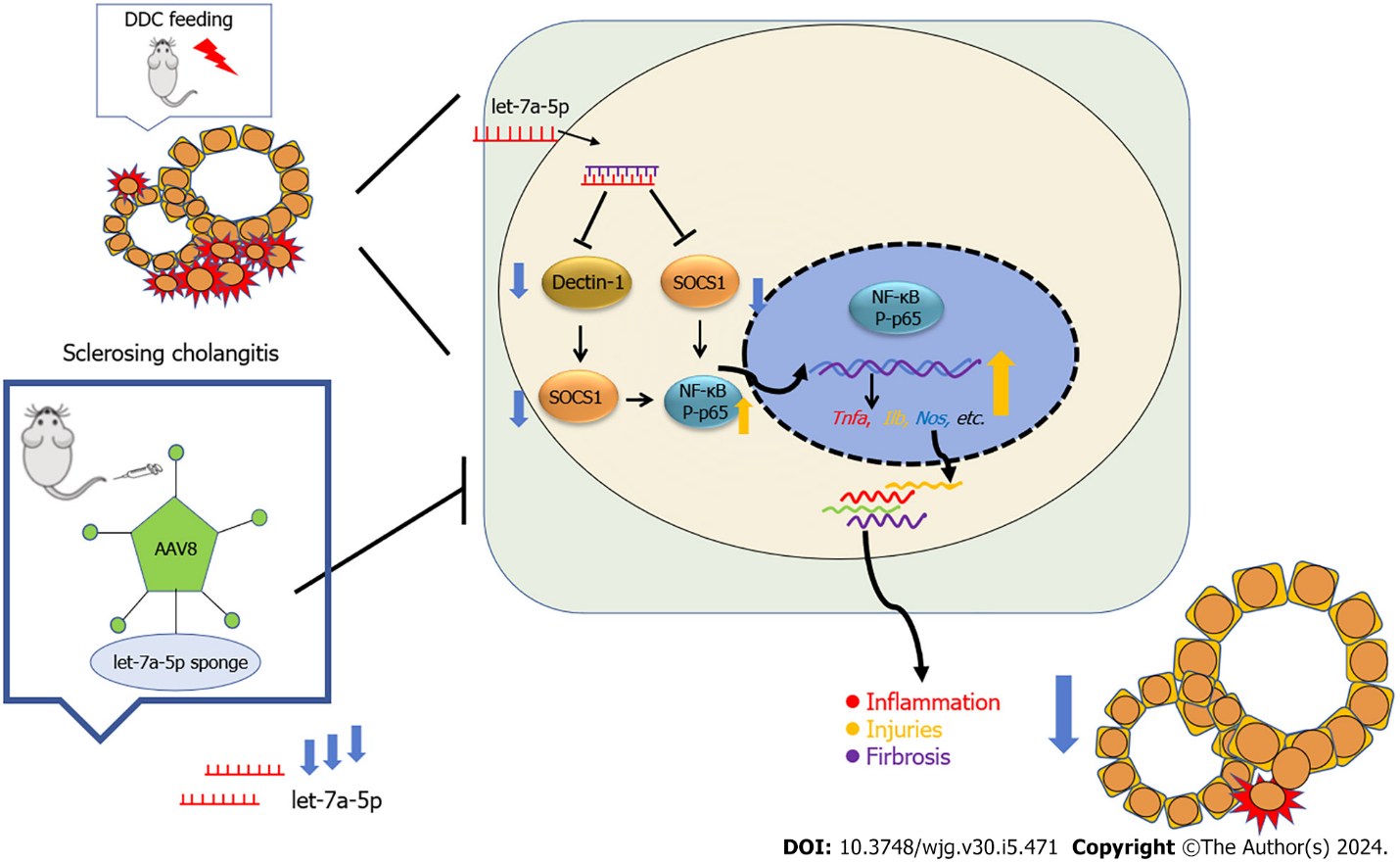
**Figure 5 Let-7a-5p inhibition mediated by recombinant adeno-associated virus 8 ameliorates biliary fibrosis in the 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine-induced cholestasis model.** A: Sirius Red staining in the liver of mice at 2 wk and 6 wk after 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine feeding; B and C: The fibrosis markers such as *Acta 2* and *Tgfb* were evaluated using qRT-PCR; D: The contents of Hyp in the liver of mice in each group were determined. DDC: 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine; SCR: Scramble control; Hyp: Hydroxyproline. a*P* < 0.05, b*P* < 0.01, c*P* < 0.001.



**Figure 6 Recombinant adeno-associated virus 8 mediated let-7a-5p inhibitor interfers suppressor of cytokine signaling 1/Dectin1 mediated NF-κB signaling pathway.** The expression of suppressor of cytokine signaling 1 (SOCS1)/Dectin1/P-p65 in each mouse of normal control group, anti-scramble control and anti-let-7a-5p group were assayed by western-blot.A:The blot of SOCS1/Dectin1/ P-p65 in each group after 2 wk 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine (DDC) feeding; B-D:the relative levels of SOCS1, Dectin1, and P-p65 in each group after 2 wk DDC feeding; E:The blot of SOCS1/Dectin1/P-p65 in each group after 6 wk DDC feeding; F-H: relative levels of SOCS1, Dectin1, and P-p65 in each group after 6 wk DDC feeding. DDC: 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine; SCR: Scramble control. a*P* < 0.05, b*P* < 0.01.



**Figure 7 Let-7a-5p inhibition mediated by adeno-associated virus 8 ameliorates local pro-inflammatary cytokines produced.** The levels of *Il6*, *Tnfa*, and *Ccl2* in the liver of mice from each group after 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine (DDC) feeding for 2 or 6 wk were evaluted by qPCR.A-C:The relative levels of *Il6*, *Tnfa*, and *Ccl2* after 2 wk DDC feeding; D-F: The relative levels of *Il6*, *Tnfa*,and *Ccl2* after 6 wk DDC feeding. DDC: 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine; SCR: Scramble control. a*P* < 0.05, b*P* < 0.01, c*P* < 0.001.



**Figure 8 Schematic** **summary of an amelioration of sclerosing cholangitis in a clinically relevant mouse model by recombinant adeno-associated virus 8-mediated microRNA let-7a inhibition.** DDC: 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine; SOCS: Suppressor of cytokine signaling.

**Table 1 The primers used in the present study**

|  |  |  |
| --- | --- | --- |
| **Target** | **Oligonucleotide sequence (5’-3’)** | |
| **Forward primer** | **Reverse primer** |
| *let-7a-5p* | GGAGGTAGTTCGTTGTGTGGT |  |
| *U6* | ATGGGTCGAAGTCGTAGCC | TTCTCGGCGTCTTCTTTCTCG |
| *Gfp* | TGCTTCAGCCGCTACCC | AGTTCACCTTGATGCCGTTC |
| *β-actin* | AACTCCATCATGAAGTGTGA | CTGCGGCTTCTATTGGGGAC |
| *Tnfa* | CTTGTTGCCTCCTCTTTTGCTTA | GACTTCAGCACTCAAGACATCC |
| *Il6* | TCACAGAAGGAGTGGCTAAGGACC | ACGCACTAGGTTTGCCGAGTAGAT |
| *Acta2* | TTCATCGGGATGGAGTCTGCTGG | TCGGTCGGCAATGCCAGGGT |
| *Tgfb* | CCACCTGCAAGACCATCGAC | CTGGCGAGCCTTAGTTTGGAC |
| *Ccl2* | TTAAAAACCTGGATCGGAACCAA | GCATTAGCTTCAGATTTACGGGT |



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