

Human Menstrual Blood-derived Stem Cell Transplantation Suppresses Liver Injury in DDC-induced Chronic Cholestasis

Ya Yang

Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of Infectious Diseases

Yanfei Chen

Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of Infectious Diseases

Yalei Zhao

Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of Infectious Diseases

Feiyang Ji

Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of Infectious Diseases

Lingjian Zhang

Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of Infectious Diseases

Shima Tang

Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of Infectious Diseases

Sainan Zhang

Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of Infectious Diseases

Qingqing Hu

Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of Infectious Diseases

Zuhong Li

Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of Infectious Diseases

Fen Zhang

Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of Infectious Diseases

Qian Li

Zhejiang University First Affiliated Hospital State Key Laboratory for Diagnosis and Treatment of Infectious Diseases

Ianjuan li (✉ ljli@zju.edu.cn)

State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, 1st Affiliated Hospital, College of Medicine, Zhejiang University <https://orcid.org/0000-0001-6945-0593>

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Abstract

Background: Cholestasis liver injury can lead to serious symptoms and prognoses in the clinic. Currently, an effective medical treatment is not available for cholestasis liver injury. Human menstrual blood-derived stem cells (MenSCs) have been considered an emerging treatment in various diseases. However, efficiency of MenSCs in cholestasis liver injury has been less investigated.

Methods: C57/BL6 mice were fed with 3,5-diethoxycarbonyl-1,4- dihydroxycholellidine (DDC) to induce chronic cholestasis liver injury model. DDC mice were injected with either phosphate buffer saline (PBS) or MenSCs at 2 and 4 weeks. Mice were sacrificed at 5 weeks. Serum and liver tissues were collected to test liver function and pathological changes. Proteomics and western blot were used to explore the related molecular mechanisms. Adeno-associated virus (AAV)9 infected mice were used for verifications of MenSC treatment target and related molecular mechanism.

Results: We found that MenSCs could protect mice against DDC-induced cholestasis by improving impaired liver function and tissue damage. MenSCs markedly prolonged survival rate of mice, decreased the mice serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), direct bilirubin (DBIL) and total bilirubin (TBIL) levels as well as intrahepatic cholestasis, bile duct dilation and fibrosis. The results further indicated that MenSCs repaired DDC-induced tight junction (TJ) and bile transport function damage and inhibited COL1A1, α -SMA and TGF- β 1 activation by upregulating liver β -catenin expression. MenSC transplantation could be an effective treatment method for cholestasis liver injury.

Background

Cholestasis liver injury, including primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), is a rare chronic liver disease characterized by the disruption of bile acid (BA) flow and increased BA concentration in the systemic circulation [1,2]. As disease progresses, nonspecific symptoms including pruritus and fatigue would appear, and eventually leads to cirrhosis or death due to liver failure [3].

Treatments for cholestasis liver injury include ursodeoxycholic acid (UDCA) and obeticholic acid (OCA), the only approved drugs, as well as symptomatic treatments and liver transplantation. Long-term application of UDCA can improve the biochemistry of serum and delay the progression of the histological stage. However, approximately 25%-40% of patients do not respond to UDCA [4,5]. OCA is applied to patients who are unsensitive to UDCA, although its administration is associated with several side effects [6,7]. Additionally, some researchers have indicated that the application of OCA might be associated with increased risk of liver failure in PBC patients [8]. PBC and PSC represent major indications for liver transplantation (LT). However, there is a contradiction between urgent clinical needs and donor liver shortage. In addition, numerous studies have clearly demonstrated that PBC and PSC recur after LT [9]. Therefore, new effective methods for treating cholestasis liver injury are necessary.

Mesenchymal stem cells (MSCs) are novel adult stem cells isolated from various tissues. MSCs could be used in various clinical applications, especially stem cell-based therapies [10]. MenSCs are derived from women's menstrual blood [11]. Unlike the MSCs obtained from other adult tissues such as bone marrow, amniotic fluid, and adipose tissue, MenSCs can be derived through a simple, safe, non-invasive procedure with fewer ethical problems. Compared with MSCs derived from adult bone marrow tissue, MenSCs show higher proliferation rate [12], which means that MenSCs may have broader clinical application prospects in the future. However, the study of the treatment efficacy of MSCs in cholestasis liver injury is limited.

In this study, we aimed to evaluate the efficacy of MenSC transplantation in mice with DDC-induced cholestasis liver injury. We further elucidated the related molecular mechanism of MenSC treatment in DDC mice. This study contributes to the potential effect of MenSCs in cholestasis liver injury, suggests MenSC transplantation a promising application for treating cholestasis in the clinic.

Materials And Methods

Animals

Six- to eight-week-old male C57BL/6 mice were purchased from the Experimental Animal Center of Zhejiang Academy of Medical Sciences and housed under standard conditions.

Cell culture

The MenSCs were cultured in DMEM/F12 (Gibco, USA) containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco).

DDC-Induced sclerosing cholangitis and MenSC transplantation

C57BL/6 mice were randomly divided into different groups and fed with either a control diet (control group) or a diet containing DDC (0.1%) for up to 5 weeks.

To evaluate the treatment effect of MenSCs in sclerosing cholangitis, 5×10^5 cells in 500 μ l of PBS were injected into mice through tail vein in the 2nd and 4th weeks of DDC treatment. The mice received MenSC transplantation was considered DDC + MenSC group. DDC-fed mice injected with equal amount of PBS were considered DDC group.

After 5 weeks of DDC feeding, mice were anesthetized and sacrificed. Mice hepatic tissues were collected immediately. Liver tissues were either fixed in paraffin or stored with frozen liquid nitrogen for further analysis. We collected peripheral blood and centrifuged for serum separation and stored at -80°C for testing.

Surface markers and differentiation of MenSCs

The expression levels of MenSC surface markers were detected by fluorescence-activated cell sorting (FACS). Collected MenSCs (2×10^7) were washed with staining buffer (BD Biosciences, USA) and incubated for 1 h in diluted antibodies including: CD29, CD34, CD45, CD73, CD90, CD105, CD117 and human leukocyte antigen-DR (HLA-DR) (Becton Dickinson, USA). Isotype antibodies IgG1 and IgG2a (Becton Dickinson) were applied as negative control. FC500 flow cytometer (Beckman Coulter, USA) and FlowJo software (Tree Star, Inc., USA) was applied for analysis.

Human mesenchymal stem cell osteogenic differentiation medium kit, chondrogenic differentiation medium kit, and adipogenic differentiation medium kit (Cyagen Biosciences, USA) were used to detect the differentiation potential of MenSCs.

Vivo tracking of MenSC

XenoLight DiR (Perkin Elmer, USA) was applied for MenSC migration tracking in vivo. XenoLight DiR solution was diluted and incubated with MenSCs at room temperature for 20 min. Then MenSCs were washed and resuspended in PBS. The resuspension was then injected into the control and DDC mice through tail veins. IVIS analysis (Caliper Life Sciences, USA) were used after 1 d, 3 d and 7 d.

Liver function tests

Mice hepatic function was evaluated by serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), direct bilirubin (DBIL) and total bilirubin (TBIL) levels, which were tested using commercial kits (Nanjing Jiancheng Bioengineering Institute, China).

Histological analysis and transmission electron microscopy

For histological analysis, fixed liver samples were embedded in paraffin and sectioned using MicromHM325 rotary microtome (Thermo Fisher Scientific Life Sciences) for H&E, Sirius Red and Masson staining. Fibrotic area (COL1A1 /total area) was quantified using ImageJ software.

For transmission electron microscopy, liver tissues were fixed in 3% glutaraldehyde and rinsed in PBS before being placed in the secondary fixative 1% osmium tetroxide solution for 2 h. Then the samples were rinsed 3 times in PBS and dehydrated in ethanol by density gradient. Samples were embedded in embedding agent and sectioned at 70 nm thickness. Samples were compared with uranyl acetate and lead citrate, then examined with transmission electron microscope (TEM).

Immunohistochemistry

Paraffin hepatic tissues were used. Samples were deparaffinized and hydrated in ethanol. 3% hydrogen peroxide was applied to block endogenous peroxidase. The samples were incubated with COL1A1 (1:1000; Cell Signaling Technology, 72026S) and α -smooth muscle actin (α -SMA) (1:1000; Cell Signaling Technology, 19245) antibodies at 4°C for 10 h. Then, samples were washed and incubated with secondary antibody (Abcam, United Kingdom) at 37°C for 1 h. Samples were stained using 3,3'-diaminobenzidine solution (DAB kit, Abcam) and scanned with NanoZoomer Digital Pathology system.

Western blot analysis

Hepatic sample lysates were extracted using RIPA buffer supplemented with cocktail protease and phosphatase inhibitor (Beyotime, China). The BCA protein assay kit (Thermo Scientific, USA) was applied to detect protein concentrations. Equal amounts of total protein were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA) after separation on 4–20% SDS-polyacrylamide electrophoresis gels (GenScript, China). Transferred membranes were blocked in diluted QuickBlock buffer (Beyotime) for 30 min, then incubated at 4°C for 10 h in the following primary antibodies: β -Catenin (1:1000; Cell Signaling Technology, 8480S), Claudin 1 (1:1000; Proteintech, 13050-1-AP), Claudin 3 (1:500; Affinity, AF0129), Claudin 5 (1:1000; Affinity Biosciences, AF5216), Claudin 7 (1:1000; Proteintech, 10118-1-AP), NTCP1 (1:1000; Abcam, ab131084), BSEP (1:100; Santa Cruz, sc-74500), OATP2 (1:100; Santa Cruz, sc-376424), Occludin (1:1000; Cell Signaling Technology, 91131), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000; Cell Signaling Technology, 5174). Then, the membranes were incubated in secondary antibodies (1:2000; Cell Signaling Technology) and detected by chemiluminescence reagents (Beyotime). ImageJ software was applied to semiquantify and analyze protein band intensities.

Protein extraction and digestion

The liver samples were separately powdered with liquid nitrogen and suspended in lysis buffer [8 M urea, 50 mM Tris 8.0, 1% NP40, 1% NaDOC (sodium deoxycholate), 5 mM dithiothreitol, 2 mM EDTA, 30 mM nicotinamide, 3 μ M trichostatin A, 1% cocktail protease (Sigma, P8215, for use with fungal extracts) and 1% phosphatase inhibitor cocktail (Solarbio, P1260)]. Samples were sonicated and centrifuged at 4°C for 10 min at 20000 g to remove tissue fragments. Protein content was detected using a 2D Quant kit (GE Healthcare). Equal amounts of protein were reduced with 5 mM dithiothreitol for 45 min at 30°C, alkylated with 30 mM iodoacetamide for 1 h in the dark at room temperature, then precipitated with acetone on ice. The precipitate was washed with acetone and suspended in 0.1 M triethylammonium bicarbonate (TEAB), digested with trypsin (Promega) overnight at 37°C. 1% trifluoroacetic acid was used to stop the digestion. Peptide obtained was cleaned using a Strata X C18 SPE column (Phenomenex) and vacuum-dried in a Scan-Vac maxi-beta (Labogene).

TMT labeling and HPLC fractionation

The peptide samples were reconstituted in 120 μ l 0.5 M TEAB and treated using a TMTsixplex label reagent kit (Pierce, 90068). One unit of TMT reagent (5 mg) was thawed and reconstituted in 420 μ l acetonitrile. Subsequently, four reconstituted samples were mixed separately with four TMT reagents (126, 127, 128, and 129), incubated for 2 h at room temperature and then vacuum-dried. Finally, labeled samples were resuspended in water and mixed. The labeled peptide samples were fractionated for proteome analysis. Fractionation was performed with an XBridge Shield C18 RP column (Waters) in an LC20AD HPLC system (Shimadzu).

Construction and infection with recombinant AAV-Ctnnb1-shRNA

To deplete β -Catenin expression in liver tissue, AAV9 that contained Ctnnb1 shRNA (shCtnnb1) and AAV-shControl was constructed by Genomeditech (Shanghai, China). Mice were injected via tail vein with 150 μ l of PBS containing 3×10^{11} VG of the AAV-shCtnnb1 or AAV-shControl. At 2 d after virus injection, mice were fed a DDC or control diet for up to 5 weeks.

Statistical analysis

All data were reported as mean \pm standard deviation (SD). Student's t-test was used for comparisons of liver function, relative protein expression and fibrotic area among the groups. Graphs were generated using GraphPad Prism 8.0.2 (GraphPad, USA). P values of less than 0.05 were considered statistically significant.

Results

Surface makers and tracking of MenSCs

We used flow cytometry to identify immunophenotype of MenSCs. The results showed positive expression of CD29, CD73, CD90 and CD105 and negative expression of CD34, CD45, CD117 and HLA-DR (Fig. 1A). MenSCs cultured in vitro were spindle-shaped and could be induced to differentiate into osteoblasts, adipocytes, and chondrocytes (Fig. 1B).

To determine the distribution and duration of MenSCs in mice, MenSCs were labeled with XenoLight DiR. Labeled MenSCs were injected into the tail veins of control and DDC-induced mice, and their distribution was evaluated by in vivo imaging. Fluorescence was detected in lungs, liver and spleen but not in heart or kidneys (Fig. 1C). Schedule of DDC feeding and MenSC transplantation was described above (Fig. 1D).

MenSCs reduce symptoms caused by DDC in mice

As we introduced above, mice were fed a DDC diet for 5 weeks before sacrifice. MenSCs were transplanted in the 2nd and 4th weeks of feeding (Fig. 1D). Compared with control mice, DDC-induced mice showed obvious weight loss, fur loss and jaundice, which could be significantly reduced by MenSC transplantation (Fig. 2A, B). Additionally, MenSCs decreased the high BA concentration in the systemic circulation of DDC mice (Fig. 2C). Grossly, the livers of DDC mice were dark red, smaller and stiffer and the gallbladders were enlarged compared with those of control mice, while MenSC treatment improved these symptoms (Fig. 2D). The survival study showed that 4 mice died 9 weeks after modeling (6/10). MenSC transplantation improved the survival rate up to 100% (Fig. 2E). To evaluate the liver functions, we tested the AST, ALT, ALP, DBIL and TBIL levels in mouse serum. The results showed that MenSCs could significantly reduce the levels of AST, ALT, ALP, DBIL and TBIL, which means that MenSC transplantation could restore DDC-induced mouse liver damage (Fig. 2F).

MenSC transplantation improved DDC-induced pathological changes in mouse livers

Histological assessment using H&E staining of hepatic tissues exhibited periportal ductular reactions, including intrahepatic bile duct dilation and cholestasis, as well as inflammatory cells infiltration in the DDC group liver, which were significantly relieved in the MenSC group (Fig. 3A). Masson and Sirius Red staining showed fibrotic changes in the hepatic sections of the DDC mice (Fig. 3A, B). Additionally, the expressions of α -SMA and COL1A1, the markers of hepatic fibrosis, were measured by immunohistochemistry. The results indicated that α -SMA and COL1A1 expressions were increased in the DDC mice and significantly reduced after MenSC transplantation (Fig. 3C, D). Transmission electron microscopy of control liver sections revealed a TJ structure, which is a thin double-stranded electron-dense structure of defined diameter (Fig. 3F). The TJ structure in the DDC livers showed less electron-dense and poorly defined margins and was partly restored in the MenSC group.

MenSC transplantation altered the liver proteomics profile of DDC-fed mice

To further explore the molecular mechanism of treatment efficacy of MenSC transplantation in DDC-induced cholestasis liver injury, a proteomics analysis was performed. Principal component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were used to visualize proteomic differences in different groups. The PCA score plot revealed no outliers (Fig. 4A). Additionally, no discernible clustering was observed between DDC and DDC + MenSC group. The PCA score plot was characterized by the following parameters: $R^2X = 0.896$, $Q^2 = 0.842$. The DDC and DDC + MenSC groups were clearly distinguished in the OPLS-DA score plot (Fig. 4B, C). After the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO) and Gene Set Enrichment Analysis (GSEA) pathway analyses of the differentially expressed proteins, the TJ signaling pathway caught our attention (Fig. 4D and E).

MenSCs promoted TJ- and bile transport function-related protein expression and reduced fibrosis-related proteins expression

To verify our hypothesis, western blot was applied to detect the related proteins expression. The results indicated that Claudin-1, Claudin-3, Claudin-5, Claudin-7 and Occludin were reduced after DDC feeding but restored by MenSC transplantation (Fig. 5A). Considering the significant efficiency of MenSC transplantation in DDC-induced hyperbilirubinemia in mice, the transport function-related proteins BSEP, OATP2 and NTCP1 were also analyzed by western blot. The results indicated that compared with control mice, BSEP, OATP2 and NTCP1 were decreased in the DDC group and restored in the DDC + MenSC group (Fig. 5B). Next, we detected expressions of COL1A1, TGF- β 1 and α -SMA, which are proteins related to the fibrosis pathway. According to the results, COL1A1, TGF- β 1 and α -SMA levels were increased in DDC group but decreased in DDC + MenSC group (Fig. 5C). It was previously reported that liver-specific β -catenin knockout mice had intrahepatic cholestasis, which resembled DDC-induced symptoms. Thus, we

measured the β -catenin levels in liver tissues. β -catenin was significantly inhibited in DDC group and upregulated in DDC + MenSC group (Fig. 5D).

Liver β -Catenin deficiency inhibited the therapeutic efficacy of MenSC transplantation in DDC-induced models

To verify this hypothesis, β -catenin knockdown mice were used. Mice were infected with AAV-shControl or AAV-shCtnnb1. Compared with the liver tissues of normal mice, green fluorescence could be detected in the liver tissues of AAV-infected mice (Fig. 6A). To evaluate the β -catenin knockdown efficiency, the liver β -catenin expression levels were detected by western blot. Compared with AAV-shControl mice, AAV-shCtnnb1 mice showed lower liver β -catenin levels (Fig. 6B). Liver function tests showed that MenSC transplantation failed to downregulate serum AST, ALT, ALP, DBIL and TBIL levels (Fig. 6C). According to the H&E staining of the liver tissues, MenSCs did not significantly improve DDC-induced intrahepatic cholestasis or bile duct dilation in AAV-shCtnnb1 mice (Fig. 6D). Additionally, MenSC failed to reduce the fibrotic area in DDC-fed AAV-shCtnnb1 mice (Fig. 6E, F). The results indicated that liver β -Catenin deficiency could extremely inhibit the treatment efficacy of MenSC transplantation in DDC-induced liver injury.

β -Catenin deficiency inhibited the regulatory effect of MenSC transplantation on related proteins

To further explore the regulatory effect of β -catenin on related pathways and proteins, western blot was used to assess the expression of proteins. According to the results, MenSC transplantation could not restore the DDC-induced damage to β -Catenin, Occludin, Claudin-1, Claudin-3, Claudin-5 and Claudin-7 expression in AAV-shCtnnb1 mice (Fig. 7A, B). Similar results were also detected in BSEP, OATP2 and NTCP1 levels (Fig. 7C). Subsequently, we detected TGF- β 1 and α -SMA expression levels. We found that MenSCs failed to inhibit the expression of TGF- β 1 and α -SMA in AAV-shCtnnb1 mice (Fig. 7D). These results showed that MenSCs regulated the related proteins and pathways by upregulating β -Catenin expression

Discussion

Cholestasis liver injury is a pathophysiological process induced by bile secretion and excretion disorders. A variety of factors, such as drugs, oxidative stress, inflammatory injury and immune disorders, are considered as the causes of cholestasis [13]. Currently, adults with different phenotypes of cholestasis have increasingly been evaluated for variants in these genes to identify specific cholestasis-related genes [14,15]. Excessive accumulation of bile components, including bile acid, cholesterol and bilirubin in hepatic and systemic circulation is considered as the major driver of liver injury. Long-term continuous cholestasis could develop into liver fibrosis and even cirrhosis [16].

Although UDCA could slow disease progression in some cholestasis patients, UDCA unsensitivity and intolerance remain outstanding issues [13]. The limited treatment effect of UDCA cholestasis indicates

that novel therapeutic approaches are required.

Currently, MSC transplantation is increasingly applied in researches due to its therapeutic potential in a variety of liver diseases. Investigators suggested the effect of MSCs in promoting liver tissue repair and survival rates in acute liver failure, hepatectomy, hepatitis B virus-related acute-on-chronic liver failure, ischemic-type biliary lesions, liver fibrosis, liver transplantation and related graft-vs-host disease, et al [17–24]. Although rash and fever (37–38°C) that resolved without additional treatment were observed in several patients [25], no MSC transplantation-related safety issues were detected in either short- or long-term follow-up [26,27]. All these previous studies indicate that MSC transplantation is an ideal candidate for cholestasis treatment.

In this study, we demonstrated that MenSCs could attenuate the development of DDC-induced liver injury. Injected MenSCs could accumulate in mouse livers and significantly improve emaciation, jaundice and mortality. Intrahepatic bile duct dilation, cholestasis and concomitant fibrosis are the main pathological changes in DDC mice and could be reduced by MenSC therapy.

However, previous studies indicated that although transplanted MSCs were recruited to injured liver sites, few cells differentiated into HLCs [12]. Most researchers believe that MSCs exhibit treatment effects mainly through paracrine activities [28]. Thus, we mainly focused on identifying the target of MenSC treatment in DDC mouse models.

According to TEM images, MenSCs could repair TJ structural injury in the liver caused by DDC feeding. The blood–bile barrier (BBIB) is primarily composed of TJs [29], represents a physical barrier formed by liver epithelial cells and hepatocytes, which separates bile from blood sinusoids [30]. Loss of BBIB is believed to be the main cause of cholestasis liver injury [31]. To determine the mechanism of MenSC treatment in DDC-induced cholestasis liver injury, TMT-based quantitative proteomic analysis was used to select target pathways and molecules. After the GSEA, GO and KEGG analysis, TJ pathway is selected as one of the regulators involved in liver damage and treatment. Combined with western blot verification, we found that MenSCs could restore the expression of claudins (including Claudin-1, Claudin-3, Claudin-5 and Claudin-7) and Occludin, the two major families of tetraspanins at TJs in DDC mouse livers. Despite the proteomics results, considering that cholestasis could be partly attributed to bile transport function disorder [32,33] we detected expression changes in OATP2, BSEP and NTCP1 to evaluate bile transport functions in different groups. We found that MenSC could also restore bile transporter levels inhibited by DDC feeding.

Liver fibrosis is believed to be the most important pathological change caused by cholestasis. Our results showed that MenSC transplantation could reduce liver fibrosis and downregulate TGF- β 1 and α -SMA expressions in DDC mice. We hypothesize that MenSCs may downregulate fibrosis-related pathways by improving cholestasis, thereby inhibiting the progression of liver fibrosis.

Furthermore, we demonstrated that β -Catenin is a key target of MenSC treatment. MenSCs promote the repair of TJs and bile transport function damage by upregulating the expression of liver β -Catenin, which

is inhibited by DDC, thereby inhibiting the progression of liver fibrosis. We found that hepatic β -Catenin knockdown did not cause significant liver damage in normal mice but inhibited the therapeutic effect of MenSC transplantation in DDC-induced hepatic cholestasis and fibrosis. Furthermore, β -Catenin knockdown could inhibit MenSCs' regulation of TJ and bile transport function related proteins and pathways in DDC mice.

Thompson et al. reported that the upregulation of β -Catenin in mice could enhance the resolution of intrahepatic cholestasis after chronic DDC administration for 150 d [34]. According to Tao et al., mice with β -catenin-deficient hepatocytes demonstrated increased liver injury following the DDC diet [35]. These studies are consistent with our results.

However, the research by Saggi et al. suggested that β -catenin might play an opposite role relative to that in our study in the DDC-induced liver injury model [36]. According to Saggi et al., in the mice fed a DDC diet for 2 weeks, inhibiting β -catenin could result in decreased liver injury. Conflicting results were also noticed by Utiey et al. [37]. In their study, β -catenin upregulation did not improve liver injury based on the assessment of AST and ALT levels, which is consistent with the results of Thompson et al. After two weeks of DDC feeding, excessive β -catenin expression and activation failed to improve serum biochemical markers of hepatic injury [34]. However, researchers observed that β -catenin was over-activated and the liver function tests improved at 150 d. We hypothesized that β -catenin may play different regulatory roles at different stages of DDC-induced liver injury. This hypothesis needs further explorations, and the efficiency of MSC treatment at different stages of injury on the efficacy requires further investigation.

In addition, although we hypothesized that MenSCs transplantation could inhibit liver fibrosis induced by cholestasis, an in-depth study of the mechanism was not conducted. To date, a series of studies have indicated that β -catenin could be a protective factor against cholestasis-induced fibrosis.

Interestingly, we noticed that β -catenin aggravates hepatic fibrosis in carbon tetrachloride (CCl₄)-induced models. Li et al. reported that inhibiting the Wnt/ β -catenin signaling pathway could attenuate CCl₄-induced hepatic fibrosis in rats [38]. According to Rao et al., β -catenin promotes hepatic fibrosis by activating hepatic stellate cells in CCl₄-induced mouse models [39]. The different regulatory effects of β -catenin in different liver fibrosis models attracted our attention. The role of β -catenin in hepatic fibrosis induced by a variety of pathological reasons is worthy of further exploration.

Conclusion

In summary, the current work investigated the treatment effect of MenSC transplantation in DDC-induced liver injury, and the results suggest cellular therapy a promising strategy for cholestasis treatment.

Abbreviations

MenSCs: Menstrual blood-derived stem cells; DDC: 3,5-diethoxycarbonyl-1,4- dihydroxycollidine; PBS: Phosphate buffer saline; AAV: Adeno-associated virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; DBIL: Direct bilirubin; TBIL: Total bilirubin; TJ: Tight junction; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis; BA: Bile acid; UDCA: Ursodeoxycholic acid; OCA: Obeticholic acid; LT: Liver transplantation; MSCs: Mesenchymal stem cells; HLA-DR: Human leukocyte antigen-DR; α -SMA: α -smooth muscle actin; GAPDH: Anti-glyceraldehyde- 3-phosphate dehydrogenase; TEAB: Triethylammonium bicarbonate; PCA: Principal component analysis; OPLS-DA: Orthogonal partial least-squares-discriminant analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology; GSEA: Gene Set Enrichment Analysis; BBIB: Blood–bile barrier; CCl₄: Carbon tetrachloride.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

L. L., Y. Y., and Y. C. performed study concept and design. L. Z., S. T., S. Z. and Q. H. performed mice feeding. Z. L., F. Z. and Q. L. performed mice sacrifice and tissue collection. Y. Y. and Y. C. performed the experiments and underlying data verification. Y. Y., F. J. and Y. Z. provided analysis, interpretation of data and statistical analysis. Y. Y. and Y. Z. revised the manuscript. All authors approved the final manuscripts as submitted.

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Availability of data and materials

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD028425. All data will be made available upon reasonable request to the corresponding authors.

Ethics approval and consent to participate

Animal experiments in this study were conducted under ethical conditions approved by the Animal Care Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University, and the approval number is 2019-0002. MenSCs were provided by Professor Charlie Xiang (the First Affiliated Hospital, School of Medicine Zhejiang University). Isolation and expansion of MenSCs were approved by Ethics Committee of the First Affiliated Hospital, Collage of Medicine, Zhejiang University, and the approval number is 2017-623.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University.310003, Qingchun Road, Hangzhou, China.

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Figures

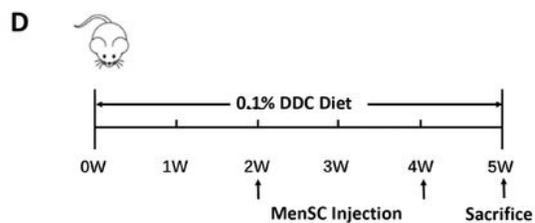
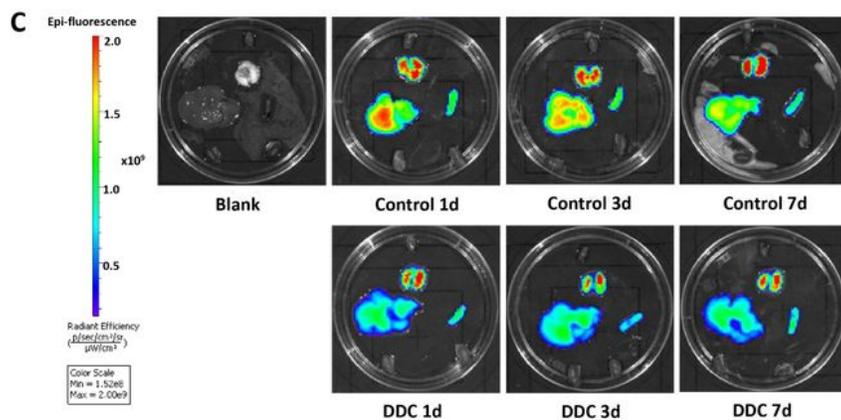
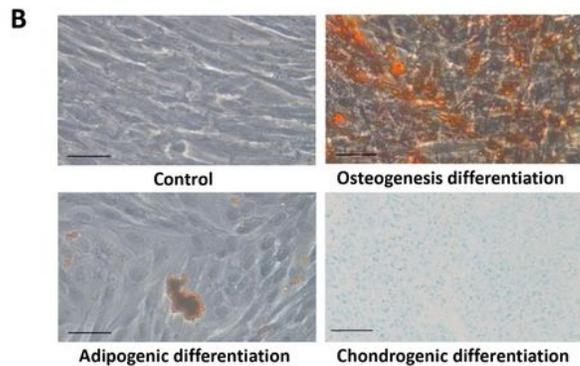
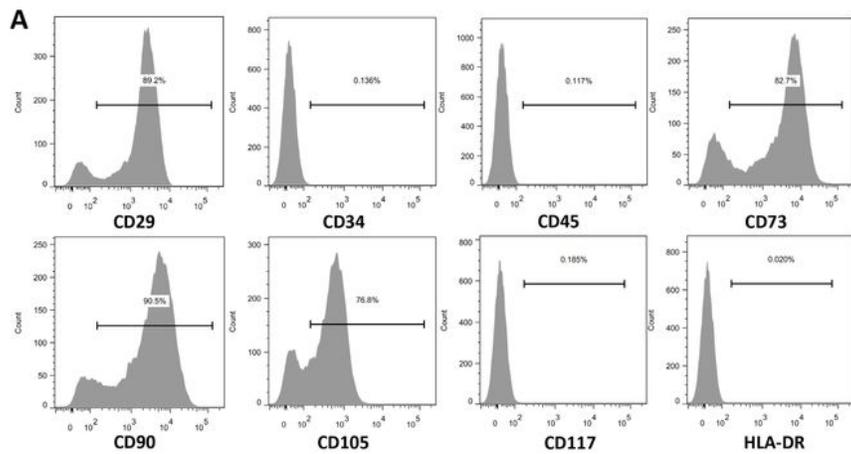


Figure 1

Surface markers and tracking of MenSCs. (A) Surface markers of MenSCs were determined using flow cytometer. (B) Cultured MenSCs and differential potential in vitro, Alizarin red staining of osteogenesis differentiation, Oil Red O staining of adipogenic differentiation, Alcian blue staining of chondrogenic differentiation. Scale bar: 50 μm . (C) Analysis of DiR-labeled MenSCs after systemic administration. (D) Schedule of DDC feeding and MenSC transplantation.

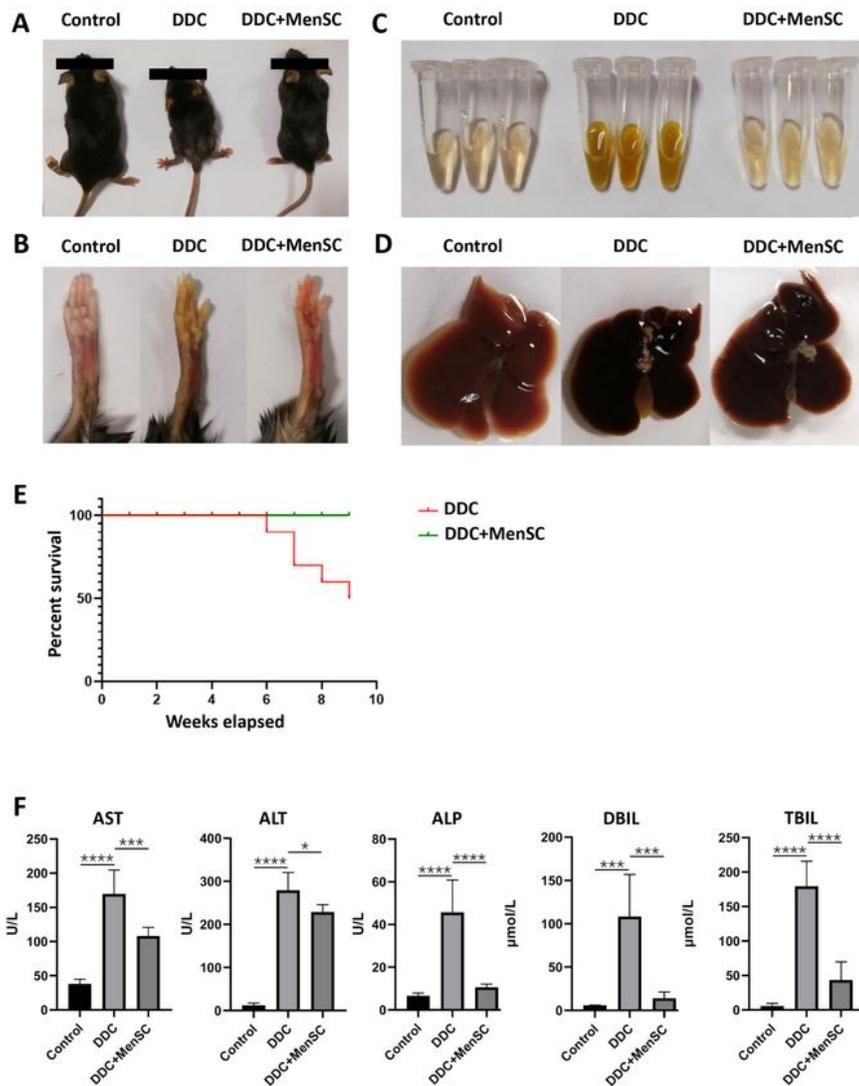


Figure 2

MenSCs reduce symptoms caused by DDC in mice. (A) The general condition of the mice in different groups. (B) The jaundice of mice in different groups. (C) The serum of mice in different groups. (D) The livers and gallbladder of mice in different groups. (E) The survival rate was analyzed in the DDC group and DDC+ MenSC group for 9 weeks after modeling ($n = 10$ for each group). (F) Liver function tests in the different groups: AST, ALT, ALP, DBIL and TBIL. ($n = 7$ for each group). *** $p < 0.001$, **** $p < 0.0001$.

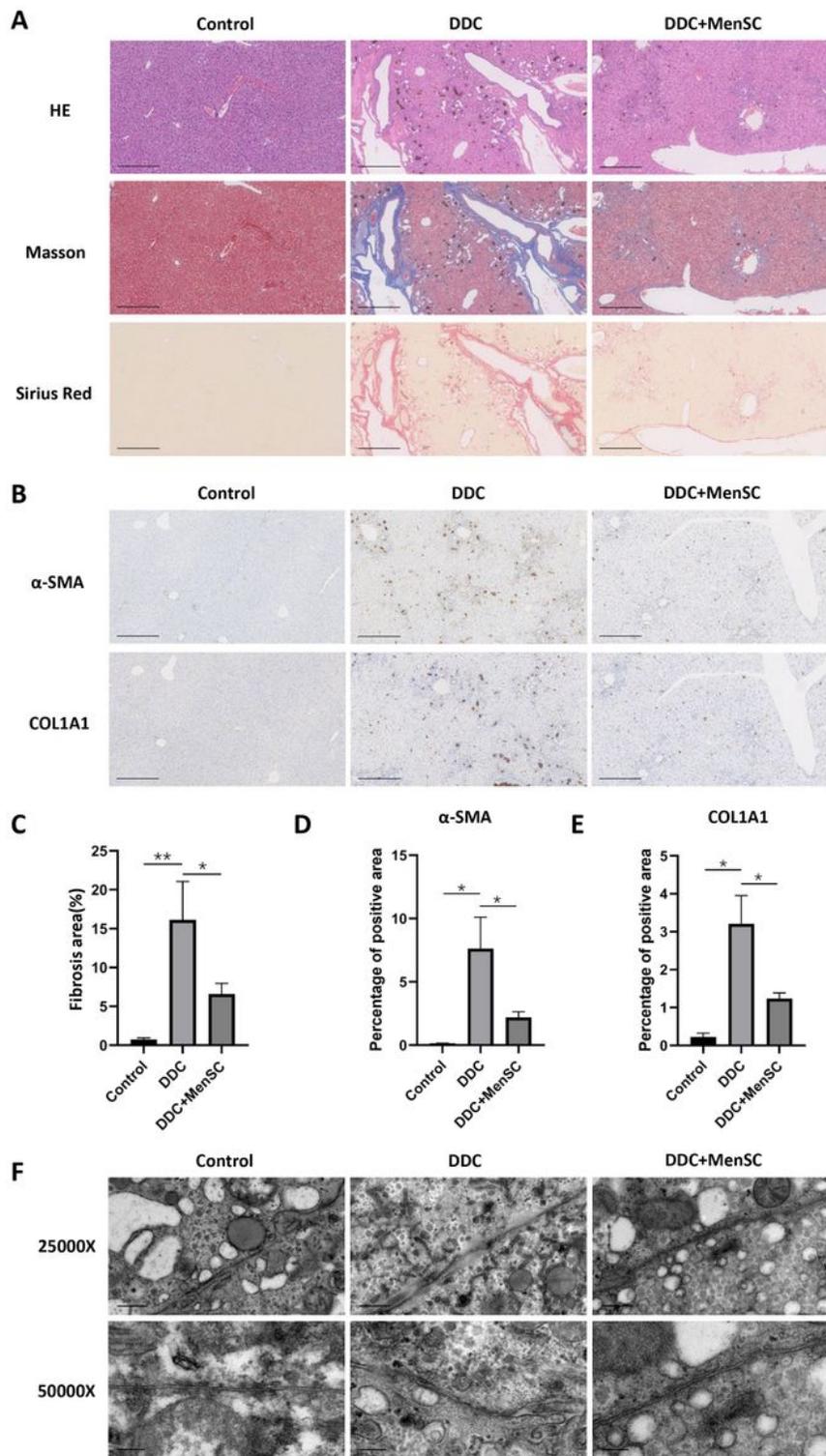


Figure 3

MenSC transplantation improved DDC-induced pathological changes in mouse livers. (A) H&E staining, Masson staining and Sirius Red staining of liver sections (n = 3 for each group). Scale bar: 250 μ m. (B) Representative immunohistochemistry images for α -SMA and COL1A1 (n = 3 for each group). Scale bar: 250 μ m. (C) Fibrosis area analysis. (D, E) Semi-quantitative analysis of the expression of α -SMA and

COL1A1 in liver. (F) Transmission electron microscopy of TJ in different groups. Scale bar: 0.5 μm for 25000x and 200 nm for 50000x. * $p < 0.05$, ** $p < 0.01$.

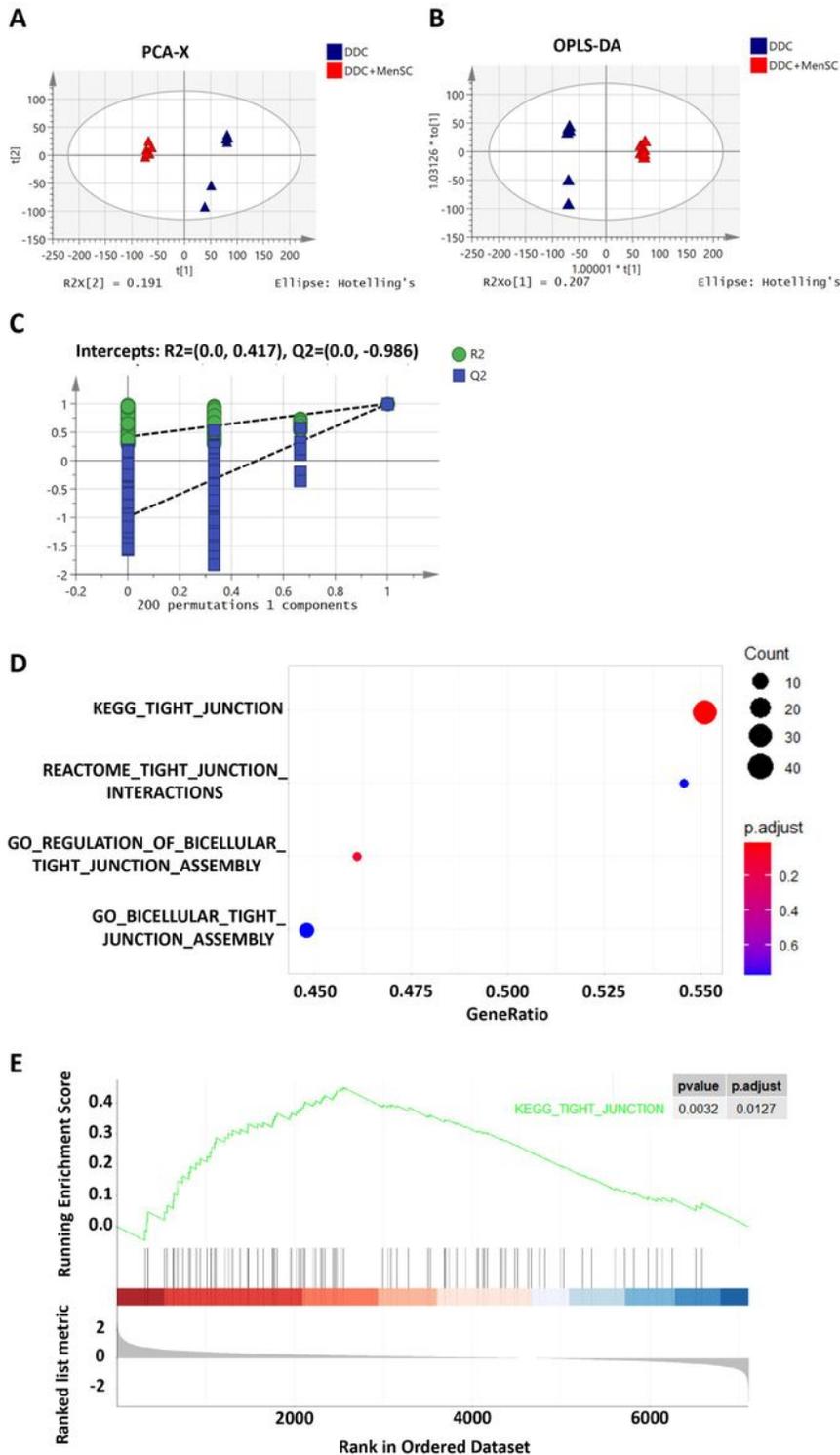


Figure 4

MenSC transplantation altered the liver proteomics profile of DDC-fed mice. (A) Model of a PCA plot comparing between DDC and DDC+MenSC groups. (B) OPLS-DA score scatter plot comparing the DDC

and DDC+MenSC groups. Each symbol represents one liver sample (n=6 for each group). (C) Permutation test of the OPLS-DA model. (D, E) KEGG, GO and GSEA pathway enrichment analysis.

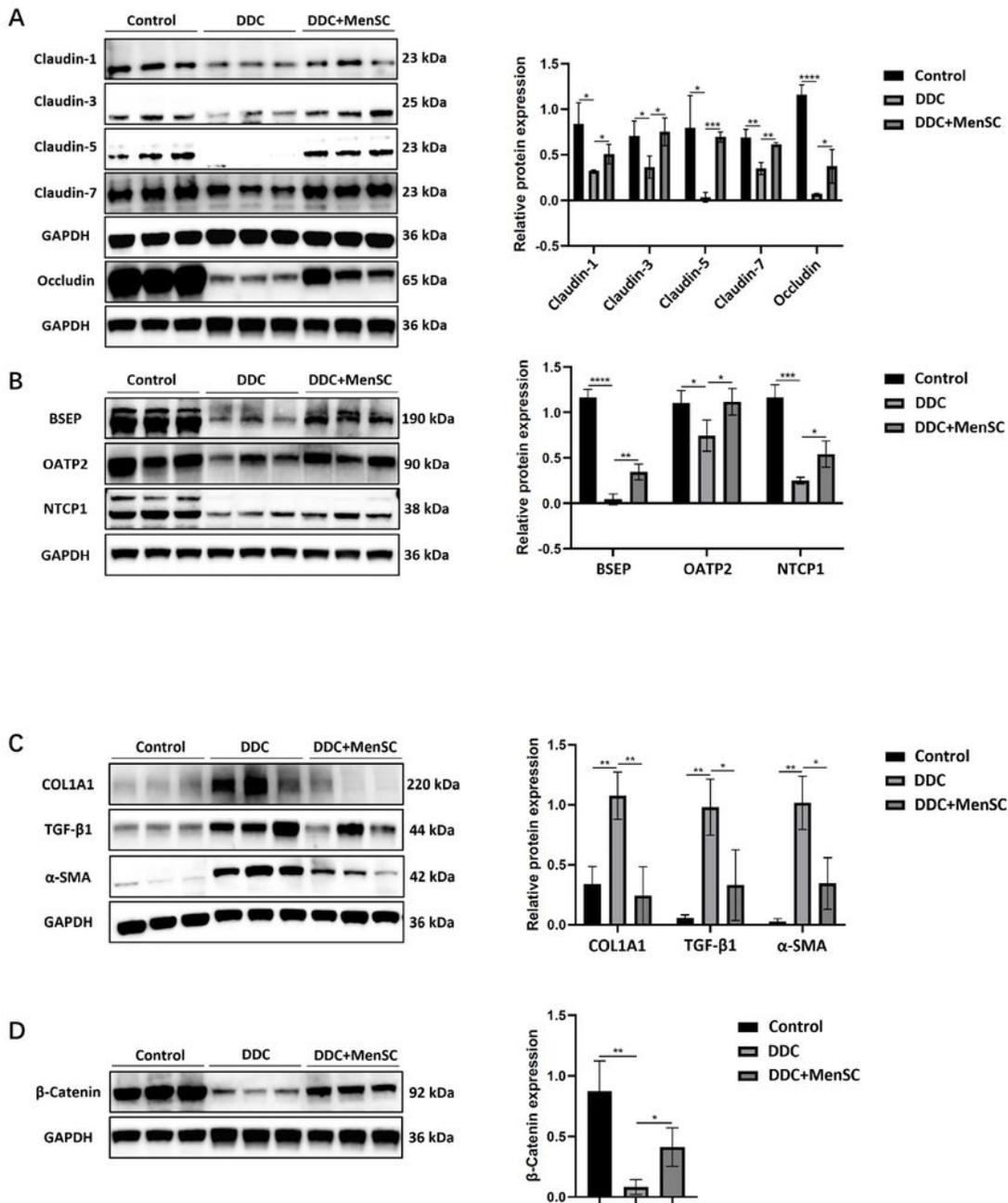


Figure 5

MenSCs promoted TJ- and bile transport function-related protein expression and reduced fibrosis-related protein expression. (A) Western blot analysis protein levels of Claudin-1, Claudin-3, Claudin-5, Claudin-7 and Occludin in the liver of different groups (n=3 for each group). (B) BSEP, OATP2 and NTCP1

expression in liver tissues of different groups (n=3 for each group). (C, D) Liver COL1A1, α -SMA, TGF- β 1 and β -Catenin expression among different groups (n=3 for each group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

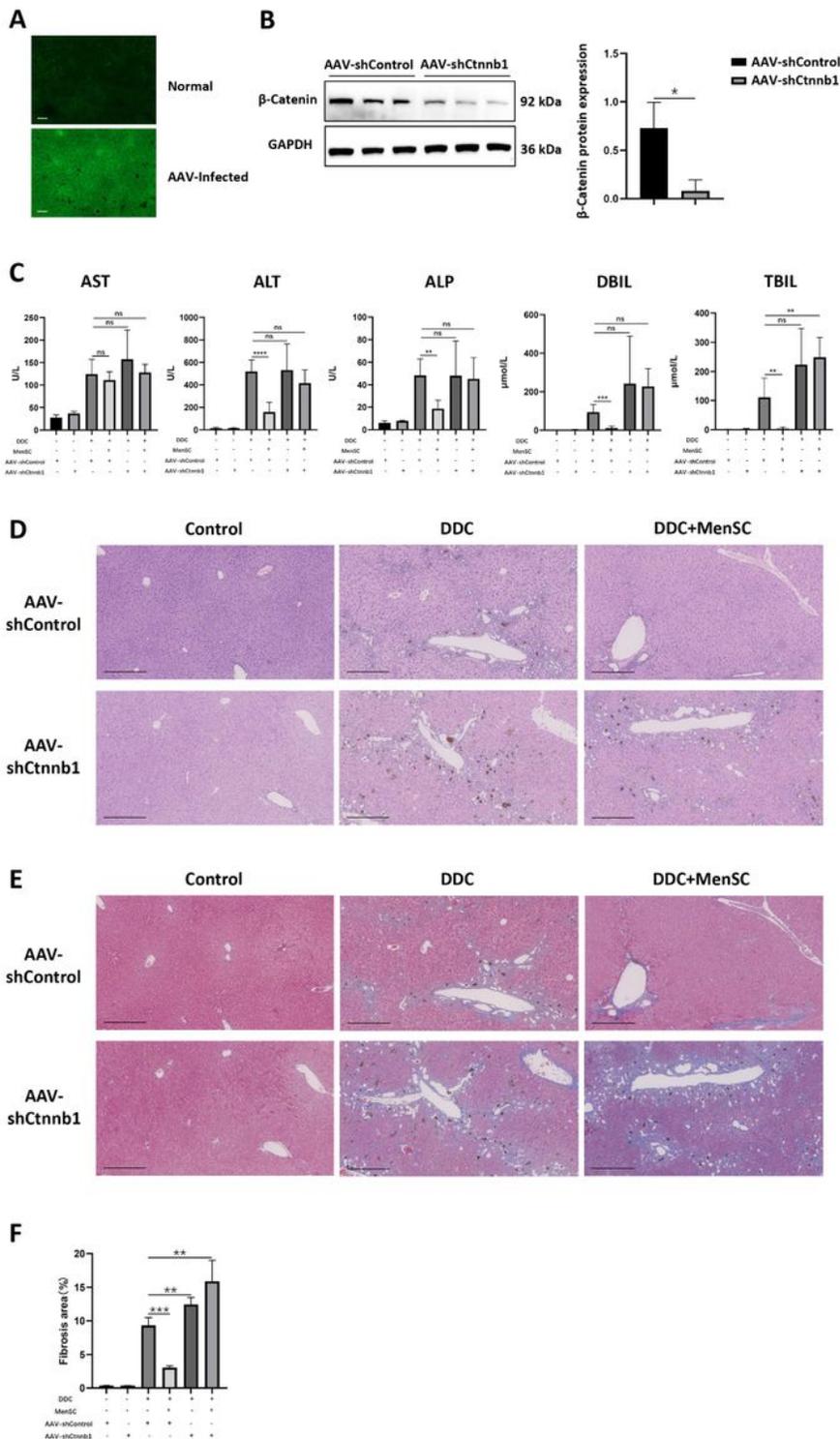


Figure 6

Liver β -Catenin deficiency inhibited the therapeutic efficacy of MenSC transplantation in DDC-induced models. (A) Representative images of sections from AAV-infected mouse livers. Scale bar, 50 μ m. (B)

Expression of β -Catenin in liver tissues of AAV-shControl and AAV-shCtnnb1 mice (n=3 for each group). (C) AST, ALT, ALP, DBIL and TBIL levels in peripheral blood of AAV-shControl and AAV-shCtnnb1 mice of Control, DDC and DDC+MenSC groups (n=6 for each group). (D, E) Representative images of H&E and Masson staining of liver tissues from AAV-shControl and AAV-shCtnnb1 mice in different groups. Scale bar, 250 μ m. (F) Fibrosis area analysis (n=5 for each group). **p < 0.01, ***p < 0.001, ****p < 0.0001 and n.s. indicates no significance between the two indicated groups.

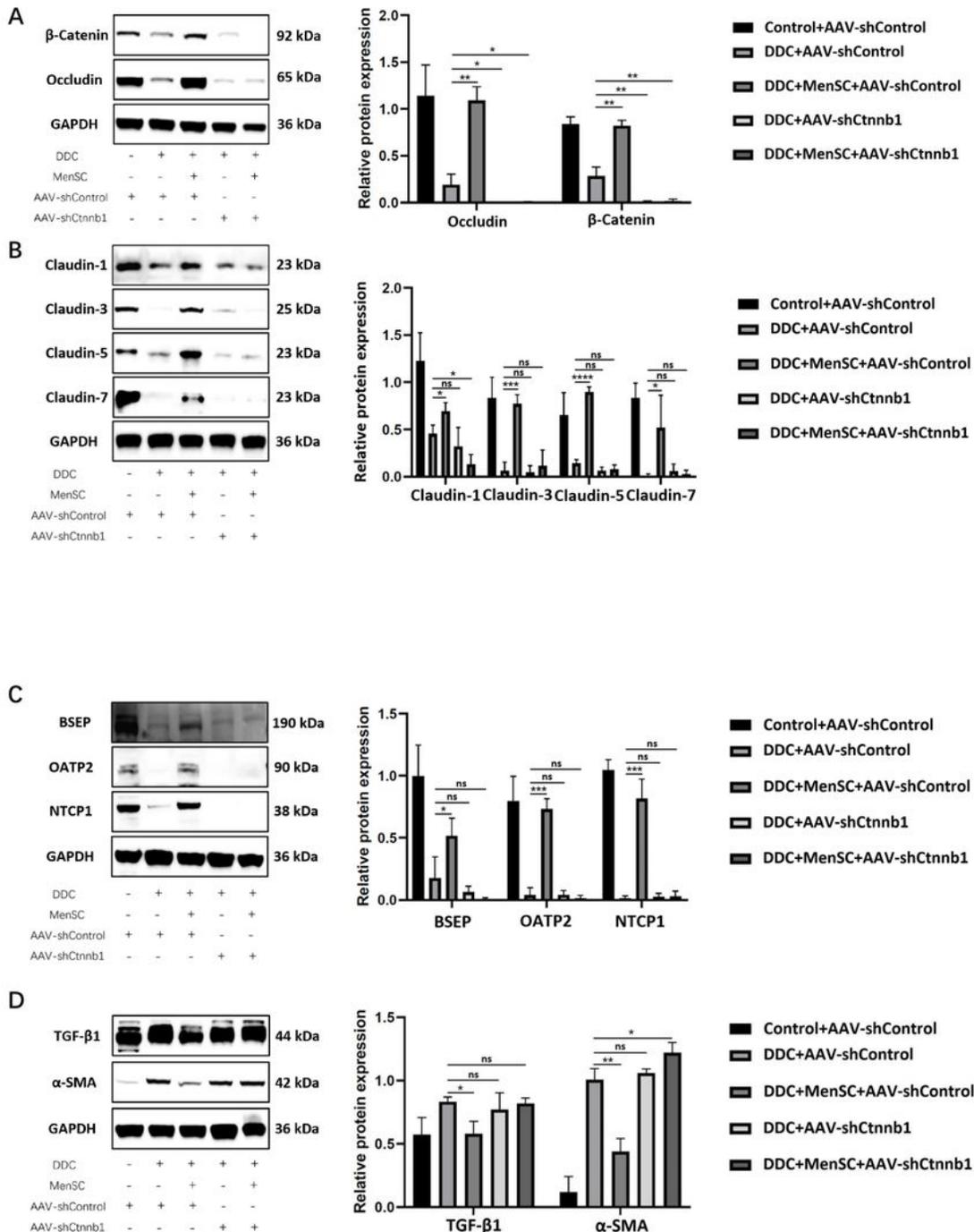


Figure 7

β -Catenin deficiency inhibited the regulatory effect of MenSC transplantation on related proteins. (A) Western blot analysis protein levels of β -Catenin and Occludin in the AAV-shControl and AAV-shCtnnb1 liver tissues of different groups (n=3 for each group). (B) Expression of Claudin-1, Claudin-3, Claudin-5 and Claudin-7 in liver tissues (n=3 for each group). (C) Protein levels of BSEP, OATP2 and NTCP1 in liver tissues (n=3 for each group). (D) Expression of TGF- β and α -SMA in liver tissues (n=3 for each group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 and n.s. indicates no significance between the two indicated groups.