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Human umbilical cord mesenchymal stem cells ameliorate experimental cirrhosis through activation of keratinocyte growth factor by suppressing microRNA-199

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Abstract. – OBJECTIVE: Keratinocyte growth factor (KGF) has a demonstrated role in the prevention of cirrhosis during liver regeneration. Previous studies have shown that transplantation of human umbilical cord mesenchymal stem cells (HUCMSCs) reduces the development of cirrhosis after liver injury. However, whether KGF may be involved in the underlying molecular mechanisms remains unknown. Here we addressed this question.

MATERIALS AND METHODS: We did HUCMSC transplantation in mice that had developed cirrhosis by carbon tetrachloride (CCl_4). The effects of UCMSC transplantation on KGF levels and liver damage were examined. The level of a KGF-targeting microRNA, miR-199, was examined. The regulation of KGF by miR-199 was studied by bioinformatics analyses and luciferase reporter assay.

RESULTS: HUCMSC transplantation significantly ameliorated the severity of liver fibrosis, reduced portal hypertension and sodium retention that were induced by CCl4. HUCMSC transplantation significantly increased the levels of KGF in the injured liver, seemingly through suppression of miR-199, which targeted 3'-UTR of KGF mRNA to inhibit its protein translation.

CONCLUSIONS: HUCMSCs may ameliorate cirrhosis through activation of KGF by suppressing miR-199.

Key Words:
Human umbilical cord mesenchymal stem cells (HUCMSCs), Cirrhosis, miR-199, Keratinocyte growth factor (KGF).

Introduction

Cirrhosis is a prevalent disease[1-4], and the pathology of cirrhosis includes injury-induced chronic inflammation, portal hypertension due to impaired blood flow, and disruption of normal hepatic architecture, resulting in liver dysfunction[5,6].

In the research of cirrhosis, carbon tetrachloride (CCl_4) intraperitoneal injection has been most commonly applied to an animal model, due to its low toxicity to mice and being highly reproducible[7-12]. Keratinocyte growth factor (KGF), a potent mitogen for different types of epithelial cells, is predominantly produced and secreted by hepatic stellate cells (HSCs)[13]. KGF is a member from fibroblast growth factor (FGF) family and is known as FGF7[14]. KGF functions via binding to its high-affinity receptor, a splice variant of FGF receptor 2 (FGFR2-IIIb), in different cell types[15]. Moreover, KGF expression is strongly up-regulated after acute injury to various tissues, including skin, bladder, kidney and liver[16]. Recently, KGF has been shown to play a critical role in the prevention of cirrhosis and enhancement of liver regeneration[13,17].

Previous studies[18-26] also show that transplantation of human umbilical cord mesenchymal stem cells (HUCMSCs) has many therapeutic effects on a variety of diseases. Moreover, HUCMSC transplantation reduces the development of cirrhosis after liver injury[27-29]. However, whether HUCMSCs may function partially through KGF as well as the mechanisms has not been studied.

MiRNAs are small RNA species that range from 19 to 25 nucleotides in length, and are often found to be dysregulated in various diseases[30-32]. Recent findings[5,6,33-35] have implicated the involvement of miRNAs in the HCV infection and development of cirrhosis. These studies have shown up-regulation of a number of miRNAs in the fibrotic liver, which may imply a possible involvement of miRNAs in the pathogenesis of cirrhosis. However, the exact underlying regulatory molecular pathways have not been completely elucidated.

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Here, we performed HUCMSC transplantation to mice that had developed cirrhosis by CCl_4, and examined the effects on KGF levels and liver damage. Furthermore, the level of a KGF-targeting microRNA, miR-199, was checked. The regulation of KGF levels by miR-199 was studied using bioinformatics analyses and confirmed by luciferase reporter assay.

Materials and Methods

Experimental Protocol Approval

All the experimental methods in the current study have been approved by the Research Committee at No. 1 Hospital of Zibo Municipality. All the experiments have been carried out following the guidelines from the Research Committee at No 1 Hospital of Zibo Municipality. All mouse experiments were approved by the Institutional Animal Care and Use Committee at No 1 Hospital of Zibo Municipality (Animal Welfare Assurance). Surgeries were performed following the Principles of Laboratory Care, supervised by a qualified veterinarian.

Isolation, Culture and Differentiation of HUCMSCs

HUCMSCs from healthy donors were isolated and cultured in DMEM culture medium (Dulbecco's Modified Eagle's Medium, Gibco, San Diego, CA, USA) containing inactivated 10% fetal bovine serum (FBS, Gibco), 3.7 g/l HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid, Sigma-Aldrich, St. Louis, MO, USA), 1% 200 mmol/l L-glutamine 100x (Gibco) and 1% PSA (Gibco). After 72 hours' culture, the adherents HUCMSCs were maintained until 80% confluence. After confirmation of MSC properties, a positive clone from the HUCMSCs was picked up and subjected to chondrogenic, osteogenic and adipogenic differentiation assays.

Isolation of Mouse Hepatic Satellite Cells (HSCs)

Mouse hepatic HSCs were separated from non-parenchymal liver cells by arabinogalactan gradient ultracentrifugation, as has been previously described[36]. The procedure yielded HSCs that were more than 85% pure and 90% viable, as determined by phase contrast and UV-excited fluorescence microscopy by the aid of trypan blue exclusion. HSCs were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA, USA), 100 IU/ml penicillin, and 100 µg/ml streptomycin, in the incubator with 95% air and 5% CO_2. Growth medium was changed daily during the first 4 days in culture and every second day after that.

Plasmid Preparation

Plasmids were successfully constructed using molecular cloning technology. Target sequences [miR-199, miR-199 antisense (as-miR-199), and null sequence] were inserted into the pGL3-Basic vector (Promega, Beijing, China). Sequencing was performed to confirm the correct orientation of these new plasmids. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Luciferase-reporter Activity Assay

MiRNAs targets were predicted with the algorithms TargetScan, as previously described[37]. The candidates were analyzed for a context+ score, which is the sum of the contribution of 6 features (including site-type contribution, 3' pairing contribution, local AU contribution, position contribution, TA contribution and SPS contribution). The KGF 3'-UTR reporter plasmid (pRL-KGF) and the KGF 3'-UTR reporter plasmid with a mutant at miR-199 binding site (pRL-KGF-mut) were purchased from Creative Biogene (Shirley, NY, USA). HSCs were collected 36 hours after transfection for dual-luciferase reporter assay (Promega), according to the manufacturer's instructions.

Animal Manipulations

All animal procedures were conducted according to the guidelines for the care and use of laboratory animals approved by Chinese PLA General Hospital. Female C57BL/6 mice (Charles River Laboratories, China) of 10 weeks of age were given free access to tap water and pelleted mouse diet. Cirrhosis was induced by CCl_4 intraperitoneal administration. Briefly, CCl_4 solution of 50% (v/v) in paraffin oil (Sigma-Aldrich, St. Louis, MO, USA) was administered at a dose of 2 ml/kg body weight of the mice, by intraperitoneal injections twice a week for 8 weeks. Each experimental group contained 10 mice. During cirrhosis induction, the mice were provided with 0.3 g/l phenobarbital in drinking water to enhance CCl_4 hepatotoxicity.

Evaluation of Liver Fibrosis

The liver sample was fixed with 10% phosphate-buffered formalin and embedded in paraffin. Sirius red staining technique was performed, which stains collagen. Fibrotic areas were counted on 200 random selected fields corresponding to approximately 6 mm^2, using an unbiased counting frame, as has been described previously[14]. The percentage of the fibrotic area was expressed as the number of fibrotic fields divided by total fields, and then multiplied by 100.

Sodium Balance

The urine sodium concentration (UNa) was assayed by flame photometry (Roika 2000, Roika, UK), and renal sodium excretion (UNaV) was calculated with the following formula: UNaV = UV × UNa, where UV is urine volume. The intake of sodium was assessed by measuring the amounts of food and water consumed. Sodium balance was calculated as (Na+ provided by food and water)-UNaV.

Portal Pressure

After evaluation of sodium metabolism, under anesthesia, a PE-50 polyvinyl catheter was placed in the cecal vein. The other end of the catheter was connected to a highly sensitive transducer (ADInstruments Shanghai Trading Co. Shanghai, China) to assess portal pressure.

Quantitative Real-time PCR (RT-qPCR)

Total RNAs of liver or cultured cells were extracted with miRNeasy mini kit (Qiagen, Hilden, Germany) for cDNA synthesis. Quantitative real-time PCR (RT-qPCR) was performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed, using $2^{-\Delta\Delta Ct}$ method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to the experimental controls to get relative values.

Western Blot

Primary antibodies were rabbit anti-KGF and anti-α-tubulin (Cell Signaling, San Jose, CA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). α-tubulin was used as protein loading controls. The protein levels were first normalized to α-tubulin, and then normalized to the experimental control.

Statistical Analysis

Statistical analyses were performed with SPSS 19.0 software (SSPS Inc., Chicago, IL, USA). All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher's Exact test for comparison of two groups. All values are depicted as a mean ± standard deviation, and are considered significant if $p < 0.05$. Each group contained 10 individuals.

Results

HUCMSC Isolation and Quality Controls

We studied the molecular mechanisms underlying the therapeutic effects of HUCMSCs on experimental cirrhosis. For this aim, the HUCMSCs were thus isolated (Figure 1A), and then confirmed the MSC properties of a selected clone by differentiation assay (Figure 1B-D).

HUCMSCs Significantly Attenuate Severity of Cirrhosis Induced by CCl4

The experiments to evaluate the effects of HUCMSCs on experimental cirrhosis are shown in a schematic (Figure 2A). CCl_4 was injected to induce cirrhosis in mice. After 8 weeks, the mice received a transplantation of 10^6 HUCMSCs, or saline as a control. The mice were then kept for another 4 weeks before analyses (Figure 2A).

We found that transplantation of HUCMSCs significantly decreased the percentage of the fibrotic area (Figure 2B). Portal hypertension and sodium metabolism were also assessed, showing that transplantation of HUCMSCs significantly decreased the portal pressure (Figure 2C), and significantly improved sodium balance (Figure 2D), probably through an increased sodium excretion (Figure 2E). These data suggest that transplantation of HUCMSCs may alleviate cirrhosis induced by CCl_4.

HUCMSCs Increase KGF Protein Instead of mRNA in the Injured Mouse Liver

We found that HUCMSC transplantation significantly increased KGF protein (Figure 3A) instead of mRNA (Figure 3B) in the injured mouse liver. These data suggest that HUCMSC transplantation may affect KGF protein translation.

MiR-199 Targets 3′-UTR of KGF mRNA and Suppressing its Expression

Since our data show that HUCMSC transplantation inhibits KGF protein translation, we per-

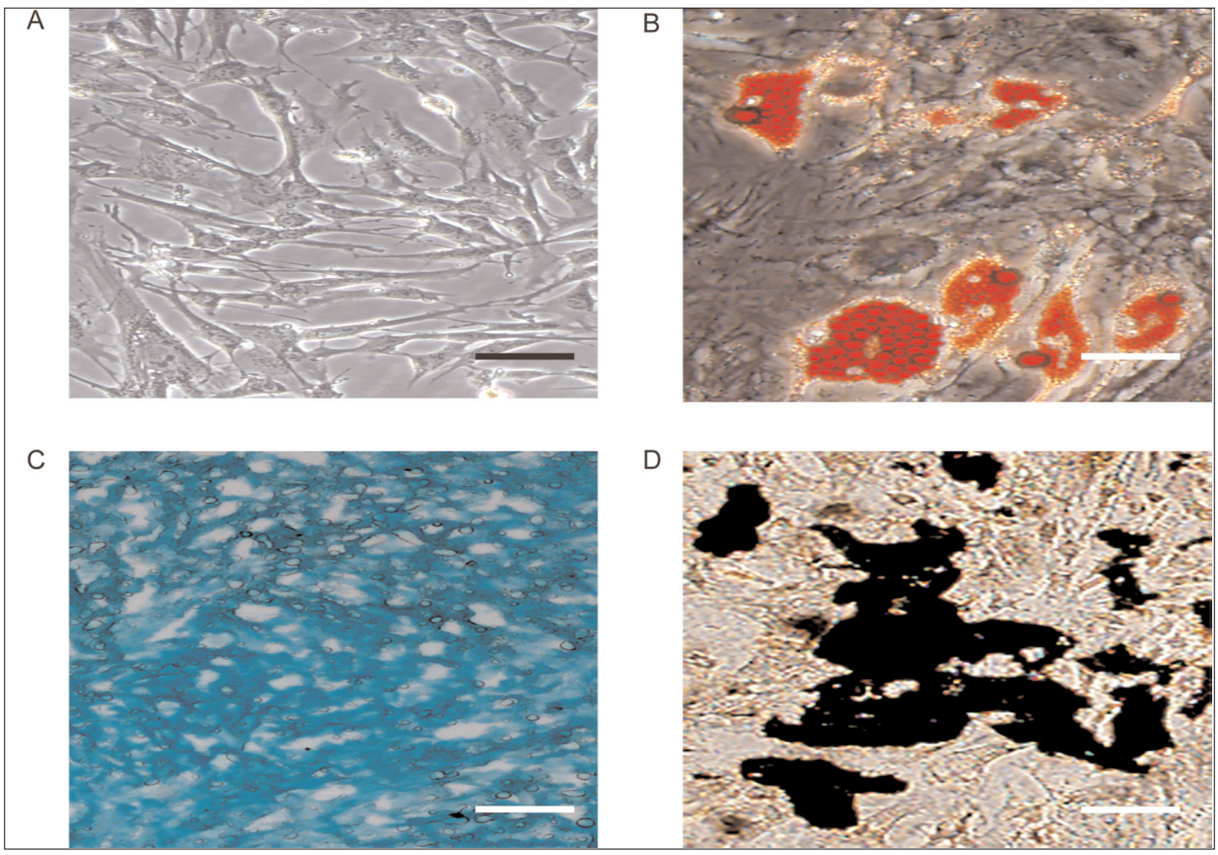

Figure 1. HUCMSC isolation and quality controls. ***A,*** HUCMSCs in culture. ***B,-D,*** A selected clone of HUCMSCs was examined for differentiation properties by Von kossa staining to evaluate osteogenic induction ***(B)***, Oil red O staining to evaluate adipogenic induction ***(C)*** and Alcian blue staining to evaluate chondrogenetic induction ***(D)***. Scale bars are 50 μm.

formed bioinformatics analysis to check whether any miRNAs may target KGF mRNA, since miRNAs are key regulators of protein translation. From all candidates, we found that miR-199 bound to 3'-UTR of mouse KGF mRNA sequence ranged from 130th to 137st base site (Figure 4A). Moreover, the levels of miR-199 were significantly decreased in the mouse liver after HUCMSC transplantation (Figure 4B). Next, we prepared plasmids of miR-199 modification and confirmed the quality of these plasmids by RT-qPCR (Figure 4C). Afterward, the intact 3'-UTR of mouse KGF mRNA (KGF 3'-UTR), together with a 3'-UTR with mutant at miR-199-binding site of mouse KGF mRNA (KGF 3'-UTR mut), was then cloned into luciferase reporter plasmids. Mouse HSCs were co-transfected with 1 g miR-199/null/as-miR-199 plasmids and 1 g mouse KGF 3'-UTR or mouse KGF 3'-UTR mut plasmids, after which the luciferase activity was measured, showing that miR-199 specifically targets 3'-UTR of KGF mRNA to inhibit its translation in mouse HSCs (Figure 4D). Hence, our study suggests that HUCMSCs may ameliorate cirrhosis through activation of KGF by suppressing miR-199 (Figure 5).

Discussion

The upregulation of KGF levels has been associated with the successful healing of injured epithelia [38]. The activation of HSCs is considered as the key event of hepatic fibrosis and cirrhosis. Moreover, KGF has been found to be predominantly expressed by activated HSCs, rather than by quiescent HSCs and other liver cells[13]. Indeed, KGF regulates epithelial-mesenchymal interaction that is critical for the multiple paracrine signaling, which was mediated by cytokines and extracellular matrix components resulted from the liver injury. A recent study on lung fibrosis found that miR-155 was significantly induced by inflammatory cytokines while it was downregulat-

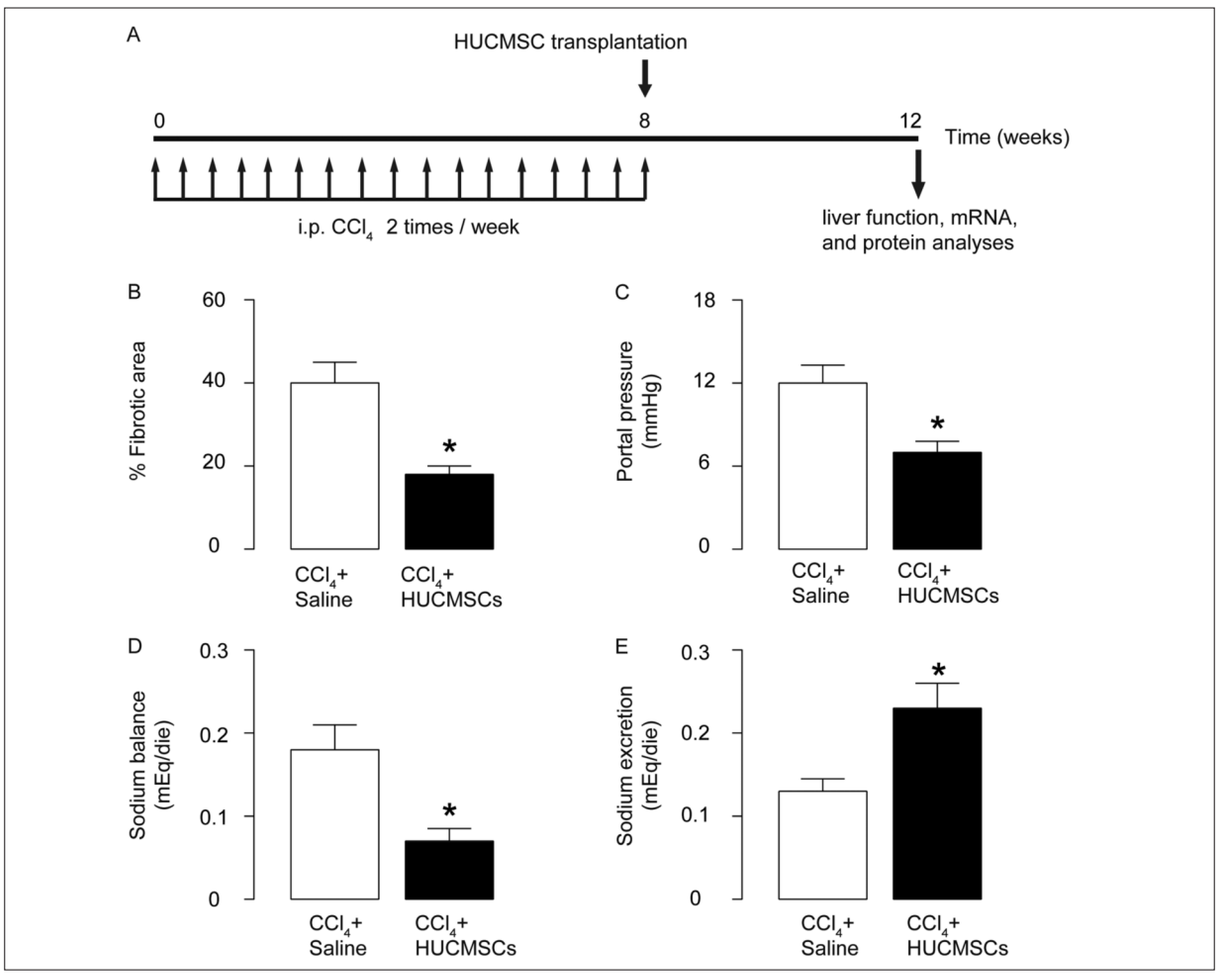

Figure 2. HUCMSCs significantly attenuate severity of cirrhosis induced by CCl_4. ***A,*** Schematic of the experiment: CCl_4 was injected to induce cirrhosis in mice. After 8 weeks, the mice received transplantation of 106 HUCMSCs, or saline as a control. The mice were then kept for another 4 weeks before analyses. ***B,*** The fibrotic area at sacrifice was evaluated after Sirius red staining, shown by the percentage of the fibrotic area. ***C,*** Portal pressure. ***D,*** Sodium balance. ***E,*** Sodium excretion. $*p < 0.05$. n = 10. Statistics: one-way ANOVA with a Bonferroni correction.

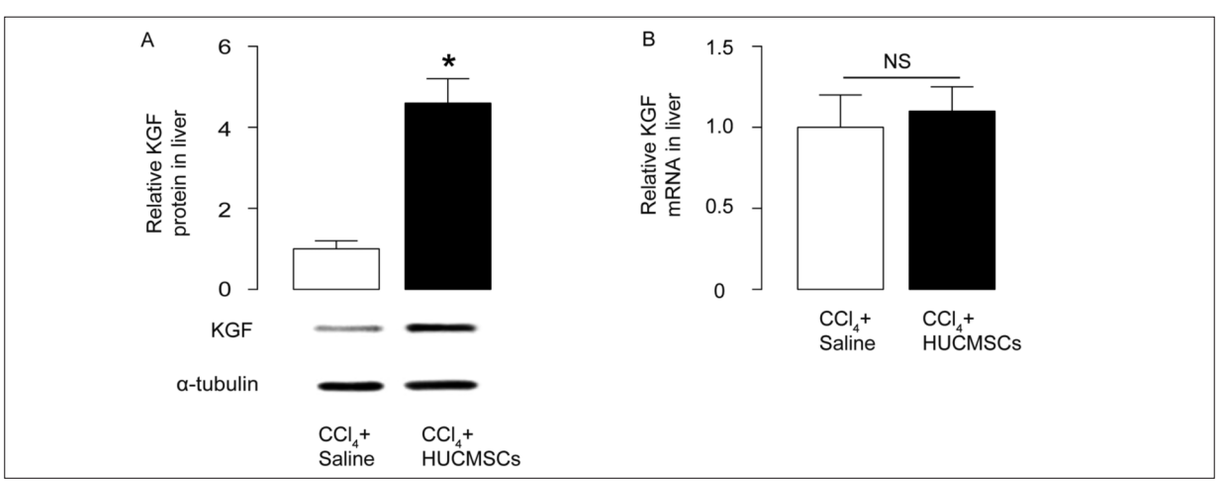

Figure 3. HUCMSCs increase KGF protein in the injured mouse liver. ***A,*** KGF protein by Western blot. ***B,*** KGF mRNA by RT-qPCR in the injured mouse liver. $*p < 0.05$. NS: non-significant. n = 10. Statistics: one-way ANOVA with a Bonferroni correction.

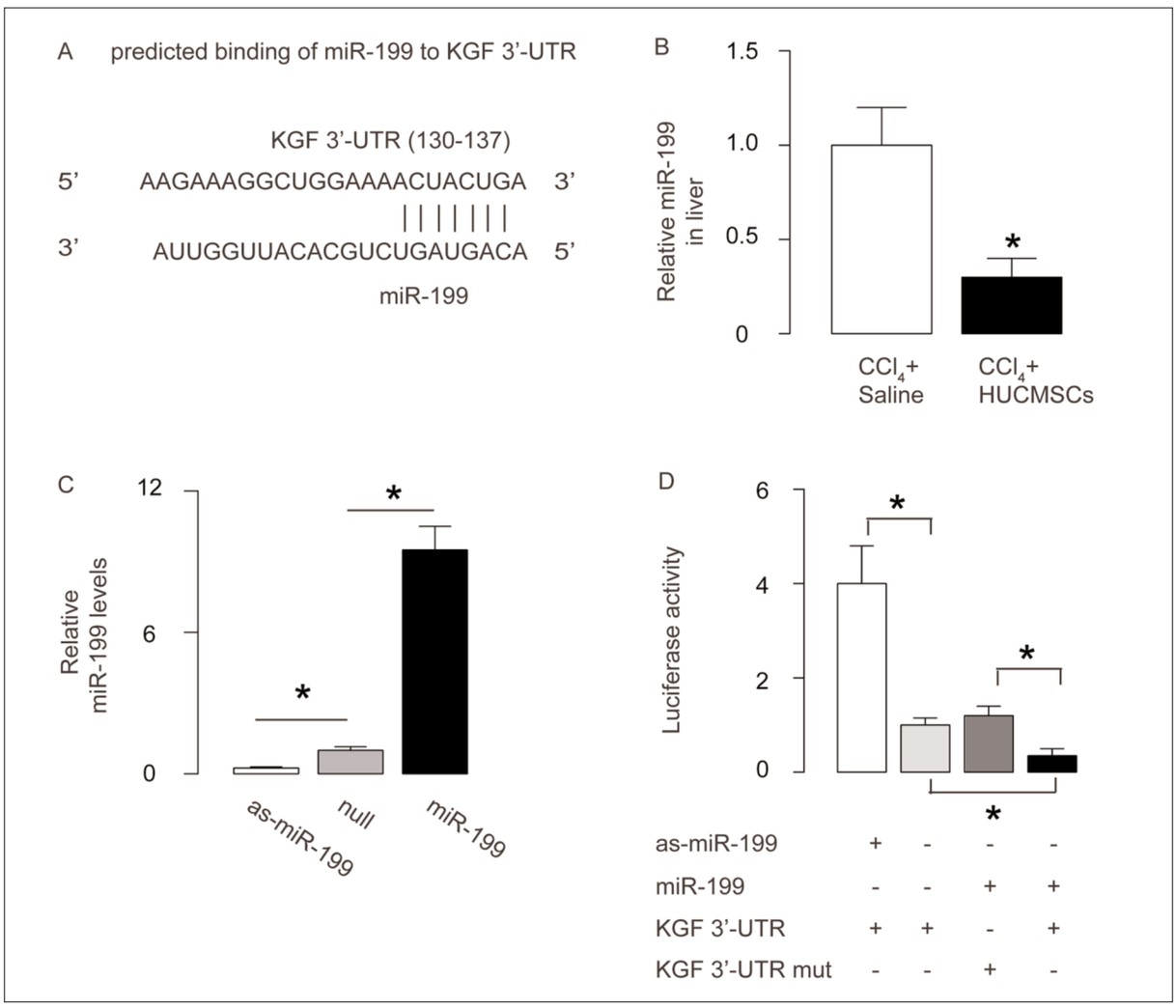

Figure 4. MiR-199 targets 3'-UTR of KGF mRNA and suppresses its expression. ***A,*** Bioinformatics show that miR-199 bound to 3'-UTR of KGF mRNA sequence in mouse ranged from 130th to 137st base site. ***B,*** The levels of miR-199 by RT-qPCR in the injured mouse liver after HUCMSC transplantation. ***C,*** We prepared plasmids of miR-199 modification and confirmed the quality of these plasmids by RT-qPCR. ***D,*** The intact 3'-UTR of mouse KGF mRNA (KGF 3'-UTR), together with a 3'-UTR with mutant at miR-199-binding site of mouse KGF mRNA (KGF 3'-UTR mut), was then cloned into luciferase reporter plasmids. Mouse HSCs were co-transfected with 1 μg miR-199/null/as-miR-199 plasmids and 1 μg mouse KGF 3'-UTR or mouse KGF 3'-UTR mut plasmids, after which the luciferase activity was measured. *$p < 0.05$. n 10. Statistics: one-way ANOVA with a Bonferroni correction.

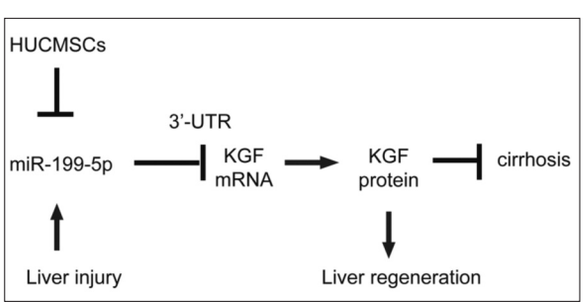

Figure 5. Schematic of the model. HUCMSCs may ameliorate cirrhosis through activation of KGF by suppressing miR-199.

ed by TGFβ in the setting of lung injury[39-43]. Ectopic expression of miR-155 in human fibroblasts induced modulation of a large set of associated genes including KGF. miR-155 was experimentally validated to be the functional target to 3'-UTR of KGF *in vitro*, while the *in vivo* experiments with the mouse model showed that miR-155 expression level was correlated with the degree of lung fibrosis[44].

In the current study, HUCMSCs significantly attenuated the levels of hepatic fibrosis, portal hypertension and sodium retention in the mouse

model, seemingly through upregulation of KGF protein, instead of mRNA. We screened all KGF-targeting miRNAs and specifically found that the levels of miR-199 were affected by HUCMSC transplantation and the regulation of KGF protein translation by miR-199 was then proven in HSCs by luciferase reporter assay.

However, our data doesn't exclude a possibility that miR-199 may target genes other than KGF, which may also contribute to its effects during liver injury and recovery. Future studies should address these questions.

Conclusions

We suggest that HUCMSCs may ameliorate cirrhosis through activation of KGF by suppressing miR-199. Thus, inhibition of miR-199 may be an important mechanism for the therapeutic effects of HUCMSCs, and may be a promising treatment during the therapeutic approaches for liver injury.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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