

Overexpression of CCR2 in mesenchymal stem cells contributes to homing and liver regeneration*

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Summary

Mesenchymal stem cells (MSCs) are emerging as a new therapeutic strategy for liver diseases. However, the inability of stem cells to efficiently return to the target tissue is a major obstacle to therapy. The purpose of this study was to clarify the effects of chemokine CCL2 and its receptor CCR2 on homing of bone mesenchymal stem cells (BMSCs) after partial hepatectomy and the mechanism by which they enhance liver regeneration. A mouse model of partial hepatectomy was established. Transcriptome sequencing was used to detect the expression of CCL2 in liver tissue. CCR2 was transfected with lentivirus into bone marrow MSCs (BMSCs), and the expression of CCR2 in BMSCs was detected by qRT-PCR. The effect of BMSCs overexpressing CCR2 on liver regeneration in mice with partial hepatectomy was detected by a liver index, HE staining and serum ALT and AST contents. The migration ability of BMSCs modified by CCR2 was observed by a transwell test and PKH26 staining. The influence of CCL2-CCR2 on the homing signalling pathway of BMSCs was detected by western blot and scratch experiments. It was found that CCL2 expression was up-regulated during the whole process of liver regeneration. BMSCs could express the CCR2 receptor, but lentivirus-transfected BMSCs could express more CCR2. The BMSCs modified by CCR2 showed strong migration ability. At 72 h after partial hepatectomy, compared with KM-BMSCs group, the liver index of the KM-BMSCs-CCR2 group was higher, the liver tissue structure was more normal, and the levels of ALT and AST were lower. Expression of the Rho/ROCK signalling pathway proteins increased in the liver of the KM-BMSCs-CCR2 group, while migration ability of BMSCs decreased after treatment with the ROCK inhibitor Y-27632. In summary, this study suggested that the expression of CCR2 on BMSCs enhanced their targeted migration to the liver of partially hepatectomized mice and promoted liver regeneration. The Rho/ROCK signalling pathway plays an important role in the homing of BMSCs.

Keywords: Mesenchymal stem cells, CCR2, CCL2, Rho/ROCK signalling pathway

The liver is an important organ for metabolism and detoxification. It has a unique dual blood supply: 20% of the blood comes from the hepatic artery, and 80% is from the portal vein. It is rich in food antigens, bacterial products and environmental toxins. The liver is one of the most vulnerable organs in the body due to its anatomical site and functional characteristics. A variety of chemical, physical and biological factors can cause liver damage. Although the liver has a strong regenerative ability, the self-repair ability of liver cells is far from sufficient compared with various liver diseases. Therefore, improving the efficiency of liver regeneration is a key step in the treatment of liver diseases.

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Mesenchymal stem cells (MSCs), an important member of the stem cell family, originate from the mesoderm and ectoderm. Given their easy separation, chemotaxis, multidirectional differentiation potential, immunomodulation and other biological characteristics, MSCs have attracted people's attention and become a popular seed cell in the treatment of various refractory diseases in cell therapy. Homing is a key step for stem cells to play a role in tissue repair. The inability of stem cells to efficiently home to the target tissue is a major obstacle affecting the repair effect (7). Therefore, improving the homing rate of stem cells is crucial in the application of stem cells in disease treatment.

Chemokine families and their receptors not only mediate the migration of white blood cells but also play an important role in the homing of MSCs (3).

C-C chemokine ligand 2 (CCL2) was first extracted and purified from human glioma cell lines and bone marrow mononuclear cell lines in 1989 (11). CCL2 is secreted by liver parenchyma cells, Kupffer cells and sinus endothelial cells. Wang et al. (10) found that 44.6% of MSCs express CCL2 receptor CCR2, and the combination of CCL2 and CCR2 promotes the homing of MSCs to ischemic brain. Belema-Bedada et al. (2) also confirmed that CCR2 can promote the homing of MSCs to an injured heart caused by ischemia/reperfusion. However, the role of CCR2 in promoting MSCs homing remains unclear. Therefore, the study of the role and possible mechanism of the CCL2/CCR2 molecular axis in MSCs homing is of great significance for further understanding the mechanism by which MSCs promote liver injury repair and exploring new therapeutic methods for liver injury.

Material and methods

Groups of bone marrow mesenchymal stem cells (BMSCs). SPF male Kunming mice weighing 20-25 g were used in this study. The animal experiment program was reviewed and approved by the Animal Ethics Committee of Guilin Medical College. The experiment was carried out in strict accordance with the National Standard of the People's Republic of China (GB/T 35892-2018). Three male Kunming mice were sacrificed by cervical dislocation. The femur and tibia of both lower extremities were separated under sterile conditions. The bone marrow was washed into a centrifuge tube with DMEM/F12 containing penicillin 100 U/mL and streptomycin 100 U/mL + 10% FBS and then made into a single cell suspension. Centrifugation was performed at 350 g for 5 min, and the supernatant was discarded. The cells were inoculated in a culture flask and cultured in a 5% CO₂ incubator at 37°C. Half the amount of fluid was changed for 48 h, and then the full amount was changed every 3 days. When the culture flask was about 80% full, the cells were passed in a ratio of 1 : 3.

BMSCs were divided into C-BMSCs and C-BMSCs-CCR2 groups: C-BMSCs group was BMSCs isolated by the above method, and C-BMSCs-CCR2 group was CCR2 transgenic cells constructed by the following method.

The CCR2 gene sequence was synthesized from the mouse CCR2 gene information provided by GenBank. The CCR2 gene sequence was digested with pGMLV-PA4 vector to obtain a CCR2 over-expressed recombinant lentiviral vector (PGMLV-PA4-CCR2). 293T cells were co-transfected with the above lentivirus vectors and virus packaging helper plasmid, and the corresponding lentivirus was obtained by culture and concentration of supernatant. When the third generation of mouse BMSCs grew to 80% fusion, BMSCs were infected with the virus following the virus infection ratio of 50 : 1. After infection for 6-8 h, fresh medium was replaced to continue culture. After 48 h, the stable expression strains were screened with puromycin medium with the final concentration of 0.1 mg/L.

Model and groups of partial hepatectomy in mice. Kunming mice (KM) were anesthetized with 0.3% pentobarbital sodium at 0.1 mL/10 g by intraperitoneal injection.

After the abdomen was disinfected with 70% ethanol, a central vertical incision of the lower abdomen of xiphoid process was made, with a length of 1.5-3 cm. The epidermis, muscularis and peritoneum were cut successively to fully expose the liver. The middle lobe of the liver and the left lobe were ligated and removed.

Model mice were divided into three groups: RNA-Seq group (n = 21), KM-BMSCs group (n = 20) and KM-BMSCs-CCR2 group (n = 20). The RNA-Seq group was designed to understand the expression of CCR2 ligand CCL2 in liver regeneration. The right lobe of regenerated liver was taken at 24, 48, 72, 96, 120, 144 and 168 h after partial liver resection, respectively, and the transcriptome of the liver was sequenced using Illumina HiSeqTM2500 high-throughput sequencing platform. In the KM-BMSCs group, 0.1 mL (1 × 10⁷/mL) of C-BMSCs cells were injected through caudal vein immediately after partial hepatectomy. In the KM-BMSCs-CCR2 group, 0.1 mL (1 × 10⁷/mL) of C-BMSCs-CCR2 cells were injected through caudal vein immediately after partial hepatectomy.

Expression of CCR2 in BMSCs. In order to detect the expression of CCR2 in BMSCs, the BMSCs of C-BMSCs and C-BMSCs-CCR2 groups were analyzed by qRT-PCR. The BMSCs culture medium in the 6-well plate was discarded and BMSCs were washed with PBS precooled at 4°C. Next 1 mL Trizol was added into the crushed cells, and the liquid in the well was transferred into a 1.5 mL centrifuge tube. Then 250 µL chloroform was added, mixed well, and stood for 3 min. Centrifugation was performed at 13,000 × g at 4°C for 8 min. Transfer the supernatant to a new centrifugal tube, add 0.8 times the volume of isopropyl alcohol, and mix inversely. -20°C for 15 min. At 4°C, centrifuged at 13,000 g for 10 min, the white precipitate at the bottom of the tube was RNA. Add 20 µL of RNA enzymes-free water to dissolve RNA. The RNA was reversely transcribed into cDNA according to the Invitrogen reverse transcription kit instructions. Using cDNA as template and β-actin as reference gene, quantitative PCR was performed at the QuantiTect SYBR Green RT-PCR Kit (Qiagen) Detector under PRISM 7900 Sequence. PCR reaction conditions were as follows: 95°C for 5 min; 95°C 10 s, 60°C 30 s, 72°C 10 s; 40 cycles. The experimental data were analyzed by 2^{-(ΔΔCt)} method. The sequence of primers is as follows:

Primer	Sequence (5' to 3')
CCR2-F	GGGCTCACTATGCTGCAAAT
CCR2-R	CGAAACAGGGTGTGGAGAAT
β-actin-F	AACAGTCCGCCTAGAAGCAC
β-actin-R	CGTTGACATCCGTAAGACC

Detection of migration ability of BMSCs. The migration ability of BMSCs was detected by transwell assay and PKH26 staining. BMSCs of C-BMSCs and C-BMSCs-CCR2 groups at logarithmic growth stage were prepared into cell suspension with a cell density of 2 × 10⁶ cells/mL. 100 µL cell suspension was added to the upper chamber of Transwell, and 100 ng/mL CCL2 was added to the lower chamber of Transwell. After a 48 h culture, the BMSCs remaining on the upper surface were wiped with cotton swabs, fixed with 4% paraformaldehyde for

15 min, and stained with 0.1% crystal violet for 15 min. They were washed with PBS twice, and observed under microscope.

BMSCs of C-BMSCs and C-BMSCs-CCR2 groups were digested by trypsin to form a single cell suspension. Centrifugation at 400 g for 5 min, supernatant was aspirated, and 1 mL diluent was added to resuspended cells. $1 \text{ mL } 2 \times 10^7$ BMSCs were quickly added to $1 \text{ mL } 2 \times 10^{-6} \text{ mol/L PKH26}$ solution and mixed quickly. Incubated at 25°C for 5 min, the same amount of serum added to stop the staining reaction, and incubated for 1 min. Centrifugation was performed at 400 g for 10 min at 25°C to remove the supernatant. BMSCs stained with PKH26 were immediately transferred into the mouse through the tail vein after liver resection. The right lobe of the liver was removed 12 h later and observed under a fluorescence microscope.

Detection of liver regeneration. The mice in the KM-BMSCs group and the KM-BMSCs-CCR2 group were sacrificed by cervical dislocation at 72, 120 and 168 h after partial liver resection. The mice were weighed, the abdominal cavity was opened, and the remaining livers were completely separated and weighed. Liver index = (liver weight/body weight) $\times 100\%$. HE staining and serological detection were performed at the point in time when there was a significant difference in the liver index between the KM-BMSCs group and the KM-BMSCs-CCR2 group. HE staining was performed on the right lobe of the liver at this time point to observe the structural changes of the liver. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) contents in serum were detected by automatic biochemical analyzer (Beckman).

Signal pathway detection of BMSCs homing. The Western blot was used to detect RhoA and ROCK expression levels of Rho/ROCK signalling pathway proteins in the right lobe of the liver of the KM-BMSCs group and KM-BMSCs-CCR2 group at the point in time when there was a significant difference in the liver index between KM-BMSCs group and KM-BMSCs-CCR2 group. RhoA rabbit anti-mouse monoclonal antibody and ROCK rabbit anti-mouse monoclonal antibody were purchased from Abcam in the UK. Goat anti-rabbit secondary antibody was purchased from Abnova Company, USA. The primary antibody was diluted at 1 : 500, and the secondary antibody was diluted at 1 : 2,000. The protein bands were detected using an ECL kit (Millipore). The gel imaging system scanned and quantitatively analyzed the optical density of the bands.

In addition, the relationship between Rho/ROCK signalling pathway and BMSCs homing was detected by a scratch test. The cells of the C-BMSCs-CCR2 group at the logarithmic growth stage were inoculated into 6-well plates. When the degree of cell fusion reached 90%-95%, the cells were starved in serum-free medium for 12 h, and then a straight and uniform scratch was drawn along the central axis of each well with the 100 μL tip. The cells were then divided into two groups: one group was added with DMEM/F12 containing 10% FBS, 10 $\mu\text{mol/L}$ ROCK inhibitor Y-27632, and the other group was only added with DMEM/F12 containing 10% FBS. Both groups were placed in an incubator for further culture for 24 h, and the scratch healing was observed under a microscope.

Statistical analysis. The results of this experiment were independently repeated for 3 times. SPSS 25.0 statistical software was used for analysis, $P < 0.05$ was considered statistically significant.

Results and discussion

MSCs can be differentiated into a variety of cell types and repair a variety of tissue and organ injuries. Studies have shown that MSCs transplantation can help repair liver injury (9, 13). At present, MSCs transplantation mainly includes two methods: local transplantation and venous transplantation. Local transplantation is a single or multi-point local direct injection at the injured site, but this method may cause local pathological reactions. Vein transplantation is the injection of stem cells through a vein to reach the site of injury. Although the intravenous route provides a relatively safe mode, the intravenous injection of MSCs is usually distributed in lung, spleen, kidney, liver, heart and other organs, and the lack of satisfactory homing efficiency to the target *in vivo* affects the therapeutic effect. Therefore, strengthening the homing of stem cells at the site of injury will help improve the effectiveness of cell therapy.

Homing of stem cells refers to the process in which autologous or exogenous stem cells can migrate directionally, cross vascular endothelial cells to target tissues, colonize and survive under the action of various factors. In 2009, Krap et al. (5) proposed to define “mesenchymal stem cell homing” as a process in which mesenchymal stem cells are captured in the vasculature of the target tissue and then migrate across vascular endothelial cells to the target tissue. Mesenchymal stem cells are similar to vascular endothelial cells and are therefore easy to migrate and implant. The homing process of mesenchymal stem cells is very similar to the homing process of leukocytes to inflammatory tissue (12), including the mobilization and migration of MSCs, endothelial adhesion of MSCs and transendothelial migration of MSCs.

The interaction between chemokine ligands secreted by damaged tissues and corresponding chemokine receptors expressed on the surface of MSCs is important for MSC mobilization (4). Schenk et al. (8) found that monocyte chemokine 3 (MCP3), also known as C-C chemokine 7 (CCL7), was greatly expressed in the infarct area of Lewis rats one month after myocardial infarction. After BMSCs transplantation, a large number of BMSCs were collected by MCP3 and returned to the site of injury, and the cardiac function was significantly improved compared with the control group. Andres et al. (1) showed that after neural stem cells (NSCs) were injected into the blood vessels of hypoxic-ischemia-induced stroke mice, they could migrate across the endothelium and return to the damaged brain region, and the mutual attraction between CCL2 and CCR2 played an important role in this process. In this study, we found that partial hepatectomy can

induce high expression of CCL2 in an injured liver, and overexpression of CCR2 in BMSCs can enhance the homing ability of BMSCs to injured liver and accelerate the repair of injured liver.

In order to understand the expression of CCR2 ligand CCL2 in liver regeneration, the right lobes of regenerated liver were taken at 24, 48, 72, 96, 120, 144, 168 h after partial hepatectomy, and transcriptome sequenc-

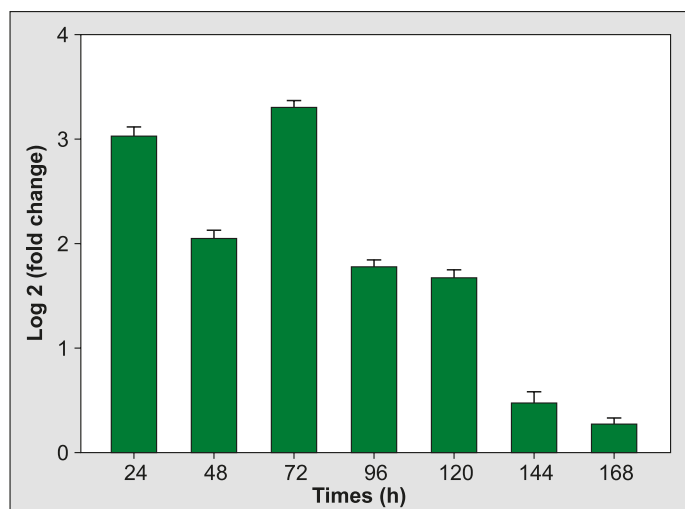


Fig. 1. Expression of the CCL2 gene in partial hepatectomy mice

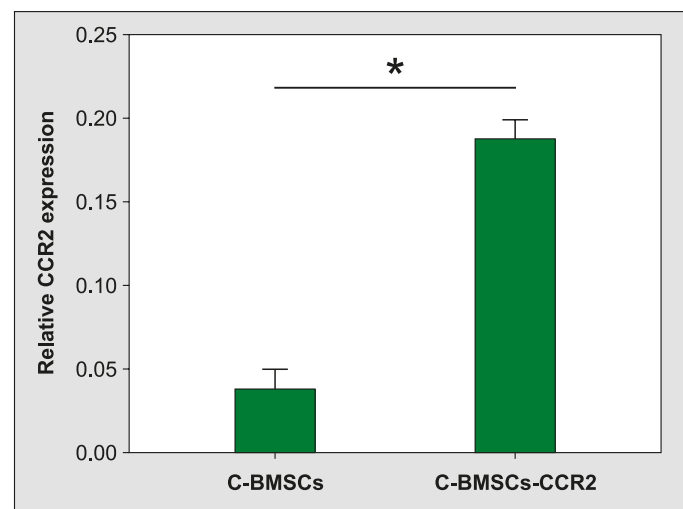


Fig. 2. Expression of CCR2 gene in cells

Explanation: * $P < 0.05$

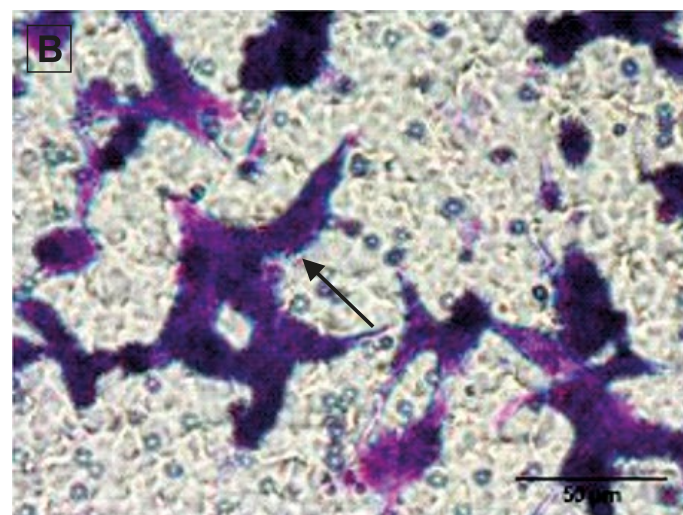
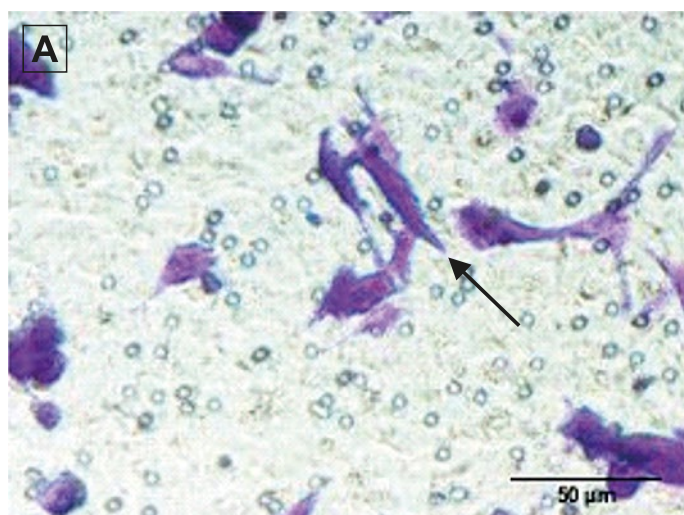


Fig. 3. Migration ability of BMSCs detected by transwell experiments. A – C-BMSCs group, B – C-BMSCs-CCR2 group

Explanation: arrows show areas of positive crystal violet staining

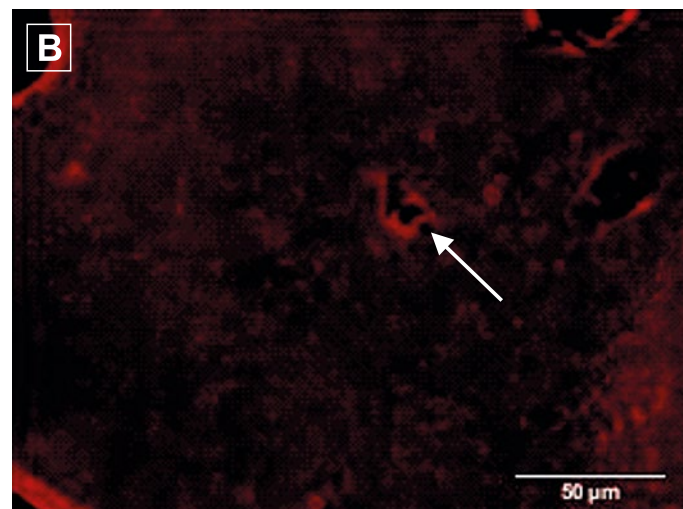
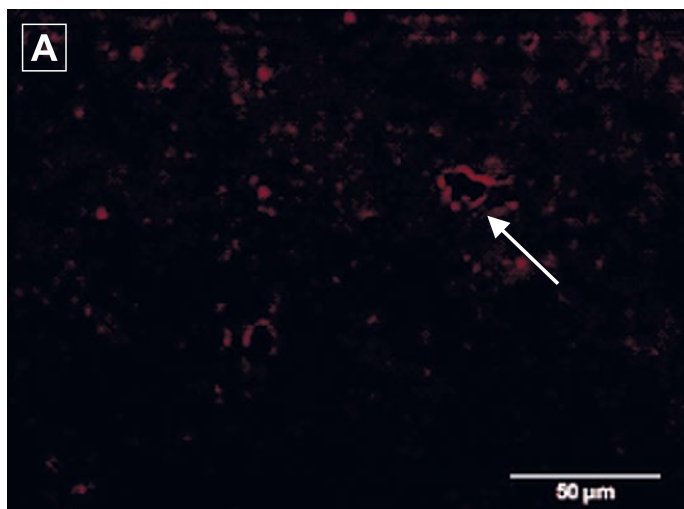


Fig. 4. Migration ability of BMSCs detected by a PHK26 staining. A – KM-BMSCs group, B – KM-BMSCs-CCR2 group

Explanation: arrows show PHK26 positive regions

ing was performed. The results showed that CCL2 expression was up-regulated during the whole process of liver regeneration (Fig. 1). In order to detect the expression of CCR2 in BMSCs transfected with lentivirus, qRT-PCR analysis was performed on BMSCs of C-BMSCs and C-BMSCs-CCR2 groups. The results showed that BMSCs transfected with lentivirus could express more CCR2, which was significantly different from those without lentivirus transfection ($P < 0.05$) (Fig. 2). BMSCs of C-BMSCs-CCR2 groups with high expression of CCR2 had higher chemotaxis and migration ability to CCL2 (Fig. 3). After the cells of C-BMSCs-CCR2 groups were stained with PHK26, they were transferred into the partially hepatectomized mice through the tail vein. 12 h later, the right lobe of the liver was removed, and fluorescence microscope observation showed that more red fluorescence appeared in the liver of mice in the KM-BMSCs-CCR2 group, indicating that the CCR2-modified BMSCs had a stronger ability to migrate to the damaged liver (Fig. 4). CCR2-modified BMSCs can also accelerate the repair of damaged liver. At 72 h after partial hepatectomy, the liver index of KM-BMSCs-CCR2 group was significantly higher than that of KM-BMSCs group (Fig. 5). We picked this time for further research. At this time, HE staining showed that the hepatic lobule structure of KM-BMSCs-CCR2 group was clear, liver cell plate was double row, liver cells were significantly enlarged, liver cells and nuclei were different in size, and vacuoles appeared in cytoplasm. In the KM-BMSCs group, the hepatic cells were disordered locally, and some hepatic cells were edema and mitotic (Fig. 6). The content of ALT and AST in KM-BMSCs-CCR2 group was $145.28 \pm 20.19 \text{ U} \cdot \text{L}^{-1}$ and $79.83 \pm 25.23 \text{ U} \cdot \text{L}^{-1}$, respectively, which were significantly lower than those in KM-BMSCs group ($P < 0.05$) (Tab. 1).

Rho/ROCK is a universal signal transduction pathway in all tissues of the body; it is closely related

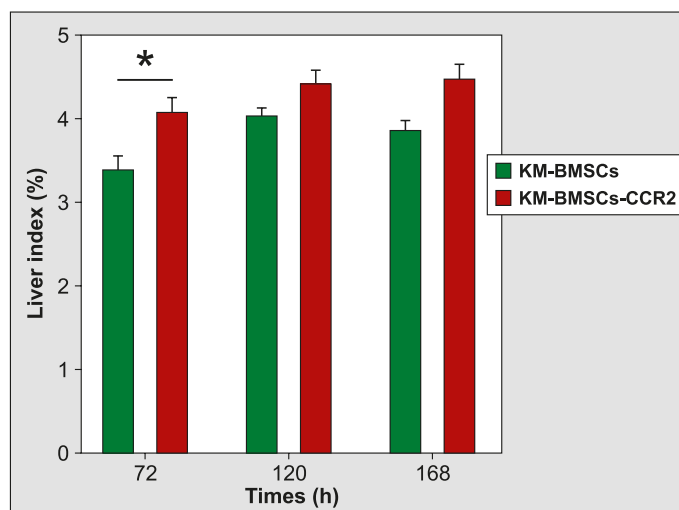


Fig. 5. Liver index

Explanation: * $P < 0.05$

Tab. 1. Liver function comparison

Group	ALT ($\text{U} \cdot \text{L}^{-1}$)	AST ($\text{U} \cdot \text{L}^{-1}$)
KM-BMSCs	198.79 ± 23.48	153.42 ± 20.15
KM-BMSCs-CCR2	$135.85 \pm 19.68^*$	$89.64 \pm 24.35^*$

Explanation: * $P < 0.05$

to a variety of biological behaviours such as cell contraction, adhesion, migration, proliferation and cytoskeleton formation (6). The key molecules of the Rho/ROCK signalling pathway include Rho GTP enzyme, Rho-associated kinase (ROCK) and their acting substrates. Lee et al. (15) proved that lysophosphatidic acid induction of MSC migration requires Rho activation. The binding of CCL2 to its receptor CCR2 can activate the intracellular Rho GTP enzyme (6). In our study, western blot analysis showed that after BMSCs overexpressing CCR2 were transferred into partially hepatectomized mice, RhoA and ROCK expression of Rho/ROCK signalling pathway proteins in the liver of mice in the KM-BMSCs-CCR2 group were increased

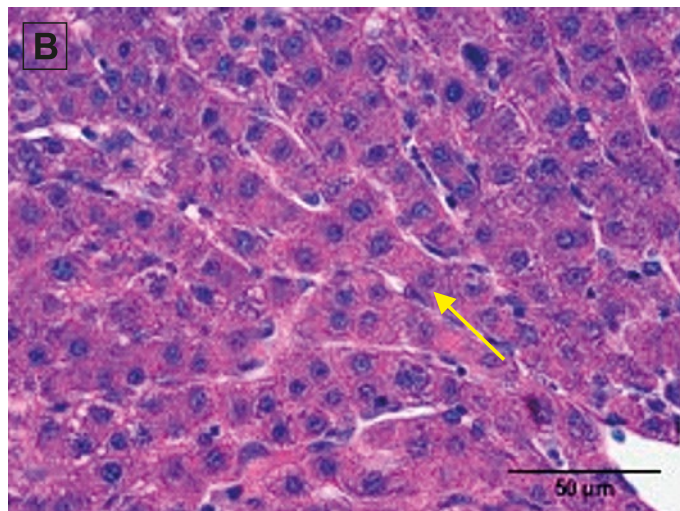
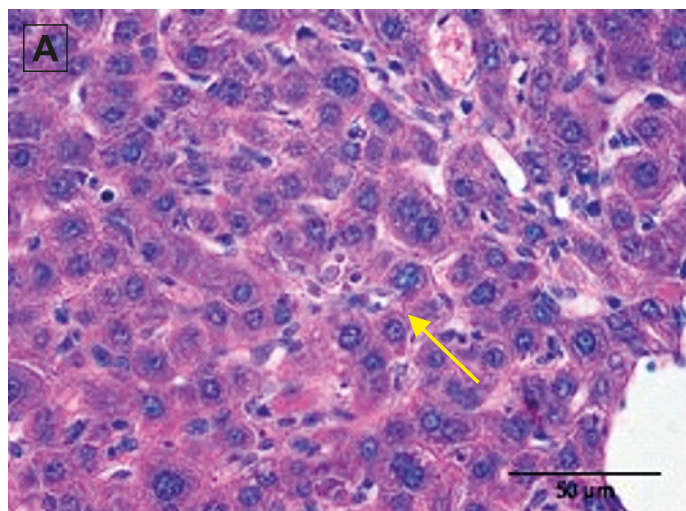


Fig. 6. Liver structure. A – KM-BMSCs group, B – KM-BMSCs-CCR2 group

Exlnation: arrows show the lobule region of the liver. HE staining

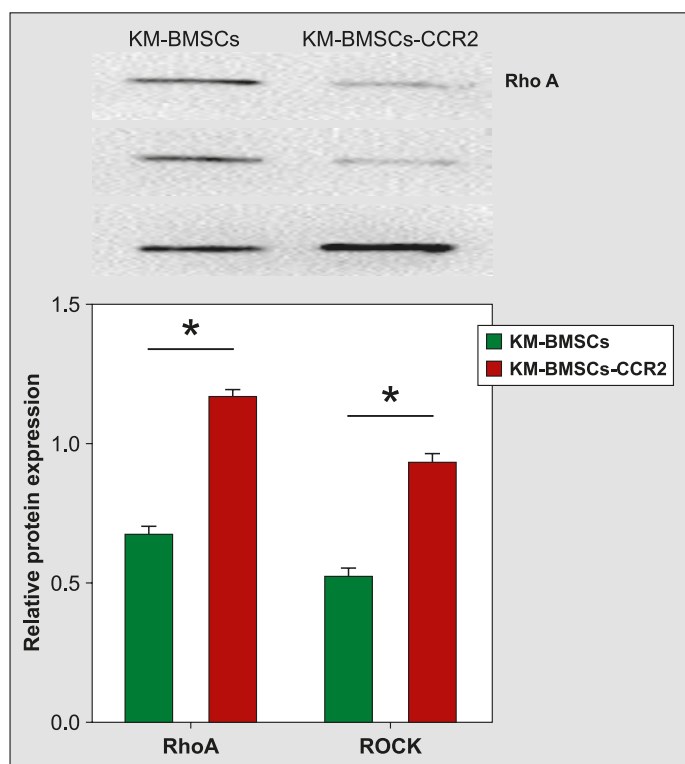


Fig. 7. Western blot detects proteins expression of Rho/ROCK signalling pathway

Explanation: * $P < 0.05$

compared with those in the BMSCs group (Fig. 7). When the C-BMSCs-CCR2 group was treated with the Rho/ROCK signalling pathway inhibitor Y-27632, the scratch test showed the migration ability of the C-BMSCs-CCR2 group was weaker than that of the untreated group, indicating that the Rho/ROCK signalling pathway affected the homing of BMSCs (Fig. 8).

In summary, CCL2 expression is induced after liver injury, and overexpression of CCR2 in BMSCs can lead to migration of BMSCs to the injured liver and homing to the injured site through Rho/ROCK signalling pathway, thus promoting liver regeneration.

References

- Andres R. H., Choi R., Pendharkar A. V., Gaeta X., Wang N., Nathan J. K., Chua J. Y., Lee S. W., Palmer T. D., Steinberg G. K., Guzman R.: The CCR2/CCL2 interaction mediates the transendothelial recruitment of intravascularly delivered neural stem cells to the ischemic brain. *Stroke* 2011, 42, 2923-2931.
- Belema-Bedada F., Uchida S., Martire A., Kostin S., Braun T.: Efficient homing of multipotent adult mesenchymal stem cells depends on FROUNT-mediated clustering of CCR2. *Cell Stem Cell* 2008, 2, 566-575.
- Chamberlain G., Fox J., Ashton B., Middleton J.: Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 2007, 25, 2739-2749.
- Hocking A. M.: The role of chemokines in mesenchymal stem cell homing to wounds. *Adv Wound Care (New Rochelle)* 2015, 4, 623-630.
- Karp J. M., Leng T. G. S.: Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell* 2009, 4, 206-216.
- Murali A., Rajalingam K.: Small Rho GTPases in the control of cell shape and mobility. *Cell Mol. Life Sci.* 2014, 71, 1703-1721.
- Naderi-Meshkin H., Bahrami A. R., Bidkhori H. R., Mirahmadi M., Ahmadiankia N.: Strategies to improve homing of mesenchymal stem cells for greater efficacy in stem cell therapy. *Cell Biol. Int.* 2015, 39, 23-34.
- Schenk S., Mal N., Finan A., Zhang M., Kiedrowski M., Popovic Z., McCarthy P. M., Penn M. S.: Monocyte chemoattractant protein-3 is a myocardial mesenchymal stem cell homing factor. *Stem Cells* 2007, 25, 245-251.
- Sungkar T., Putra A., Lindarto D., Juwita Sembiring R.: Anti-fibrotic effect of intravenous umbilical cord-derived mesenchymal stem cells (UC-MSCs) injection in experimental rats induced liver fibrosis. *Med. Glas. (Zenica)* 2021, 18, 62-69.
- Wang L., Li Y., Chen J., Gautam S. C., Zhang Z., Lu M., Chopp M.: Ischemic cerebral tissue and MCP-1 enhance rat bone marrow stromal cell migration in interface culture. *Exp. Hematol.* 2002, 30, 831-836.
- Yoshimura T.: The production of monocyte chemoattractant protein-1 (MCP-1)/CCL2 in tumor microenvironments. *Cytokine* 2017, 98, 71-78.
- Zachar L., Bacenkova D., Rosocha J.: Activation, homing, and role of the mesenchymal stem cells in the inflammatory environment. *J. Inflamm. Res.* 2016, 9, 231-240.
- Zhang J., Gao J., Lin D., Xiong J., Wang J., Chen J., Lin B., Gao Z.: Potential Networks Regulated by MSCs in Acute-On-Chronic Liver Failure: Exosomal miRNAs and Intracellular Target Genes. *Front Genet.* 2021, 12, 650536.

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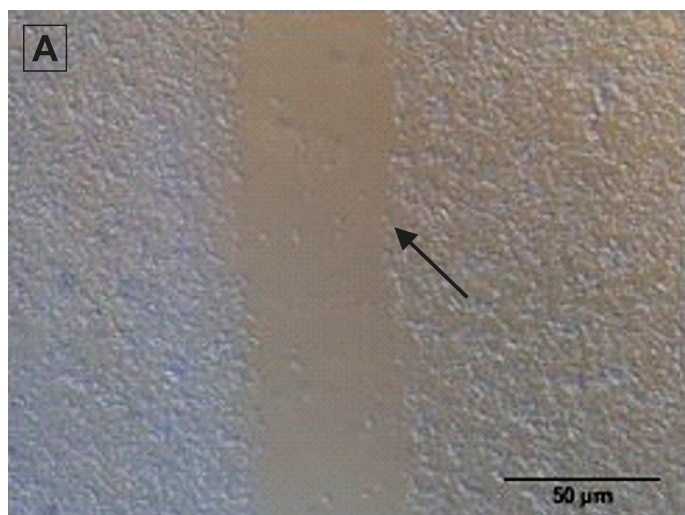


Fig. 8. Effect of Y-27632 on BMSCs migration ability. A – C-BMSCs-CCR2 + Y-27632 group, B – C-BMSCs-CCR2 group
Explanation: arrows shows the scratch areas