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Original Article

Bone mesenchymal stem cells overexpressing FGF4 contribute to liver regeneration in an animal model of liver cirrhosis

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Abstract: It is recognized that Fibroblast Growth Factor 4 (FGF-4) could not only increase the proliferation of bone marrow mesenchymal stem cells (BMSCs), but also induce BMSCs into hepatocyte-like cells in vitro. However, the role of FGF4 played in liver regeneration in vivo is unclear. This study constructed FGF4 overexpressing BMSCs and then transplanted them into cirrhotic rats to investigate the role of FGF4 played in liver regeneration. The results showed that FGF4 promoted the location of the BMSCs only at the early stage, and more proliferating cell nuclear antigen (PCNA), epithelial cell adhesion molecule (EpCAM) and Jagged-1 positive hepatocytes were found in the cirrhotic rats. This study indicated that FGF4 transduced BMSCs contributed to liver regeneration might by the transplanted microenvironment.

Keywords: BMSCs, transplant, migration, proliferation, microenvironment Introduction

Liver cirrhosis is one common disease in China, because of the large population of HBV-related liver diseases [1]. Liver transplant is the only effective therapy for the end stage liver diseases. However, the scarcity of liver donor organs limited its widespread application. BMSCs contribute to liver regeneration via directly differentiation, and/or paracrine mechanism and immunosuppressive activities [2-4]. However, their recruitment to the injured tissues, survival and function are yet to be investigated [5, 6]. Our previous study showed that FGF4 could induce bone marrow derived monocytes into hepatocytes in vitro [7], which demonstrated that the hepatic differentiation of BMSCs might be induced by the cytokines secreted from the injured liver cells. However, which and how cytokines direct hepatic fate of specification of BMSCs still remains unclear.

Fibroblast growth factors (FGFs) elicit a variety of biological responses, including cell proliferation, differentiation and migration [8]. FGF-4 represents one of the FGFs with the highest mitogenic activity in several cell lines in vitro [9-11]. However, in vivo, the effect of FGF4 impact on the proliferation and differentiation of the injured liver is still unclear.

In the present study, we achieved a remarkable expression of FGF4 in BMSCs by gene transfection, which was accompanied by enhanced recruitment of BMSCs to the cirrhotic liver at the early stage of the transplantation of FGF4 transduced BMSCs. The liver function of the cirrhotic rats was improved, accompanied with more PCNA, EpCAM and Jagged-1 positive hepatocytes scattering in the cirrhotic liver. We hope our findings will not only advance our understanding of the role of BMSCs in liver regeneration, but also might improve cytokine-based therapy for liver cirrhosis.

Materials and methods

Animals

Female and male SD rats (6-8 wk) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences of

Table 1. Kinetics of liver function of the cirrhotic in the different groups (n = 6)

		Pre-transplant	48 h after transplant	72 h after transplant	7 days after transplant
ALB (g/L)	Group-FGF4	31.06	32.02	44.40	48.28
	Group-BMSCs	29.98	30.89	45.61	50.02
	Group-GFP	30.02	31.08	48.48	50.96
	Group-LC	30.22	30.68	32.40	31.26
CHO (mmol/L)	Group-FGF4	0.86	1.48	1.58	2.38
	Group-BMSCs	0.90	1.39	1.72	2.40
	Group-GFP	0.88	1.40	1.70	2.46
	Group-LC	0.91	0.98	1.02	1.00
TG (mmol/L)	Group-FGF4	0.36	0.46	0.67	0.84
	Group-BMSCs	0.32	0.48	0.65	0.88
	Group-GFP	0.33	0.51	0.66	0.89
	Group-LC	0.35	0.36	0.39	0.40
TBIL (umol/L)	Group-FGF4	17.40	14.68	10.84	4.79
	Group-BMSCs	18.26	16.26	12.48	6.67
	Group-GFP	17.86	16.90	13.02	6.98
	Group-LC	18.06	17.03	17.21	17.00
ALT (U/L)	Group-FGF4	80.88	78.82	68.89	64.68
	Group-BMSCs	82.79	80.40	74.02	65.58
	Group-GFP	82.38	79.91	74.53	65.69
	Group-LC	81.97	80.80	78.97	69.91
AST (U/L)	Group-FGF4	312.02	278.04	188.62	170.99
	Group-BMSCs	308.14	286.22	178.46	174.05
	Group-GFP	298.86	290.91	186.74	179.24
	Group-LC	310.56	310.89	300.90	276.18

*$P < 0.05$ was considered to have significantly differences. ALB: Albumin; CHO: Cholesterol; TG: Triglyceride; TBIL: Total Bilirubin; ALT: Alanine aminotransferase; AST: Aminotransferase aspartate.

China (Beijing). All studies were performed under approval of the Ethics Committee of the Animal Facility of Chinese PLA General Hospital and were in agreement with the guidance suggestion of caring for laboratory animals [12]. Rats were group-housed under controlled temperature (25°C) and a 12-h light/dark cycle, fed standard rat chow and tap water and maintained for 2 times per wk in our animal facilities before the experiment.

Lentivirus vector for pGC-FU-FGF4

The FGF4 gene sequence was amplified by polymerase chain reaction (PCR) from a pGC-FU Vector (Genechem Company), and the specific primer sequences 5'-GGGTCAATATGTAATTTTCAGTG-3' and Reverse primer: 5'CGTCGCCGTCCAGCTCGACCAG-3'. After digesting the pGC-FU-GFP lentivirus with AgeI (R0552v, NEB Company), the amplified PCR product was inserted into the AgeI restriction site using an In-Fusion™ PCR Cloning Kit (639626, Clontech) to produce the recombinant vector pGC-FU-FGF4. The vector pGC-FU-FGF4 and the viral packaging system were cotransfected into293 cells to produce the recombinant FGF4 lentivirus (Lv-FGF4) and the null green fluorescent protein lentivirus (Lv-null-GFP). The lentiviral titers averaged 2×10^8 TU/ml.

Preparation of BMSCs and FGF4 transfection

The BMSCs from the male SD rats were prepared and characterized as described previously [13]. They were transduced with Lv-FGF4 or Lv-null-GFP at the passage 3. For transduction, cells were seeded at 2000 cells/cm^2 in a T-75 cm^2 flask. The following day virus particles were added at a multiplicity of infection (m.o.i.) of 100 for 18 hours. Then cells were washed and cultured with fresh medium continually. Because the recombinant lentivirus expressed GFP, the transfection efficiency of BMSCs with the viral system was evaluated via fluorescence microscopy.

RNA isolation and real-time PCR (qPCR)

In brief, total RNA was isolated using Trizol (Invitrogen) according to manufacturer instructions, and was quantified by spectrophotometry. One-microgram total RNA samples were reverse-transcribed using the PrimeScriptTM RT reagent kit (TaKaRa, Dalian, China) according to manufacturer protocols. RT-PCR was performed using a SYBR PrimeScriptTM RT-PCR Kit (TaKaRa) in a real-time thermal cycler system. Gene-specific primers for FGF4, EpCAM, Jagged-1 and B-actin were designed using the

Premier software with the primers listed in **Table 1** (Sangon Biotech, Shanghai, China). The cycle conditions were set as follows: initial template denaturation at 95°C for 30 s, followed by 38 cycles of denaturation at 95°C for 10 s, and combined primer annealing/elongation at 72°C for 30 s. The cycle was followed by a melting curve analysis, ranging from 54°C to 95°C, with temperature increasing by steps of 0.5°C every 10 s. The relative concentration of cDNA was determined by the standard dilution curve and normalized against that of β-actin.

Animal experimental design and transplant procedure

The CCl4-induced liver cirrhosis SD rat model was established previously [17]. The BMSCs from male donor rats were infused into the tail vein of female cirrhotic recipient rats. The female rats were randomly assigned to four experimental groups (n = 6 for each group): group-LC, group-BMSCs, Group-GFP, and the Group-FGF4. Female rats were sacrificed at 48 h, 72 h, and 7 day after cell transplant. Peripheral blood samples and the median lobe of the liver were collected. The liver tissues were fixed with 4% paraformaldehyde and were analyzed by immunohistochemistry.

Migration assay in vivo

To determine whether FGF4 could promote the engraftment of BMSCs in cirrhotic liver, the sex-determining region Y (SRY) protein was detected for tracing the location of transplanted cells by immunohistochemistry. The 4-μm liver tissue sections were deparaffinized using xylene andalcohol andrehydrated with water and were treated with a goat anti-rat primary antibody to SRY (1:50, sc-8233, Santa Cruz) in PBS at 4°C overnight. After 3 washes in PBS, the sections were incubated with a rabbit anti-goat second antibody (1:1000, Beijing Zhongshan Biotechnology) at 37°C for 2 hours. Finally, the immunoreactivity of the sections were visualized by incubation in 3, 3'-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 (Liquid DAB+ Substrate Chromogen System; Dako) for 3 minutes to induce a color reaction. The expression and localization of SRY was examined under a light microscope (Olympus, Japan), and the presence of a brown color reaction product indicated a positive result. Immunostained sections were evaluated by two investigators in a double blind fashion. Ten non-overlapping fields of view (400× magnification) were selected, and the mean value of the proportion of SRY-positive cells for each section was used for statistical analysis.

Liver function assay

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin (ALB), total bilirubin (TBIL), cholesterol (CHO), and triglyceride levels were measured with an automated analyzer (Beckman DXC 600, Beckman Counter, Inc., Brea, CA).

Expression of PCNA, EpCAM and Jagged-1

To determine whether FGF4-transduced BMSCs could promote the proliferation of hepatocytes, immunohistochemical staining for PCNA (1:100, sc-7909, Santa Cruz.) was performed with an ABC staining kit (Vector Laboratories) according to the manufacturer's recommendations. PCNA-positive cells were quantified by counting the number of positive hepatocytes in 10 random fields with a 20× objective.

To determine whether the engrafted BMSCs could promote the proliferation of hepatic progenitor cells, immunohistological staining was performed for Jagged-1 (1:150, sc-6011, Santa Cruz.) and EpCAM (1:100, sc-66020, Santa Cruz.). Jagged-1 and EpCAM-positive cells were quantified by counting the number of hepatocytes in 10 random fields with a 20× objective.

Statistical analysis

Results are expressed as the mean ± SD. Student's t-test was performed to analyze the differences between two groups. Multiple-group comparisons were performed using one-way ANOVA followed by a Student-Newman-Keuls test. SPSS 17.0 statistical software was used for analysis. $P < 0.05$ was considered statistically significant.

Results

FGF4-Transduced BMSCs improved their stemness

Nightly-six hours after transduction, 92% of FGF4-BMSCs were GFP-positive, and the q-PCR showed that a 4.6-fold increase in FGF4 expres-

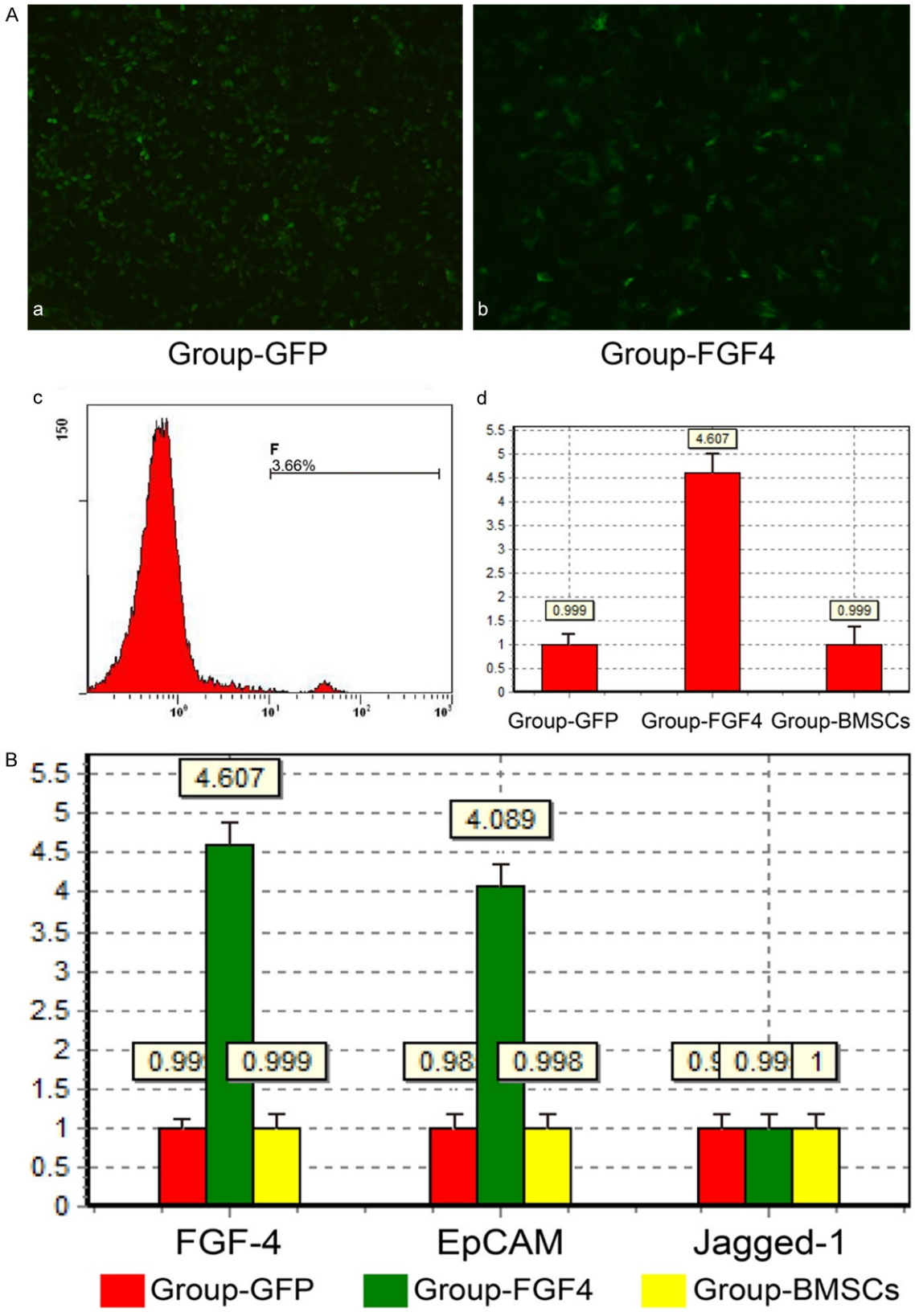

Figure 1. Expression of FGF4, EpCAM and Jagged-1 on FGF4 transduced BMSCs. A. Nearly 92% of FGF4-BMSCs were GFP-positive 96 hours after transduction, and 4.6-fold increase in FGF4 expression in FGF4-transduced BM-

SCs relative to the null-BMSCs evaluated by Q-PCR. B. Compared with the Group-GFP, FGF4 transduced BMSCs displayed a similar expression of Jagged-1, and a higher expression of EpCAM. Expression standard errors are expressed within brackets at the top of each bar. *means $P < 0.001$.

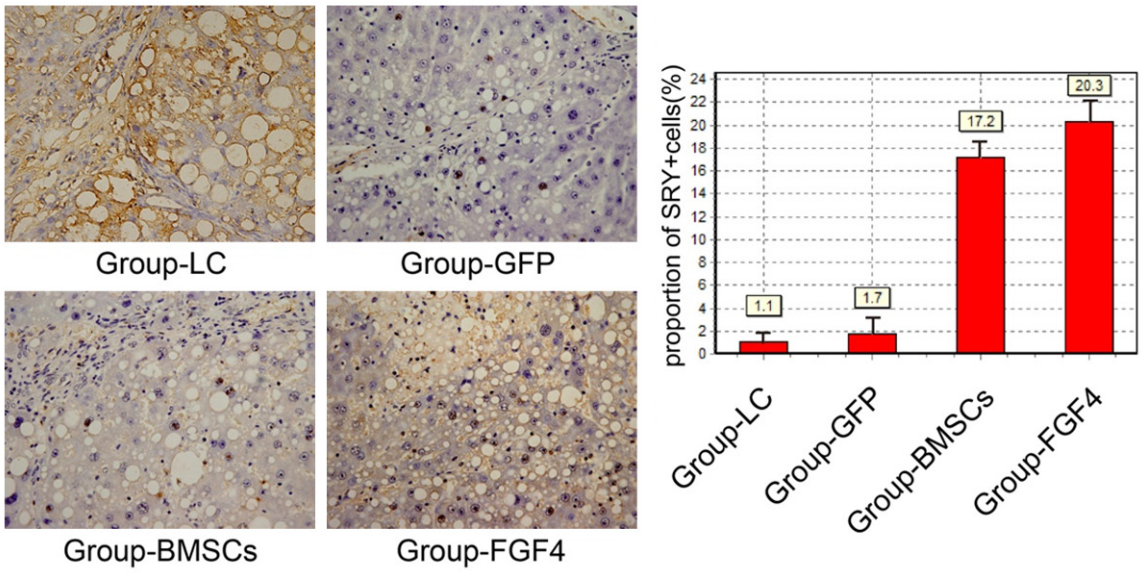

Figure 2. Effects of FGF4 on the homing of BMSCs to cirrhotic liver. Left: homing of BMSCs to the cirrhotic liver of different experimental groups was observed, 48 hours after transplantation. A brown nuclear signal in some cells indicated the presence of SRY-positive cells (200× magnification). Right: quantitative analysis shows that the proportion of SRY cells in the Group-BMSCs and the Group-GFP is not significantly different ($P > 0.05$). However, the proportion of SRY cells in the Group-GFP and the Group-FGF4 are significantly different ($P < 0.05$). Results are expressed as the mean ± SD.

sion in FGF4-transduced BMSCs relative to the null-BMSCs (**Figure 1A**).

To evaluate the impact of FGF4 on BMSCs, the stem cell related markers of EpCAM and Jagged-1 were detected by q-PCR. The result showed that Group-FGF4 displayed a similar expression of Jagged-1, and a higher expression of EpCAM, compared with the Group-GFP (**Figure 1B**).

The FGF4 promotes the engraftment of BMSCs in the cirrhotic liver

To validate the homing of BMSCs to the injured tissue, SRY was detected. At 48 hour of transplantation, rats were sacrificed, and the SRY positive cells were counted. Some cells in the liver showed a brown nucleus in the experimental group, indicating that the BMSCs were homing to the cirrhotic livers. The proportion of SRY positive cells in the Group-GFP was similar to that in the Group-BMSCs. However, transplantation of FGF4-BMSCs further increased the proportion of SRY cells in the cirrhotic livers (**Figure 2A**, **2B**). But, after 72 h of cells transplantation, the different groups of BMSCs nearly could not be detected in the cirrhotic livers.

Dynamic of liver functions of the animal model of liver cirrhosis

At the 48 h, 72 h and 7 day of transplantation, rats were sacrificed, and the liver function related factors including ALB, CHO, TG, TBIL, ALT, and AST were assayed. The liver function related factors have been improved in the cirrhotic rats that accepted cells transplant, contrasted with the non-transplant group rats. However, there were no significant differences among Group-BMSCs, Group-GFP, and Group-FGF4 (**Table 1**).

Effect of FGF4 on the proliferation of hepatocytes of the cirrhotic liver

To evaluate the proliferation of hepatocytes, PCNA was detected by immunohistochemistry.

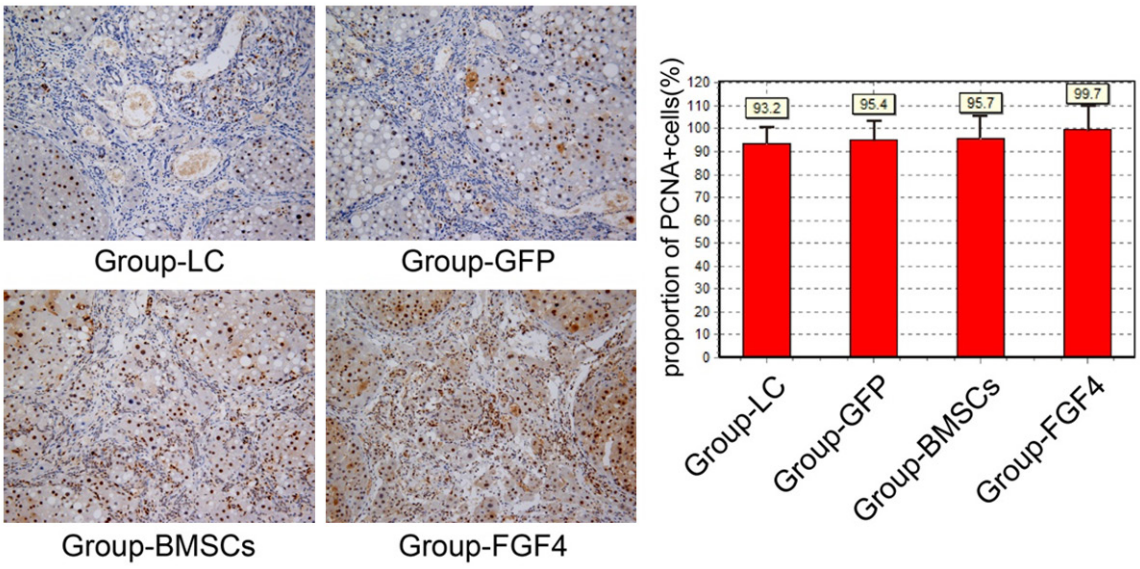

Figure 3. Determination of the proliferation of hepatocytes. Left: PCNA expression in hepatocytes was observed in different experimental groups 48 hours after transplantation. A brown cytoplasmicsignal in some cells indicates the presence of PCNA positive cells (200× magnification). Right: quantitative analysis shows that the proportion of PCNA cells in the Group-LC and Group-BMSCs is significantly different ($P < 0.05$), whereas the proportion of PCNA cells in the Group-GFP and the Group-BMSCs are not significantly different ($P > 0.05$). A significant difference ($P < 0.05$) is observed in the Group-GFP and Group-FGF4. The results are expressed as the mean ± SD.

At 48 hours of transplantation, rats were sacrificed, and the PCNA-positive cells were counted. Some cells in the liver showed a brown nuclear signal in the experimental group, indicating that BMSCs could promote liver regeneration. The proportion of PCNA-positive cells in the Group-GFP was similar to that in the Group-BMSCs. However, the transplantation of FGF4-BMSCs further increased the proportion of PCNA cells in the cirrhotic livers (**Figure 3A**, **3B**).

Effect of FGF4 on the proliferation of the HPCs in the cirrhotic liver

The Jagged-1 and EpCAM were identified as markers of hepatic progenitor cells (HPCs). At 48 hours of transplantation, some biliary duct cells in the portal area of the liver showed a brown nuclear stain in the experimental group, indicating that the BMSCs could promote the proliferation of HPCs. The proportion of Jagged-1-and EpCAM-positive cells in the Group-GFP was similar to that in the Group-BMSCs. However, the transplantation of FGF4-BMSCs further increased the proportion Jagged-1 and EpCAM cells in the cirrhotic livers (**Figure 4A**, **4B**).

Discussion

In this study, we first recombined a lentiviral vector of FGF4, and then successfully modified BMSCs with the vector. We also firstly confirmed that the FGF4 promoted the stemness of BMSCs with EpCAM expression. Our study also showed that FGF4 transduced BMSCs promoted the engraftment of BMSCs in cirrhotic liver only at the early stage of cells transplant. Furthermore, as compared to transplantation of the unmodified BMSCs, or BMSCs modified by GFP gene, there were more PCNA positive cells were observed in the cirrhotic liver, accompanied with more Jagged-1, and EpCAM positive cells scattering in the portal areas of the cirrhotic rats that accept FGF4 modified BMSCs transplant. Our results indicated that BMSCs overexpressing FGF-4 might contribute to liver regeneration of the animal model of liver cirrhosis by promoting the proliferation of BMSCs and modulating the microenvironment of the cirrhotic liver.

BMSCs transplantation is generally recognized to be the most promising stem cell therapy in the treatment of liver cirrhosis due to its advantages of self-replicating ability, multi-directional differentiation potential, easily obtaining, no

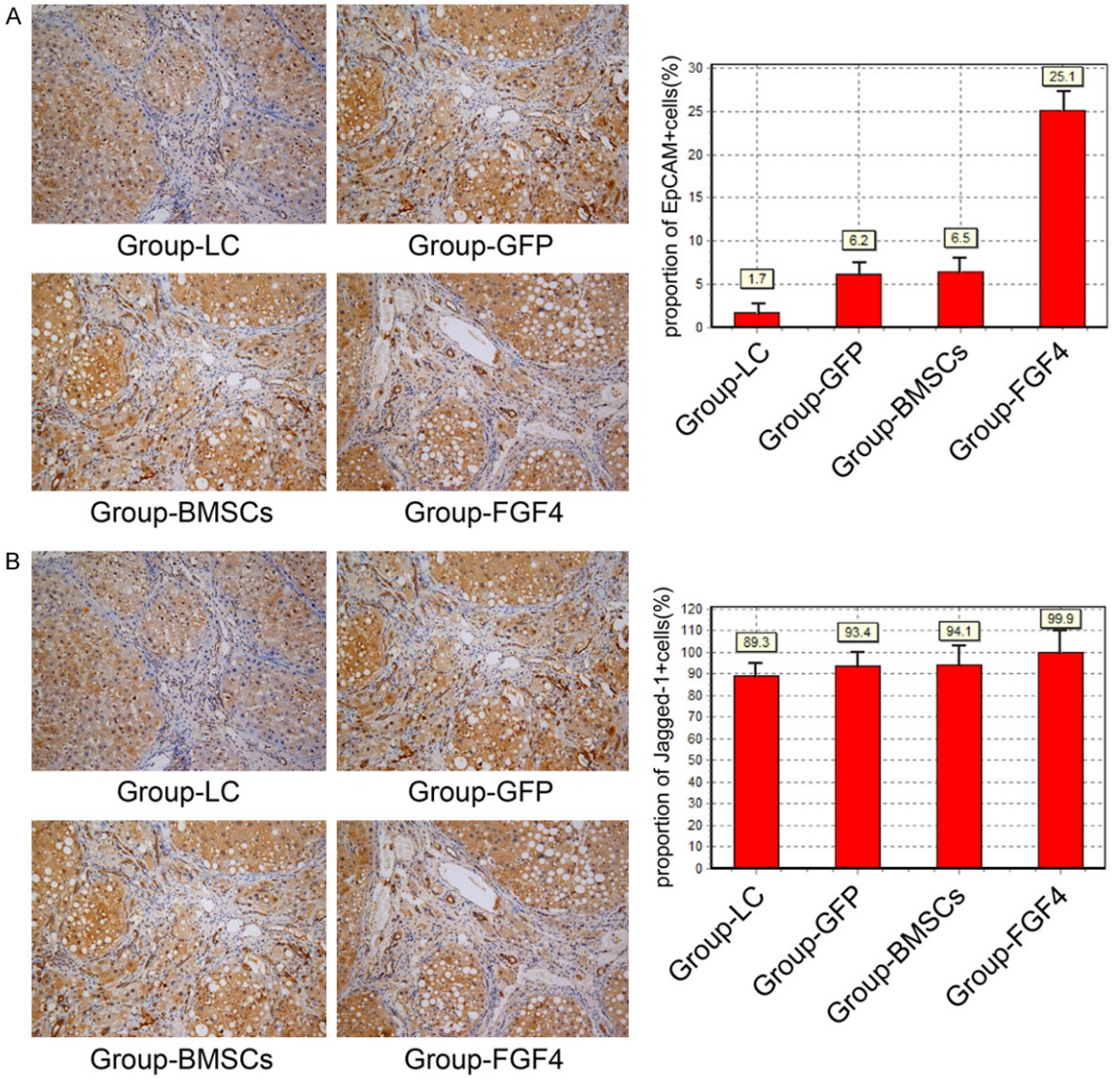

Figure 4. Effect of FGF4 on the proliferation of the HPCs of cirrhotic liver. A. Left: EpCAM expression in the liver tissues was observe in different experimental groups. A brown membranous and cytoplasmic signal in some cells indicates the presence of EpCAM-positive cells (200× magnification). Right: quantitative analysis shows that the proportion of EpCAM cells in the Group-LC and Group-BMSCs are significantly different ($P < 0.05$), whereas the proportion of EpCAM cells in the Group-GFP and the Group-BMSCs are not significantly different ($P > 0.05$). A significant difference ($P < 0.05$) was observed in the Group-GFP and Group-FGF4. The results are expressed as the mean ± SD. B. Left: Jagged-1 expression in the liver tissues was observed in different experimental groups 48hours after transplantation. A brown membranous signal in some cells indicates the presence of Jagged-1-positive cells (200× magnification). Right: quantitative analysis shows that the proportion of Jagged-1 cells in the Group-LC and in the Group-BMSCs is significantly different ($P < 0.05$), whereas the proportion of Jagged-1 cells in the Group-GFP and the Group-BMSCs is not significantly different ($P > 0.05$). A significant difference ($P < 0.05$) is observed in the Group-GFP and Group-FGF4. The results are expressed as the mean ± SD.

immune rejection, and no ethical conflicts [15, 16]. However, simple BMSCs transplantation has limitations because of limited survival and poor differentiation of the transplanted cells [17, 18]. More importantly, the adverse host microenvironment in inflammatory damage or local structural alterations (e. g. liver fibrosis) may not favorable to transplanted cell surviving. Accordingly, it is desirable to explore a new approach that not only modify the donor cells but also modulate the host microenvironment as well.

FGF4, as one member of the FGFs, has multiple functions including cell proliferation, migration, survival, and differentiation in different cell types. Previous studies showed that FGF4 induced the bone marrow derived stem cells into functional hepatocytes in vitro [19, 20], but the effect of FGF4 impact on the BMSCs in vivo was unclear. The survival of transplanted cells and the interactions between the transplanted BMSCs and hepatic stem cell niche were firstly investigated in this study. As compared with Group-LC, Group-BMSCs or Group-GFP, the major findings were: (1) Group-FGF4 had a higher survival rate of the transplanted cells as shown in immunohistochemistry, evidenced by the large number of transplanted cells migrating and gathering in liver portal areas at the 48 h of transplantation. (2) More PCNA positive hepatocytes expressed in the Group-FGF4, and a significant difference was observed between Group-GFP and Group-FGF4. (3) The expressions of EpCAM and Jagged-1 in Group-FGF4 were the highest; while their expression level in Group-GFP, though higher than that in Group-LC, was similar to Group-BMSCs. Accordingly, it is reasonable to postulate that genetically modified BMSCs with FGF4 was able to express FGF4 with high efficiency and promote cell division and growth as well, a crucial role that can be transferred not only to promote the survival of the transplanted cells, but also to impact the hepatic progenitor cells niche. Although both in vitro and in vivo data have demonstrated FGF4 had above mentioned capabilities, it remained unclear whether such abilities could be translated into the therapeutic benefits. Our liver function data showed that FGF4 transduced BMSCs could improve the liver function of the cirrhotic rats, but no significant differences were observed between Group-BMSCs, Group-GFP and Group-FGF4.

In summary, the present study was partly consistent with the previous studies that the FGF4 could promote the BMSCs engraftment in the injured organs. But the definite mechanism of FGF4 modified BMSCs contributing to liver regeneration should be explored from the dynamic microenvironment of liver progenitor cell niche. Our further study will focus on the long-term amelioration of the liver function and pathological changes, which might improve the cytokine-based therapy for liver cirrhosis in the future.

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Disclosure of conflict of interest

None.

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