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ORIGINAL ARTICLE

Effects of mesenchymal stem cells and VEGF on liver regeneration following major resection

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Abstract
Purpose The study aims to determine the effects of mesenchymal stem cell (MSC) therapy and a combination therapy of MSCs transfected with vascular endothelial growth factor (VEGF) for liver regeneration after major resection.
Methods Thirty-eight rats were divided into four groups: group 1: control (sham operation); group 2: control (70 % hepatic resection); group 3: 70 % hepatic resection + systemically transplanted MSCs; and group 4: 70 % hepatic resection + systemically transplanted MSCs transfected with the VEGF gene. MSCs were injected via the portal vein route in study groups 3 and 4. Expression levels of VEGF, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor (TGF), hepatocyte growth factor (HGF), and augmenter of liver regeneration (ALR) were analyzed in the remnant liver tissue. We investigated the levels of angiogenic factors, VEGF-receptor, angiopoietin-1 (Angpt1) and Angpt2. Biochemical parameters of liver function in blood samples were measured and a histologic assessment of the livers was performed. The postoperative liver weight and volume of each rat were measured 14 days after surgery.
Results The expression levels of all measured growth factors were significantly increased in groups 3 and 4 compared to the control groups. The levels of Angpt1 and Angpt2 correlated with levels of VEGF and thus were also significantly higher in the study groups. There were significant differences between the estimated liver weights and volumes of group 4 and the resected controls in group 2. With the exception of portal inflammation, levels of all histological parameters were observed to be higher in MSC-treated groups when compared with the resected controls in group 2.
Conclusions Transplanted stem cells and MSCs transfected with VEGF significantly accelerated many parameters of the healing process following major hepatic resection. After the injection of MSCs and VEGF-transfected MSCs into the portal vein following liver resection, they were engrafted in the liver. They increased bile duct and liver hepatocyte proliferation, and secreted many growth factors including HGF, TGFβ, VEGF, PDGF, EGF, and FGF via paracrine effects. These effects support liver function, regeneration, and liver volume/weight.

Keywords Liver regeneration · Mesenchymal stem cells · Growth factors · Vascular endothelial growth factor

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Introduction

Major hepatic resection for the removal of lesions in the liver may be necessary for a wide variety of conditions. However, major hepatectomy is sometimes complicated by hepatic failure [1, 2]. A healthy liver may tolerate a resection of up to 70–80 % of its volume; however, this outcome cannot be taken for granted in extended hepatic resections. Liver failure can occur primarily as the result of an inadequate liver remnant, but it is more often precipitated by massive bleeding or septic complications [2]. Orthotopic liver transplantation remains the last resort for the treatment of acute and chronic liver failure [2–5]. Besides, transplantation is not attainable for many urgent patients because of a worldwide shortage of donor organs. Furthermore, long-term survival following transplantation can be impeded by rejection, recurrence of the original disease, and inevitable adverse effects of life-long immune suppression. Therefore, it is rational to develop alternative approaches for the treatment of liver failure. Hepatocyte transplantation and bioartificial livers are prospective methods for treating liver failure and providing temporary metabolic support of liver function [5]. In recent years, stem cell-based therapies have gained importance as a supportive therapy for this condition.

The regenerative capacity of liver is typically triggered by hepatic injuries, including partial hepatectomy or hepatocyte loss caused by viral or chemical injuries [6, 7]. The regenerative response after partial hepatectomy is mediated by a number of factors [8]. Non-parenchymal cells (Kupffer cells and sinusoidal endothelial cells) have stimulatory and inhibitory influences on hepatocyte replication after hepatectomy via paracrine effects [6]. Stem cells are involved in liver hemostasis and tissue repair after injury. Severe and persistent injuries result in transdifferentiation (epithelial-to-mesenchymal transition) or engraftment of stem cells within the liver as a final attempt to restore liver hemostasis [5]. Mesenchymal stem cells (MSCs) are self-renewing progenitors of numerous body tissues and are classified according to their origin and ability to differentiate. MSCs are functionally responsible for the development and regeneration of several tissues and organs, including the gastrointestinal tract and liver [9, 10]. MSC-based therapy presents a promising approach for regenerative medicine and tissue engineering [11, 12].

Vascular endothelial growth factor (VEGF) plays a crucial role in wound healing by increasing microvessel permeability, promoting endothelial cell growth, and facilitating endothelial cell migration through the extracellular matrix [13–16]. The aim of stem cell and growth factor therapies is to replace or facilitate the repair of damaged liver tissue. One of the most important prognostic indicators of patient survival in the case of liver failure is the regeneration period. Thus, the primary question is "how can we accelerate the regeneration period to increase patient's survival?" This study was aimed to elucidate the effects of stem cells and VEGF therapy on liver recovery and mechanisms through which these therapies work. Additionally, to determine the facilitating factors of stem cells and VEGF, which are transmitted by stem cells, during liver repair.

Materials and methods

Animals

This study was undertaken in the Institute of Experimental Medicine at Kocaeli University. The study protocol was approved by the Institutional Animal Care and Use Committee at Kocaeli University. Animal housing and experiments were approved by the local animal care committee in accordance with the institutional guidelines and national animal welfare act. The animals were housed under standard conditions (20–22 °C with 12 h light/dark cycles) before the experiments.

Isolation and culture of rBM-MSCs

A femur from each rat was excised, and the bone cavity was flushed with basal medium (LDMEM; Gibco/Life Technologies, Baisley, UK) supplemented with 10 % fetal bovine serum (FBS) (Gibco/Life Technologies) and 0.2 % primocin (Invivogen, San Diego, CA, USA) using a 21-gauge needle. The marrow plug suspension was dispersed using a pipette, filtered through a 70-μm mesh nylon filter (BD Biosciences, Bedford, MA, USA), and centrifuged at 200 g for 10 min. The supernatant containing thrombocytes and erythrocytes was discarded, and the cell pellet was resuspended in medium. The cells from each rat were seeded separately on two 25-cm^2 plastic tissue culture flasks (Falcon, BD Biosciences, San Jose, CA, USA) and incubated under standard conditions (37 °C, 5 % CO_2) for 2–3 days. Adherent cells that grew up to 70 % confluency were defined as passage zero (P0) cells. The standard culture medium was replaced every 3 days.

Flow cytometry

Undifferentiated rBM-MSCs (P3) were subjected to flow cytometry analysis (FACSCalibur; BD Biosciences). Immunostaining was performed against the following markers: CD29, CD45, CD54, CD90, CD106, MHC Class-I, and MHC Class-II. In addition, rBM-MSCs/GFP, rBM-MSCs/GFP-VEGF, and rBM-MSCs/VEG were analyzed with flow cytometry for the characterization of undifferentiated rBM-MSCs. All antibodies were supplied from BD Biosciences. Staining of more than 20 % was considered positive.

In vitro differentiation

To induce adipogenic differentiation, cells were seeded into 6-well plates (P3; 3000 cells/cm^2) and cultured with Mesencult MSC Basal Medium (StemCell Technologies, Vancouver, BC, Canada) supplemented with 10 % adipogenic supplement (StemCell Technologies) and 1 % penicillin/streptomycin for 3 weeks. The medium was refreshed every 3–4 days. The formation of intracellular lipid droplets, which indicates adipogenic differentiation, was confirmed by staining with 0.5 % Oil Red O (Sigma-Aldrich, St. Louis, MO, USA).

For osteogenic differentiation, cells (P3; 3000 cells/cm^2) were seeded on type I collagen-precoated cover slips in 6-well plates. The differentiation medium (LDMEM supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 0.05 μM ascorbate-2-phosphate (Wako Chemicals, Richmond, VA, USA), 10 mM β-glycerophosphate (Sigma-Aldrich), 2 % primocin (Invivogen), and 10 % FBS) was replaced twice a week. After 4 weeks, osteogenic differentiation was determined using Alizarin Red S staining (2 %, pH 4.2; Sigma-Aldrich). For Alizarin Red S staining, cells were fixed for 5 min in ice-cold 70 % ethanol. The stained cells were dehydrated in pure acetone, fixed in acetone-xylene (1:1) solution, and cleared with xylene.

GFP labeling

The GFP plasmid (pGFP-N1, GeneBank Acc. No. U19279) was provided by Clontech (Palo Alto, CA, USA). The MSCs were transfected using the Neon Transfection System (Invitrogen/Life Sciences) as per the manufacturer's suggestions. The transfection parameters were adjusted to 990 V, 40 ms, and 2 pulses. The stable cell lines were maintained by continuous culture of transfected cells in culture media containing G418 (Gibco/Life Sciences; 200 μg/mL).

Transfection with VEGF gene

The $VEGF_{165}$ genes were obtained from the Department of Genetics, Institute of Experimental Medicine, Istanbul University. The genes were ligated from the downstream of the CMV promoter in the pGFP-N1 vector following excision of the GFP gene via restriction digestion. The constructed plasmid was named as pVEGF. After isolating DNA with an EndoFree plasmid isolation maxi kit (Qiagen, Hilden, Germany), a plasmid mixture of pVEGF/pGFP (10:1) was transfected using electroporation (Neon Transfection System) as per the manufacturer's instructions. The transfection parameters were 990 V, 40 ms, and 2 pulses. After 48 h of culture in LDMEM supplemented with 10 % FBS, the transformed cells were selected according to their resistance against 200 μg/mL G418 under standard culture conditions. The efficacy of GFP/VEGF transfection was 92.09 ± 5.88 %, which was determined using FL1 fluorescence (525 nm green) (Suppl. Data 6).

The generated cells were analyzed for the expression of VEGF and other cell-specific markers before and after transfection (Fms-related tyrosine kinase 4/ VEGF receptor 3 (FLT4), von Willebrand factor (vWF), CD34, Neurogenin3 (Ngn3), Angiopoietin-1 (Angpt1)) using immunostaining.

Immunostaining

Cells on cover slips were rinsed briefly in phosphate-buffered saline (PBS) and fixed in ice-cold methanol for 10 min. After permeabilization with 0.025 % Triton X-100 (Merck, Darmstadt, Germany), cells were incubated with 1.5 % blocking serum (Santa Cruz Biotechnology, Heidelberg, Germany) in PBS for 30 min at 37 °C. After washing three times with PBS, the cells were incubated overnight at 4 °C with the primary antibodies listed in Table 1. After three PBS washes, the cells were incubated with secondary antibodies for 25 min. After washing, the cells were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology).

Transferase dUTP nick end-labeling (TUNEL) staining was performed to reveal apoptotic cells in tissues using an In Situ Apoptosis Fluorescein Detection Kit (S7111). Tissue sections were deparaffinized and proteinase K digestion was performed. After the addition of Tween-20, the paraffin sections were incubated for 30 min. The sections were subsequently incubated in terminal deoxynucleotidyl transferase (TdT) reaction buffer for 10 min. Following incubation of the TdT reaction mixture, the sections were incubated for a further 1–2 h at 37 °C in a humidified chamber. The rinsed sections were then placed in stop wash buffer for 10 min. Finally, counterstaining was performed using DAPI.

The cells and tissue sections were analyzed using staining against growth factors (hepatocyte growth factor, HGF; VEGF), proinflammatory and anti-inflammatory cytokines (interleukin(IL) 6, IL-1β, IL1receptor antagonist (ra), interferon gamma (IFNγ), transforming growth factor (TGFβ1), tumor necrosis factor (TNFα), macrophage inflammatory protein-2 (MIP2), myeloperoxidase (MPO), and prostaglandin E2 receptor subtype EP 3). All antibodies used in this assay were supplied by Santa Cruz Biotechnology.

Study design

MSCs were isolated from the bone marrow (rBM) of rats ($n = 4$) and labeled with GFP. The animals were randomly selected for major hepatectomy. Thirty-eight male Wistar Albino rats weighing 250–300 g were divided into four groups. The animals were housed at 21 °C, given tap water and standard rat food ad libitum.

Table 1 Measurements of growth factor concentrations in liver tissues

	Group 1 ($n=8$)	Group 2 ($n=9$)	Group 3 ($n=9$)	Group 4 ($n=9$)	p value$^{1-2}$	p value$^{1-3}$	p value$^{1-4}$	p value$^{2-3}$	p value$^{2-4}$
FGF (ng/mL)	8.4 (10–18)	7.5 (6–8.5)	15.4 (14.5–16)	16.5 (14.5–19)	NS	$p=0.001$	$p=0.001$	$p<0.001$	$p<0.001$
PDGF (pg/mL)	17 (13–22)	17.6. (13–26)	72 (56–85)	83.4 (77–88)	NS	$p=0.001$	$p=0.001$	$p<0.001$	$p<0.001$
EGF (pg/mL)	22.5 (20–25)	25.5 (22–30)	60.9 (46–68)	71.4 (65–77)	NS	$p=0.001$	$p=0.001$	$p<0.001$	$p<0.001$
TGF-β (μg/mL)	2.2 (2–2.4)	2.4 (2.1–2.7)	5 (3.8–5.9)	6 (5.8–6.1)	NS	$p=0.001$	$p<0.001$	$p<0.001$	$p<0.001$
HGF (ng/mL)	21.9 (20–24)	27.4 (18–52)	89.3 (80–99)	98 (90–114)	NS	$p=0.001$	$p=0.001$	$p<0.001$	$p<0.001$

p^{1-2}, p^{1-3}, p^{1-4}, p^{2-3}, and p^{2-4} values following comparison between groups. All values are provided as minimum, maximum, and median

FGF fibroblast growth factor, *PDGF* platelet-derived growth factor, *EGF* epidermal growth factor, *TGF-β* transforming growth factor beta, *HGF* hepatocyte growth factor, *NS* non-significant

Group 1 ($n=8$): control; sham operation
Group 2 ($n=10$): control; 70 % hepatic resection
Group 3 ($n=10$): 70 % hepatic resection + systemically transplanted MSCs
Group 4 ($n=10$): 70 % hepatic resection + systemically transplanted MSCs transfected with the VEGF gene

Surgical procedure and cell transplantation

After one night of fasting, the animals were anesthetized through intramuscular injection of ketamine hydrochloride (50–100 mg per kg of body weight). Abdominal access was achieved through a 4-cm long midline incision. The sham-operated controls (group 1) had their abdominal cavity opened and their liver manipulated without any resection. Hepatectomies were performed in groups 2, 3, and 4 as described previously [17, 18]. These groups underwent laparotomy and liver resection (70 %) in the median and left lateral lobes. After the surgical procedure, MSCs (0.5 mL, 1.0×10^6 cells) were immediately transplanted to group 3 and MSCs expressing the transfected VEGF gene (0.5 mL, 1.0×10^6 cells) were immediately transplanted to group 4, each via slow injection (30 s) into the portal vein. Hemostasis was achieved; 2 mL of 0.9 % NaCl was injected intraperitoneally, and the abdomen was closed with 3/0 continuous silk sutures. Water was given 12 h later and food was given after 24 h.

All of the animals were sacrificed on the 14th day after surgery. The livers were removed from the rats in all groups, weighed and measured. Liver biopsies were obtained, and 3–4 mL of venous blood was sampled for analysis.

Evaluation of tissue and biochemical parameters

The liver tissue was analyzed for the expression levels of growth factors, including vascular endothelial growth factor, fibroblast growth factor, platelet derived-growth factor, epidermal growth factor, transforming growth factor beta, hepatocyte growth factor, VEGF receptor (VEGFR), angiopoietin-1 (Angpt1), and Angpt2, using sandwich enzyme-linked immunosorbent assays (ELISAs). We also analyzed expressions of the hepatotropic factor augmenter of liver regeneration (hepatopoietin (HPO)) and the caspase-3 system in the tissue of the liver remnant. Rat immunoassay kits were used for analyses in accordance with the manufacturer's instructions.

Biochemical parameters of the liver, such as alanine transaminase (ALT), aspartate transaminase (AST), total protein, albumin, total bilirubin, direct bilirubin, and alkaline phosphatase (ALP) levels, were measured in the rats' venous blood samples.

Histopathological analysis of tissue sections

To perform histological and immunohistochemical assessments, the rat liver was excised, cleared of surrounding mesentery and fat, and washed with saline. Samples were obtained from the same liver lobe (specifically, the part that was regrowing) in all animals. The tissues were fixed in 10 % formalin for approximately 24 h and then embedded in paraffin. Transverse sections (3 μm thick) from the embedded tissue were stained with hematoxylin and eosin, and a histological assessment was performed by an experienced pathologist who was blinded to the identity of the groups. The presence of a double nucleus in hepatocytes, portal inflammation, and bile duct proliferation were investigated and scored from 0 to 3 (0 = none, 1 = slight, 2 = moderate, and 3 = dense).

Liver volume and weight

The postoperative wet liver weight and volume of each rat was measured 14 days after surgery. The excised liver was immediately weighed with sensitive scales. Displacement measurements were used to determine liver volumes; tissue was immersed in a measuring tube that was filled with isotonic fluid and had an opening on the side. The opening was positioned next to a glass measuring tube, which caught the fluid displaced by the volume of the tissue. Volume measurements were recorded accordingly.

Statistical analysis

Normality of data was evaluated by the Shapiro–Wilk test and histogram. Data are expressed as median, minimum, and maximum. Independent variables (for no normal) were compared between groups by Kruskal–Wallis one-way variance analysis. Post hoc comparison was performed by using the Mann–Whitney *U* test with Bonferroni correction (the threshold for statistical significance was accepted as $p < 0.0083$). The results were considered statistically significant when $p < 0.05$ (two-tailed). Data were analyzed using the software package SPSS for Windows release 21.

Results

Analyses of stem cells before transplantation

Following isolation of the mesenchymal stem cells, the cells were characterized and shown to demonstrate MSC properties (Fig. 1a, b, c). MSCs attached to the culture flasks sparsely displayed a fibroblast-like, spindle-shaped morphology during the initial days of incubation. After 3–4 days of incubation, proliferation started and the cells gradually grew into small colonies. In later passages, the MSCs exhibited a large, flattened, or fibroblast-like morphology that did not change for 25 passages. rBM-MSCs expressed CD29, CD54, and CD90, but not CD45 or CD106, and they maintained their phenotype in subsequent passages (Fig. 1d). rBM-MSCs/GFP, rBM-MSCs/GFP-VEGF, and rBM-MSCs/VEGF transfection samples were also analyzed using flow cytometry; there were no significant differences between the samples (Suppl. Data 7). The differentiation capacity of the cells was determined by chemically inducing the cells to differentiate into chondrogenic, osteogenic, and adipogenic cell lines. The positive staining of the differentiation markers for these lineages is shown in Fig. 1e–g.

Expression of the VEGF gene in the MSCs was shown through immunostaining for VEGF after transfection (Fig. 2). Expression of VEGF in untransfected rBM-MSCs was shown using immunostaining and real-time polymerase chain reaction (RT-PCR) (Suppl. Data 1 and 5). The high expression of VEGF in transfected cells was demonstrated with RT-PCR (Suppl. Data 5). Expression of exogenous VEGF in these cells triggered the expression of other endothelial cell markers, including FLT4, vWF, and CD34. Expressions of these endothelial markers along with Angpt1 were indicated using immunostaining before transfection (Suppl. Data 1). Although these cells expressed endothelial cell markers, they did not gain endothelial cell morphology and their cell culture requirements did not change. Expression of Angpt1 was increased in VEGF+ rBM-MSCs. Expression of Angpt1 by these cells in liver tissue might be involved in the vascularization and regeneration of the tissue. Angpt1 worked synergistically with VEGF during the neovascularization process, which is required for efficient regeneration (Fig. 2). Expression of neurogenin 3 (Ngn3), an essential transcription factor for the determination of endocrine cell precursors, was observed in VEGF+ cells. However, expression of Ngn3 was not observed in the untransfected rBM-MSCs (Suppl. Data 1). Although Ngn3 expression was not sufficient to induce differentiation of the cells into endocrine precursor cells, it was one of the indicators that the cells preserved the potential to differentiate into endocrine cells (Fig. 2).

The cells growing in culture were analyzed for the expression of cytokines which are important for hepatocyte regeneration. The exogenous expression of VEGF supported the endogenous expression of other cytokines that might play a role in the regeneration process. One of the most important of these factors is hepatocyte growth factor (HGF) (Fig. 3). The cells expressed elevated levels of HGF; however, they did not express other cytokines that control cell apoptosis and inflammation, such as IFN-g, TGF-β1, IL-1ra, and IL-6 (Fig. 3). These immunostaining processes were also performed before transfection (Suppl. Data 2). During transfection, the GFP gene was also delivered and its expression was stable and sustained along with the VEGF expression. Expression of GFP in the VEGF+ rBM-MSCs provided the opportunity to track cells in the tissue after transplantation (Fig. 3).

Animals

All of the groups were sacrificed on the 14th day after surgery. There were three fatalities in our study; one rat in each group which underwent resections died because of blood loss and liver failure.

Growth factor levels and apoptosis in the liver tissue

Levels of growth factors FGF, PDGF, EGF, TGF-β, and HGF were determined in the remnant liver tissue, the results of which are shown in Table 1. The expression of all growth factors were significantly increased in groups 3 and 4 ($p < 0.001$). Levels of EGF, PDGF, and TGF-β were most significantly increased in group 4.

In our study, we analyzed the role of VEGF-transfected MSCs by quantifying changes in VEGF and VEGFR levels in the liver tissue on the 14th day after liver resection. In addition, Angpt1 and Angpt2 levels were determined in the liver tissue, because there is a close relationship between VEGF and the angiopoietin system. VEGF levels were increased sevenfold in group 3 and approximately ninefold in group 4 when compared with group 1. The increases in VEGF and VEGFR levels in group 3 and group 4 showed much greater statistical significance than in the control groups ($p < 0.001$). The

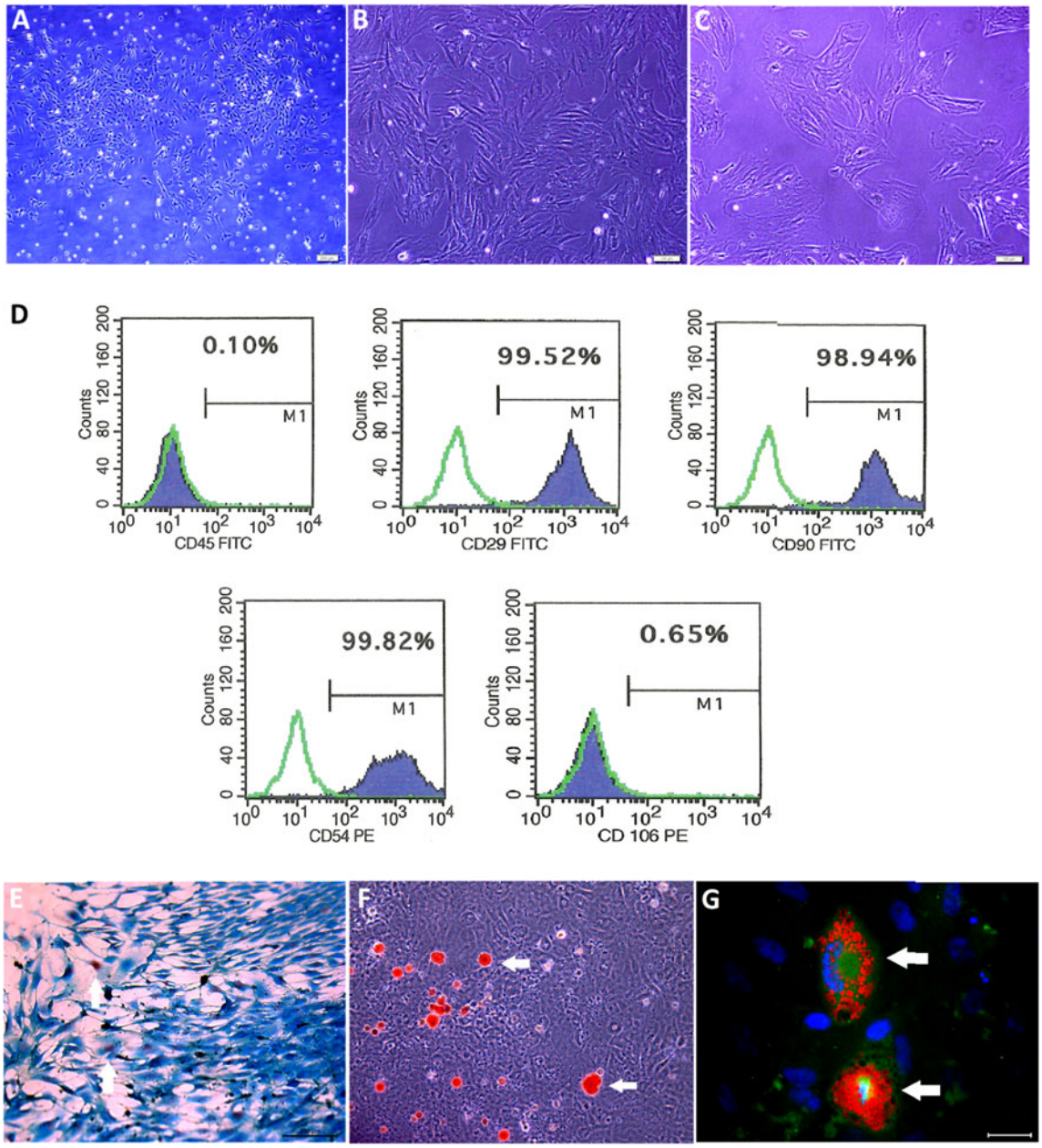

Fig. 1 Characterization of rBM-MSCs. During the onset of growth (**a** P0—6th day), MSCs showed diverse morphologies including ovoid, bipolar, and large, flattened morphology. In later passages, most of these MSCs exhibited large, flattened, or fibroblast-like morphology (**b** P2—4th day; **c** P3—2nd day). Representative flow cytometry analysis of cell-surface markers at P3 (**d**). Predefined markers that specify MSCs used to define the characteristics of cultured cells. rBM-MSCs expressed all mesenchymal stem cell markers including CD29, CD54, and CD90, but not CD45, or CD106. Differentiation of rBM-MSCs into chondrogenic (**e**), osteogenic (**f**), and adipogenic (**g**) cell lines were analyzed. After chondrogenic differentiation, the analyzed sections were positive for Alcian blue staining (**e**) (scale bar 50 μm). Osteogenic differentiation of rBM-MSCs (day 8) (**f**) after osteogenic induction. Mineral nodules were stained positive (*arrows*) with Alizarin Red S staining (original magnification ×100). Adipogenic differentiation was identified by neutral lipid vacuoles formation (stained with oil *red*, *arrows*) (*green*: Actin) in cultures (**g**) (scale bar 50 μm)

levels of Angpt1 and Angpt2, which were related to the VEGF level, were also significantly higher in the study groups. TUNEL staining was performed to determine the effects of MSCs on apoptosis. The result of TUNEL staining was significantly lower in the study groups than in the control group (Suppl. Data 3). Apoptotic cells were counted in all groups and compared with those of group 2 (Suppl. Data 4). Statistical analysis of TUNEL staining was calculated as $p < 0.001$ according to Student's t test. The ALR levels in the resected groups, groups 2, 3, and 4, were low and remained below those of group 1 ($p < 0.001$). All values are shown in Table 2.

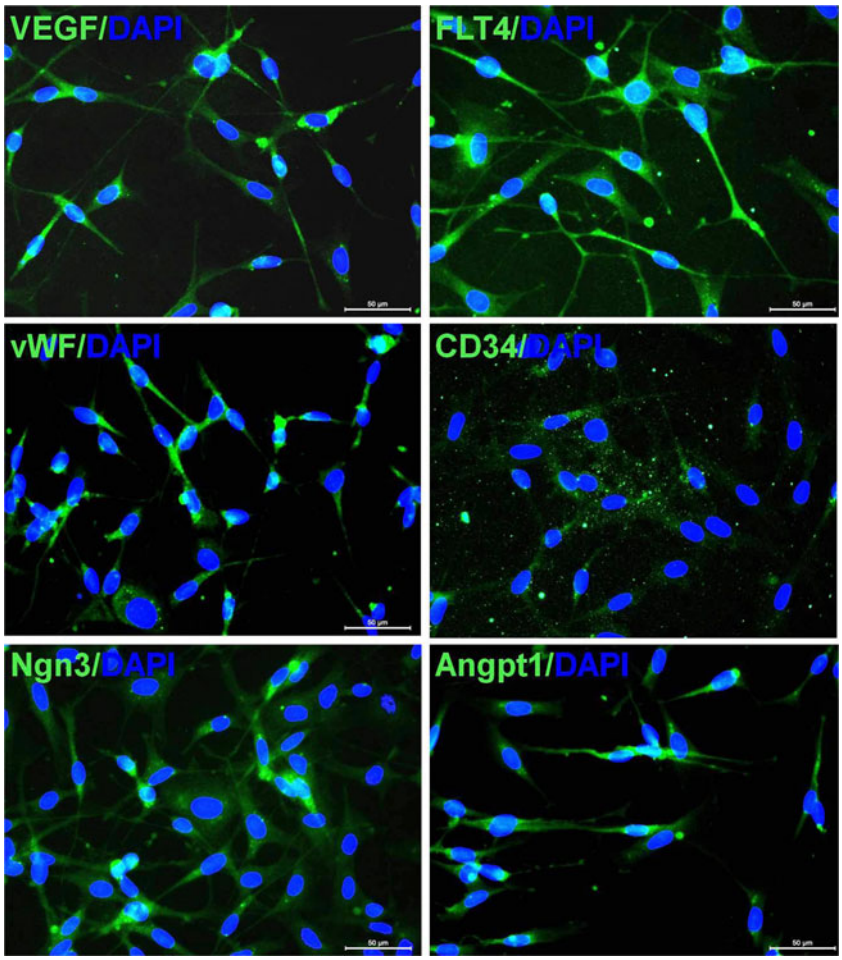

Fig. 2 After transfection of rBM-MSCs with pVEGF, VEGF expression was not the only protein observed; these cells also demonstrated expression of endothelial cell markers, such as FLT4, von Willebrand factor (vWF), and CD34. Expression of angiopoietin-1 (Angpt1) was shown in VEGF+ rBM-MSCs. Expression of Angpt1 by these cells might be involved in vascularization and support regeneration. Expression of neurogenin 3 (Ngn3), an essential transcription factor for determination of endocrine cell precursors, was observed in VEGF+ cells. The nucleus of cells was stained with DAPI (*blue*) (scale bars 50 μm)

Liver function status

Liver function parameters were measured. We observed that levels of ALT, AST, ALP, total protein, and albumin returned to normal in the study groups, whereas levels of total bilirubin and direct bilirubin were higher than normal. Levels of AST, ALT, total bilirubin, and direct bilirubin in groups 3 and 4 were significantly lower than in group 2. All biochemical values are shown in Table 3.

Histologic features

We investigated the regenerative period of the livers on the 14th day after resection. With the exception of portal inflammation, we found higher levels for all histological parameters in the resected groups compared with those in control group 2 (Fig. 4). Portal inflammation was higher in the study groups than in control group 1 and was significantly lower in the study groups than it was in the resected control, group 2. This phenomenon was likely to be the result of the suppressive effect of stem cells on inflammation. All histological features are shown in Table 4.

Liver volume and weight

We observed that liver volume in the resected study groups did not return to normal. The best result compared with the resected control group 2 was found in group 4 ($p < 0.001$). There was a significant difference in the estimated liver weight/initial body weight and the regenerated liver weight/final body weight of the animals. The rats' liver weights, assuming 100 % for group 1, reached 66, 85, and 92 % for groups 2, 3, and 4, respectively. There was a significant increase in group 4 when compared with the resected control group 2 ($p < 0.001$). Liver volumes and weight measurements are shown in Table 5.

Analysis of tissue sections for stem cell infiltration and their effect

Tissues were subsequently screened for the presence of GFP+ MSCs using immunohistochemistry, focusing on the homing and differentiation of these cells (Fig. 5). The regeneration of the liver tissue after hepatectomy was observed to be in parallel with the number of GFP+ cells. After hepatectomy, the expression of HGF was not significantly increased in the

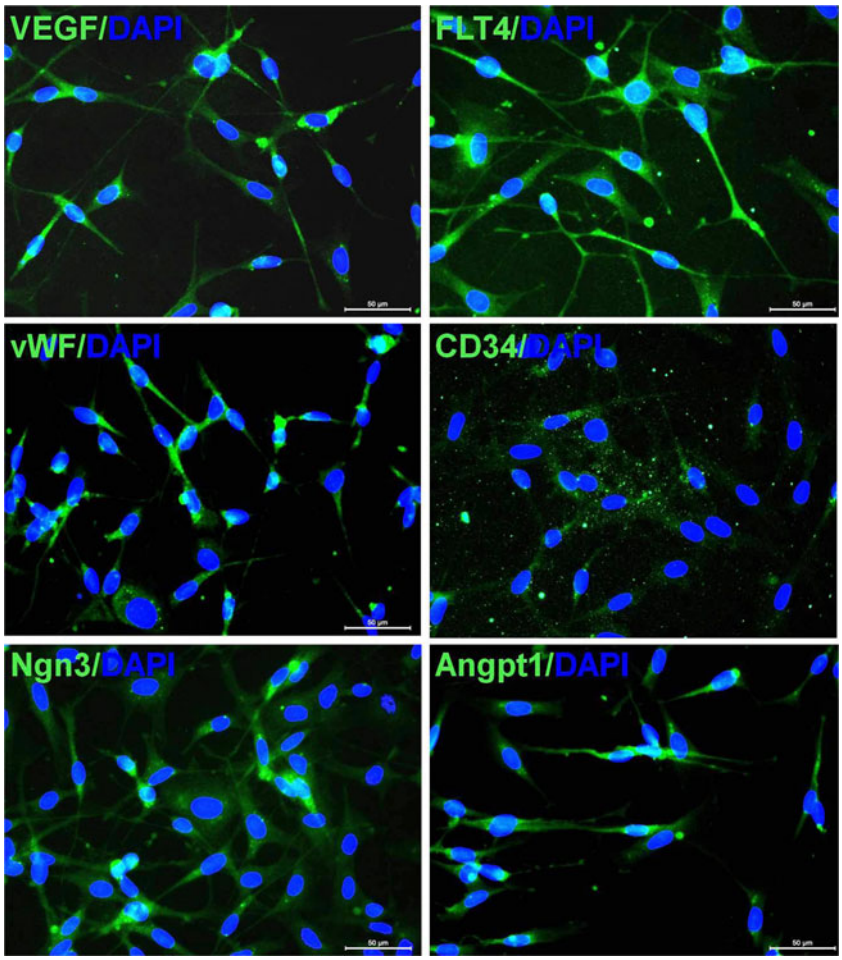

Fig. 3 After transfection of rBM-MSCs with pVEGF, VEGF expression was not the only protein observed; these cells also demonstrated the expression of endothelial cell markers, such as FLT4, von Willebrand factor (vWF) and CD34. The expression of angiopoietin-1 (Angpt1) was shown to express in VEGF+ rBM-MSCs. The expression of Angpt1 by these cells in tissue might involve in the vascularization and support the regeneration. The expression of neurogenin 3 (Ngn3), a transcription factor that is essential for determination of endocrine cell precursors, was observed in the VEGF+ cells. The nucleus of cells was stained with DAPI (*blue*) (scale bars 50 μm)

control and sham groups, in which there were no transplanted cells. The GFP-labeled BM-MSCs expressing HGF could be observed in damaged tissue, which indicated that the regeneration process had been promoted. Interestingly, higher HGF expression was detected in the VEGF+ BM-MSC-transplanted tissues compared with the BM-MSC injected group (Fig. 5).

The expression of anti-inflammatory proteins EP3 and IL1ra were increased in all tissues, but most significantly in the VEGF+ BM-MSC-transplanted group (Fig. 6a, b). The expression of these proteins might decrease inflammation and apoptosis and promote the regeneration process. The increment of EP3 and IL1ra levels was statistically significant compared with the sham and control groups, but there was no clear difference between the VEGF-transfected and untransfected cells (Fig. 6b).

The expression levels of other cytokines and inflammation-related proteins were modified in the hepatectomized group in favor of regeneration after VEGF+ BM-MSC injection (Fig. 7a, b). In this case, the difference between the stem cell-transplanted and untransplanted groups was also significant, but not within the stem cell-transplanted groups. The only exceptions were the TGFβ and IL6 levels in the VEGF+ BM-MSC group. These cytokines may protect against apoptosis in tissue, and TGFβ might also be involved in the regulation of stem cell differentiation into hepatocytes. The number of GFP+ cells in the VEGF-transfected group was slightly higher when compared with the non-transfected stem cell group, which were expected to support the regeneration process, but the level of proinflammatory (IL1β, IFNγ, TNFα, MPO) cells were also suppressed.

Discussion

After major resection or liver damage, a regenerative process is triggered, and replication of the remaining healthy hepatocytes ensues in an attempt to restore hepatic structure and function. This process is initiated or regulated, at least partially, by three major factors: cytokines, growth factors, and metabolic signaling pathways. Natural repair mechanism of the liver is dependent on endogenous pools of cells, including hepatocytes, hepatic progenitor cells/oval cells, and bone marrow stromal stem cells. If hepatocyte replication is hampered by excessive parenchymal damage or hepatocyte senescence, resident liver progenitor cells are activated to support or take

Table 2 Measurements of VEGF, VEGFR, angiopoietin 1–2 (Angpt1, Angpt2), caspase3 system, and ALR factor concentrations in liver tissues

	Group 1 ($n=8$)	Group 2 ($n=9$)	Group 3 ($n=9$)	Group 4 ($n=9$)	p value$^{1-2}$	p value$^{1-3}$	p value$^{1-4}$	p value$^{2-3}$	p value$^{2-4}$
VEGF (pg/mL)	12.4 (10–18)	14.8 (7–25)	84.6 (65–100)	114.3 (96–190)	NS	$p=0.001$	$p=0.001$	$p<0.001$	$p<0.001$
VEGFR (pg/mL)	4.4 (4–4.8)	4.3 (4–5)	12.1 (11–13.6)	17.6 (15.5–21.5)	NS	$p=0.001$	$p=0.001$	$p<0.001$	$p<0.001$
Angpt1 (pg/mL)	81.9 (75–90)	83.5 (79–89)	177.4 (145–265)	272.7 (180–400)	NS	$p=0.001$	$p=0.001$	$p<0.001$	$p<0.001$
Angpt2 (pg/mL)	43.9 (40–48)	42.6 (40–45)	184.2 (100–255)	257.1 (188–305)	NS	$p=0.001$	$p=0.001$	$p<0.001$	$p<0.001$
Caspase 3 (ng/mL)	15.7 (12.4–18.3)	17.2 (11.7–23.9)	3.4 (2.5–4)	2.3 (2–2.7)	NS	$p=0.001$	$p=0.001$	$p<0.001$	$p<0.001$
ALR (HPO) (pg/mL)	7.5 (7–8)	5.5 (4.9–6.1)	5.7 (5.5–6)	6.1 (5.5–8.1)	$p<0.001$	$p<0.001$	$p<0.001$	NS	NS

p^{1-2}, p^{1-3}, p^{1-4}, p^{2-3}, and p^{2-4} values following comparison between groups. All values are provided as minimum, maximum, and median

VEGF vascular endothelial growth factor, *VEGFR* vascular endothelial growth factor receptor, *ALR* augmenter liver regeneration, *NS* non-significant

Table 3 Liver biochemical tests in plasma

	Group 1	Group 2	Group 3	Group 4	p value$^{1-2}$	p value$^{1-3}$	p value$^{1-4}$	p value$^{2-3}$	p value$^{2-4}$
AST (IU/L)	23 (21.1–30.1)	58 (45.8–.66.4)	24.6 (21.8–29)	23.9 (21–26.7)	$p=0.001$	NS	NS	$p=0.001$	$p<0.001$
ALT (IU/L)	23.8 (21.4–28.1)	55.6 (44.3–63.3)	23.2 (21–27.8)	22.2 (18.7–25.5)	$p=0.001$	NS	NS	$p=0.001$	$p<0.001$
ALP (IU/L)	79.6 (66.4–121.4)	103.4 (98–112.4)	102.7 (95.7–121)	106.5 (99–123.5)	NS	NS	NS	NS	NS
Total Protein (g/dL)	5.6 (4.3–6.8)	5.5 (4.6–7.9)	5 (4.5–5.7)	5.2 (4.5–5.8)	NS	NS	NS	NS	NS
Albumin (g/dL)	3.2 (2.9–3.4)	3.1 (2.8–3.4)	2.9 (2.6–3.3)	3 (2.7–3.4)	NS	NS	NS	NS	NS
Total bilirubin (mg/dL)	0.7 (0.4–1.2)	11 (10–11.9)	7.8 (7.3–8.3)	7.4 (6–11.4)	$p=0.001$	$p=0.001$	$p=0.001$	$p=0.001$	$p=0.003$
Direct bilirubin (mg/dL)	0.4 (0.1–0.8)	7.3 (6.1–8)	5.2 (4.2–5.9)	4.6 (3.2–7.4)	$p=0.001$	$p<0.001$	$p=0.001$	$p=0.001$	$p=0.002$

p^{1-2}, p^{1-3}, p^{1-4}, p^{2-3}, and p^{2-4} values following comparison between groups. All values are provided as minimum, maximum, and median

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *NS* non-significant

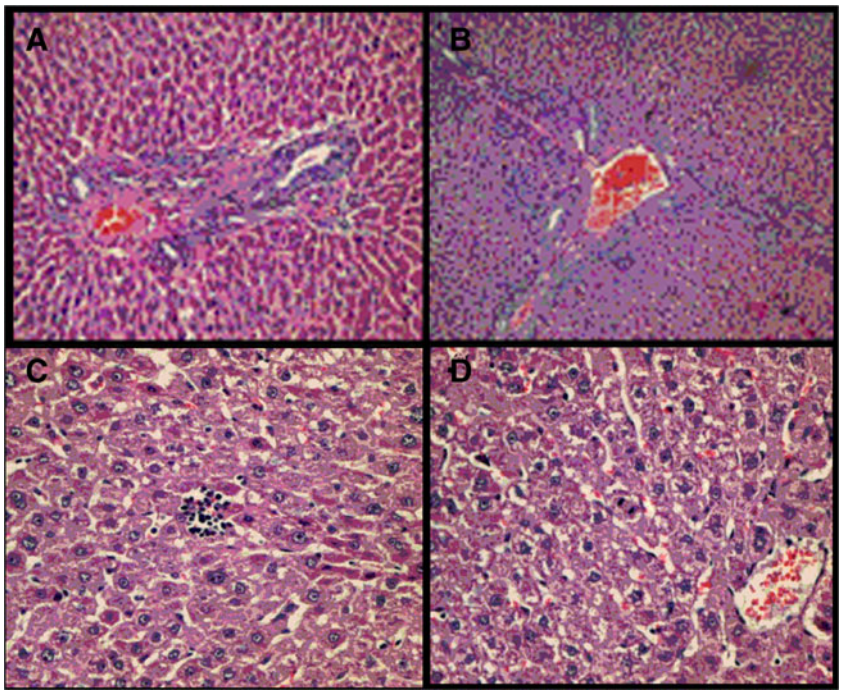

Fig. 4 **a** Mild proliferation in bile duct (HEX200), **b** middle proliferation in bile duct (HEX200), **c** extramedullary hematopoiesis zones in group 4 (HEX400), and **d** mitotic activity in hepatocytes (HEX400)

over the role of regeneration. However, this regenerative process can be inadequate at matching the rapid, concurrent loss of hepatocyte mass and function, and in these cases, liver transplantation offers the only potential hope for survival [19–21]. In the literature, many studies have addressed transplanted systemic MSC therapies for healing liver damage and injury, but MSCs + VEGF have not been investigated as a treatment following major hepatic resection. The current literature devotes limited space to the effect of stem cell therapies on growth factors and cytokines, which play a crucial role in liver regeneration. Our study was aimed to address these questions. The present study showed that MSCs engrafted safely and successfully after portal injection during major hepatic resection; labeled MSCs were found within the liver remnant. Moreover, we showed that the transplanted stem cells significantly accelerated many parameters of the healing process after major hepatic resection.

Experiments have shown that a number of growth factors such as FGF, EGF, HGF, and TGF contribute to the proliferation and differentiation of hepatocytes [1, 22–24]. Recent studies have shown that VEGF greatly contributes to the proliferation of liver sinusoidal endothelial cells (SECs) via up-regulation of VEGFR during liver injury [6, 15]. SECs compose a structurally and functionally unique capillary network that vascularizes specific organs including bone marrow and liver [25]. Treatment of liver injury with exogenous MSCs is thought to affect liver regeneration through the direct differentiation of MSCs into hepatocytes, as well as through the delivery of growth factors that promote liver regeneration and cell fusion [26, 27]. After MSCs home to sites of damaged tissue for repairing process, they interact closely with local stimuli, such as inflammatory cytokines, ligands of toll-like receptors, and hypoxic conditions, which stimulate MSCs to produce a large amount of growth factors that perform multiple functions for tissue regeneration [28–30]. In this study, we investigated the levels of various growth factors in remnant liver tissue. In doing so, we had two purposes: first, to evaluate the effect of exogenous MSCs on growth factors and second, to determine if any additional effects of overexpression of VEGF by MSCs might have on liver regeneration and levels

Table 4 Histologic features characterizing each group of rat livers (all parameters were scored from 0 to 3)

Histologic features	Group 1	Group 2	Group 3	Group 4	p value$^{1\times3}$	p value$^{2\times3}$	p value$^{1\times4}$	p value$^{2\times4}$
Double nucleus in hepatocytes	0	0.4	1.4	1.6	$p=0.001$	$p=0.001$	$p=0.001$	$p=0.001$
Portal inflammation	0.1	1.5	0.6	0.6	NS	$p=0.011$	NS	$p=0.011$
Bile duct proliferation	0	0.1	0.9	2	$p=0.001$	$p=0.001$	$p=0.001$	$p=0.001$
Apoptosis	0	0.1	1.3	2.2	$p=0.001$	$p=0.001$	$p=0.001$	$p=0.001$

p^{1-3}, $p^{2\times3}$, $p^{1\times4}$, and p^{2-4} values following comparison between groups. All values are provided as median

NS: non-significant

Table 5 Liver weight and volume for each group (postoperative 14th day)

Group 1	Group 2	Group 3	Group 4	p value$^{1\times3}$	p value$^{2\times3}$	p value$^{1\times4}$	p value$^{2\times4}$
Liver wet weight (gr)							
6.5 (6–7)	4.3 (4–4.6)	5.5 (4–8)	6.1 (5–8)	NS	NS	NS	$p=0.001$
Liver volume (cc)							
3.5 (3.2–3.8)	2.2 (1.7–2.6)	2.7 (2.3–3.2)	2.9 (2.5–3.4)	$p=0.001$	$p=0.006$	$p=0.002$	$p=0.001$

p^{1-3}, $p^{2\times3}$, $p^{1\times4}$, and p^{2-4} values following comparison between groups. All values are provided as minimum, maximum, and median

NS non-significant

of other growth factors. We found that the levels of all growth factors we examined, including FGF, VEGF, HGF, EGF, PDGF, and TGF-β, were increased in the liver remnant after major hepatectomy in groups 3 and 4 ($p<0.001$). In addition, we showed that levels of VEGF receptor, Angpt1, and Angpt2 increased in the remnant liver in groups 3 and 4 ($p<0.001$). We suggest that vascular regeneration mechanisms occur through stem cells and VEGF-transfected stem cells. Stem cells increased VEGF levels, and this in turn increased Angpt1 and Angpt2 levels by increments of VEGFR and resulted in vascular regeneration. We observed the largest positive effect in group 4. The VEGF and PDGF families are considered to constitute a VEGF–PDGF superfamily [31]. Moreover, VEGFRs are structurally related to the PDGF receptor family [32]. The VEGF and PDGF families stimulate cellular responses by binding to tyrosine kinase receptors on the cell surface [33, 34]. We hypothesized that this similarity and the use of the same receptor system facilitated VEGF's effect on PDGF levels; it is also possible that the effect of expressing VEGF on the VEGFR facilitated the effect on PDGF because they use the same pathway. TGF-β and interleukin-6 contribute to the proliferation and differentiation of hepatocytes. Interleukin-6 secreted by Kupffer cells is one of the vital factors for liver regeneration [19, 22, 24]. The interactions

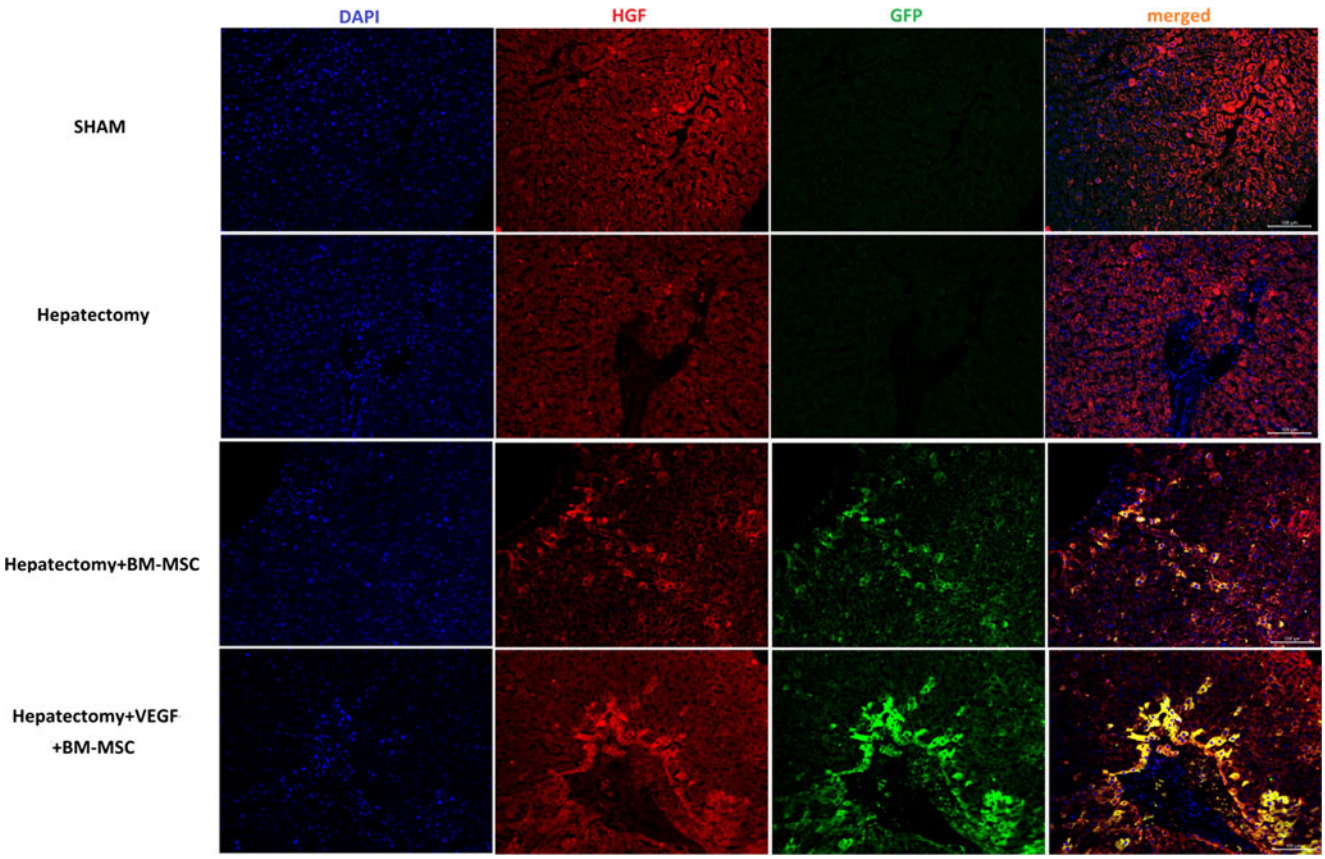

Fig. 5 Regeneration of liver tissue after hepatectomy. Expression of HGF in cells after hepatectomy was not significantly increased in the group without cell transplantation. GFP-labeled BM-MSCs expressing HGF could be observed in damaged tissue. VEGF+ BM-MSCs were also integrated in tissue with significantly high HGF expression (scale bars 100 μm)

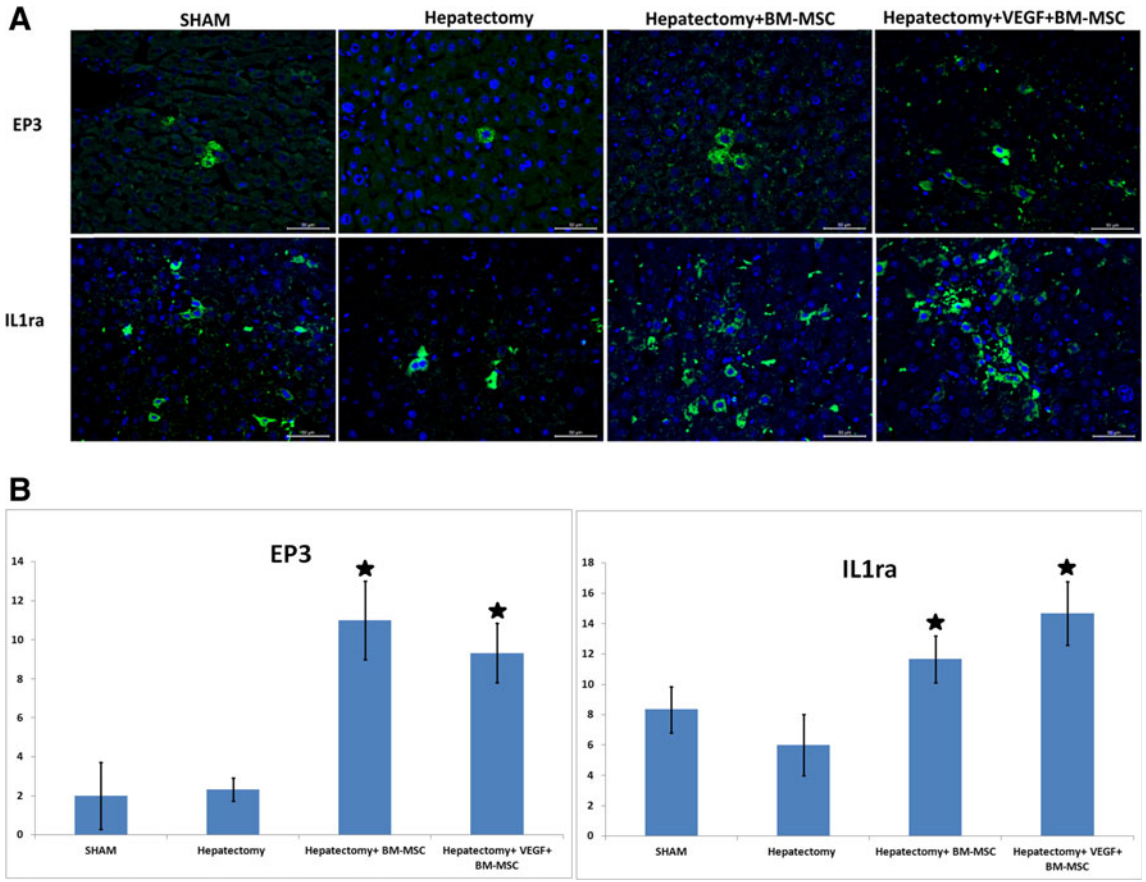

Fig. 6 **a** Expression of anti-inflammatory cytokines (EP3, IL-1ra) in liver tissues. The tissue samples in paraffin sections were stained against EP3 and IL1ra and then compared. Both EP3 and IL1ra were expressed strongly in the tissue treated with BM-MSCs, but these expressions in tissue were more significantly expressed in the group with VEGF+ BM-MSCs (scale bars 50 μm). **b** The number of cells expressing EP3 and Il1ra proteins was significantly increased after stem cell application compared with the sham or hepatectomy group without cell treatment (*$p < 0.05$)

between growth factors and signaling pathways are complex and not completely understood. It has been observed that stem cells express IL-6, IL-1β, and TGF-β. The ability of stem cells to facilitate regeneration could be explained by the use of these pathways and the induction of growth factors by stem cells. The increased level of TGF-β in our study should be considered; the highest increase was detected in group4, followed by group 3. We observed that both stem cells and the VEGF-transfected stem cells expressed TGF-β. TGF-β is a known suppressor of HGF; its expression increases early after partial resection, and stays elevated until the end of regeneration. However, the strongest evidence for the role of TGF-β in regeneration regulation comes from another observation. We believe that the level of TGF-β in the study groups increased to balance the effect of the stem cells and the VEGF-transfected stem cells on proliferation and mitosis. The highest proliferation was observed in group 4; thus, the high level of HGF in group 4 could be explained by this balance [35].

Hepatopoietin, now commonly known as augmenter of liver regeneration factor, promotes liver regeneration following partial hepatectomy [36]. The immediate release of ALR from rat liver following partial hepatectomy suggests that the released ALR may stimulate non-parenchymal cells to produce mediators of hepatic regeneration [37, 38]. In our study, there was no increase in ALR in the groups treated with stem cells. The level of ALR in group 4, which was treated with VEGF-transfected stem cells, was higher compared with the resected control group 2, but this difference was not statistically significant. This result suggests that treatment with stem cells has no effect on ALR levels. Another potential explanation for this result is that ALR levels may have increased early in the process of liver regeneration and then returned to baseline levels. Therefore, this temporary increase may not have been detected because we did not examine the early liver regeneration period in our study.

When the liver was morphologically analyzed, we observed that none of the resected groups reached the beginning weight. Assuming 100 % for group 1, groups 2, 3, and 4 reached 66, 85, and 92 % of the normal liver weight, respectively. When we measured liver volume, we observed that the resected groups did not reach the normal liver volume either. Compared with group 1, groups 2, 3, and 4 reached 61, 76,

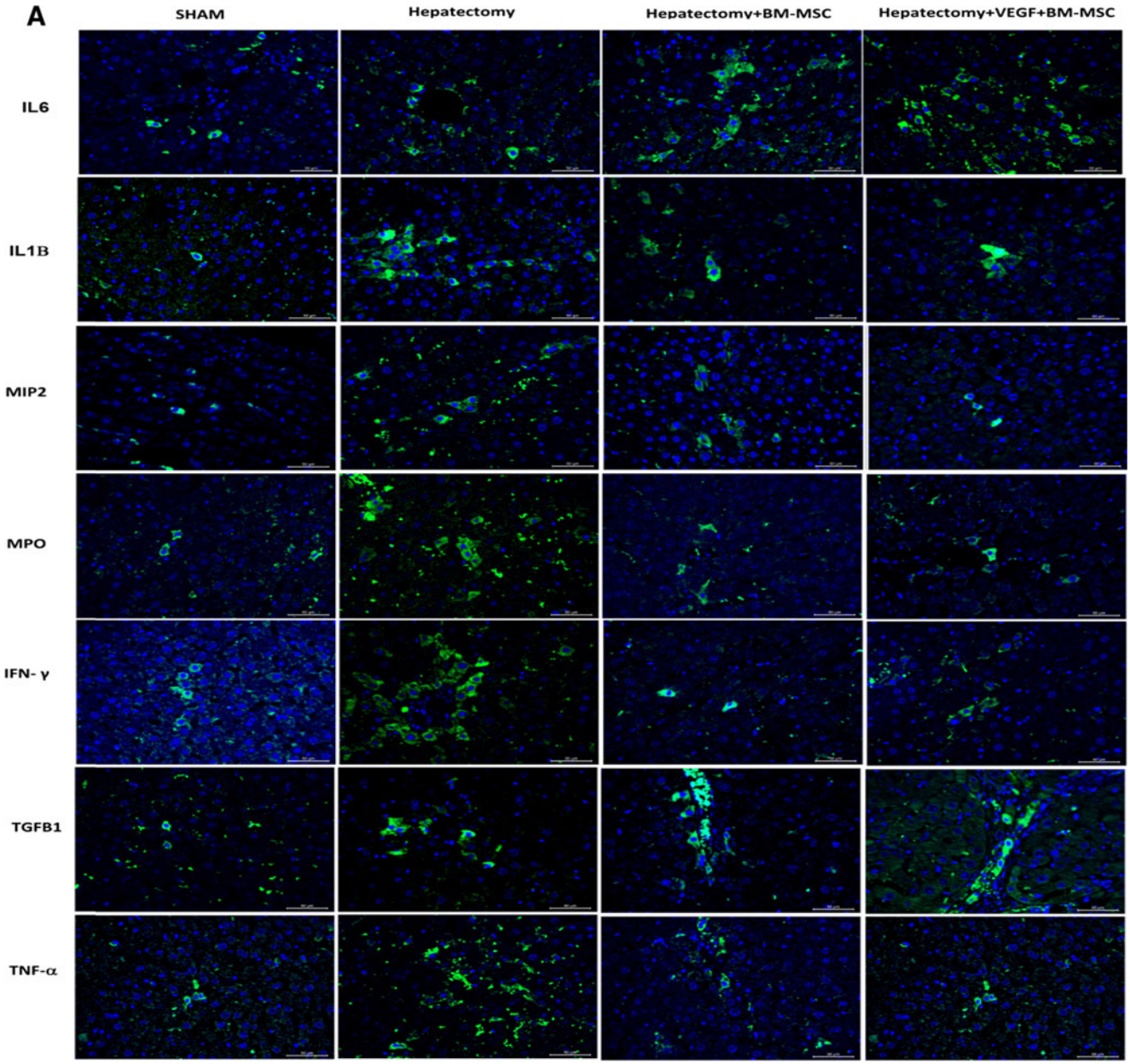

Fig. 7 **a** The expression of proteins associated with inflammation in the control and MSC-transplanted liver paraffin sections. Stem cell application greatly suppressed the expression of the inflammatory markers (IL-1β, IL6, MIP-2, MPO, IFNγ, and TNFα) in the tissue. VEGF+ BM-MSCs performed better at decreasing inflammation (scale bars 50 μm). **b** Quantification of cells expressing proteins associated with inflammation. After stem cell treatment, cells expressing proinflammatory proteins decreased in correlation with GFP-expressing cells. Only IL-6 and TGF-β1 positive cells were increased in the tissue. These cytokines might protect against apoptosis (*$p < 0.05$; **$p < 0.01$)

and 82 % of normal liver volume, respectively. The best result was observed in group 4; compared with the weight and volume of resected control group 2, both measurements were significantly higher in group 4. Group 3 had a significant increase only in liver volume compared with group 2. Previous studies have found that after a 70 % hepatectomy in rats, liver volume was 93 % between 7 and 14 days, and that by day 20, the livers had completely recovered their original volume through hyperplasia of their remaining lobes [8, 17]. Therefore, our result was slightly below the expected values for liver regeneration in group2 (61 %) at 14 days. However, the new finding in our study was the superior positive effect of stem cells, which was observed in group 4 (with an 82 % positive effect). It is clear that stem cells and VEGF-transfected stem cells increased the volume and weight of the liver of rats.

Histological observation of the livers showed that MSCs ameliorated the pathologic changes associated with 70 % hepatectomy. We observed that the presence of a double nucleus in hepatocytes increased in favor of groups 3 and 4. Portal

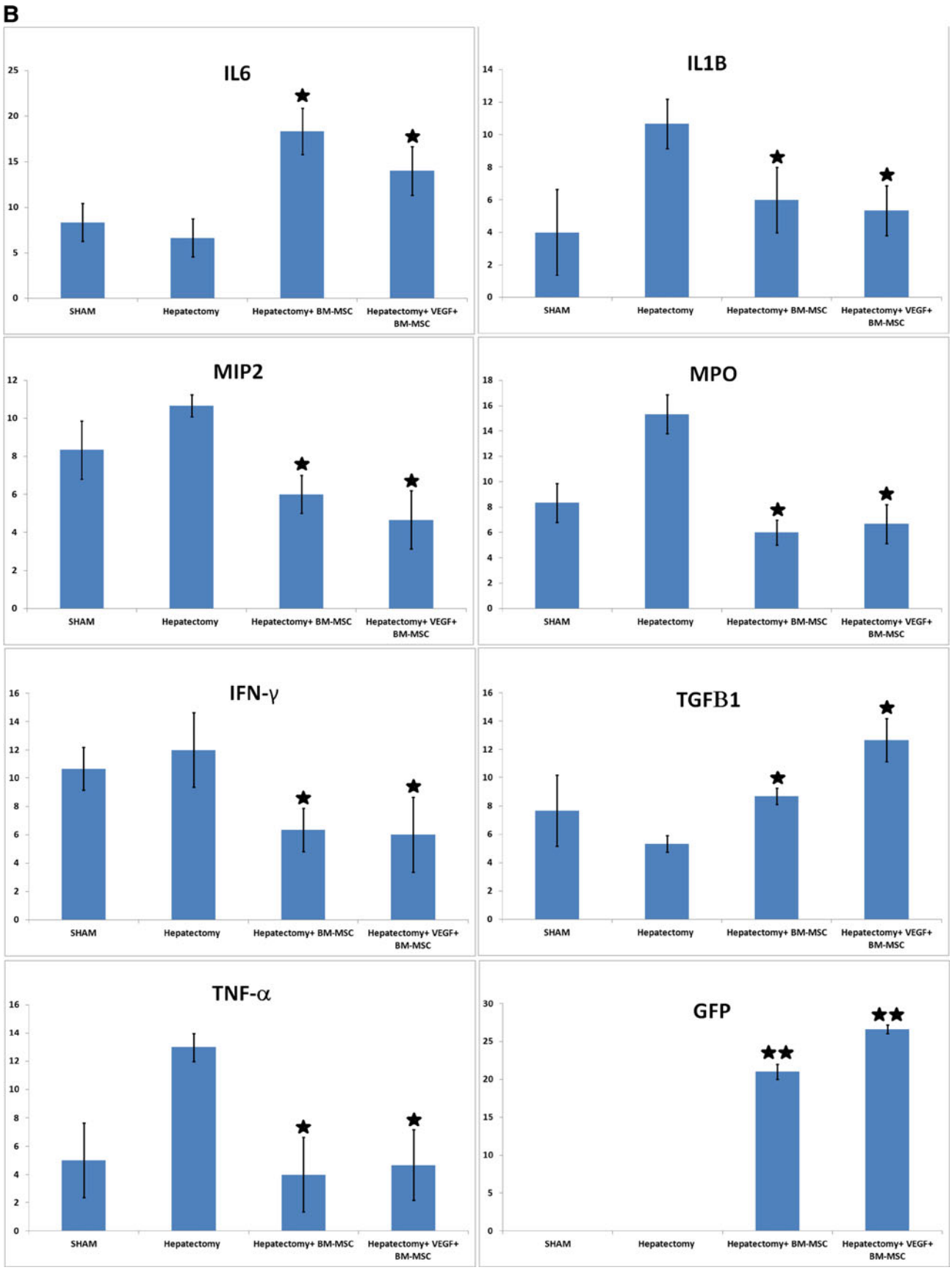

Fig. 7 continued.

inflammation decreased in groups treated with stem cells compared with resected control group 2. We assumed that stem cells decreased inflammation because of their ability to modulate the immune system. Related to this effect, MIP-2 expression decreased in the stem cell-treated groups. The proliferation of bile ducts, which occupies an important role in liver regeneration, was significantly higher in groups 3 and 4. The antiapoptotic effects of MSCs have been observed in liver injury models [39, 40]. In our study, transplanted MSCs decreased apoptosis. In addition to histopathological data, we observed that levels of caspase were lower in the liver tissue of groups 3 and 4. Furthermore, BCL3 expression was higher in the study groups. Taken together, these results indicate that apoptosis decreased in the stem cell-transplanted groups. Compared with the resected control group 2, apoptosis was fivefold lower in group 3 and sevenfold lower in group 4. There was also a significant difference in bile duct proliferation and apoptosis between groups 3 and 4. This result suggests that VEGF-transfected stem cells were more active. Taken together, these data demonstrate that stem cells affect bile duct proliferation and apoptosis during liver regeneration.

Transplantation of MSCs improves liver function of rodents undergoing acute liver damage [41, 42]. Improved liver function was shown by monitoring levels of liver enzymes such as AST and ALT in serum [43]. Studies have shown that transplantation of MSCs can restore liver function and ameliorate symptoms of liver damage [42]. The levels of liver function enzymes in groups 3 and 4 were significantly lower than in resected control group 2. When we examined other markers of liver function (total protein, ALP), we observed that there were normal levels in the stem cell-transplanted groups and the control groups. However, stem cells had a positive effect on bilirubin levels, which was significantly in favor of group 4 when compared with group 2.

In conclusion, MSCs play a specific role in the liver and have the potential to be used in tissue engineering, regenerative medicine applications, and treatment of acute and chronic liver diseases. MSCs aid in the repair of injured liver and can improve liver function. Treatment of major liver resection with exogenous MSCs and MSCs transfected with VEGF can affect liver regeneration through inducing the direct differentiation of progenitors into hepatocytes, as well as through the delivery of growth factors that improve liver regeneration. Combining stem cell therapy with VEGF therapy had a synergistic effect on liver regeneration. Additional large, prospective, randomized clinical studies are needed to achieve a greater understanding of the long-term benefits and risks of the therapeutic use of MSCs in clinical settings.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

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