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Research Paper

Construction of hepatocyte growth factor gene recombinant adenovirus vector and its expression in rat bone marrow mesenchymal stem cells☆

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Abstract

Objective: To construct the adenoviral expression vector system containing human hepatocyte growth factor (hHGF) cDNA, and to further study the transduction efficiency and the expression of HGF in mesenchymal stem cells (MSCs). **Methods:**The HGF cDNA was amplificated from the expression plasmid pCMV-HGF, and was subcloned into the adenovirus shuttle plasmid pDC316-IRES-EGFP vector containing a green fluorescence protein (GFP) reporter gene. Virus Ad-HGF was produced by homologous recombination in HEK293 package cells. Bone marrow derived MSCs were harvested and cultured, and then were transduced with Ad-HGF. The efficiency of Ad-HGF transduction was assessed by FACS analysis using GFP gene expression. And HGF/MSCs were generated. The HGF concentrations in supernatants of HGF/MSCs were determined by ELISA using anti-human HGF monoclonal antibody. **Results:** The recombinant, named pDC316-HGF-IRES-eGFP, was digested with restriction enzyme, and the DNA sequencing of HGF was identical to the report in Genebank and did not reveal any mutation. GFP expression could be observed on the second day after packing of the linearized pAd-HGF in HEK293 cells and 7.15×10^{10}pfu/ml titer of Ad-HGF was obtained. Forty-eight hours after transduction, 96.89% of HGF/MSCs were GFP positive. Peak concentration levels of hHGF(103ng/mL) in the cultured supernatants were detected on day 2 post-transduction, and the adenovirus-mediated expression of HGF by MSCs was maintained for at least 2 weeks *in vivo*. **Conclusion:**Our data demonstrated that the adenovirus expression vector system pDC316-HGF-IRES-EGFP has been constructed successfully, and their effective expressions also have been obtained in MSCs. This will provide material basis for the next study on liver regeneration after small-for-size liver transplantation.

Key words: liver transplantation; mesenchymal stem cells; hepatocyte growth factor; small-for-size graft; regeneration

INTRODUCTION

Living donor liver transplantation has been paid increasing attention because it substantially expands the availability of potential donor livers. However, the survival rate in adult recipients undergoing such transplantations is significantly lower than in children. Size mismatch of graft and recipient remains a major risk factor contributing to impaired post-operative graft function. Transient portal hypertension in small-for-size grafts and ischemia-reperfusion(I/R) associated with small-for-size liver transplantation impairs liver graft regeneration. So small-for-size liver grafts show delayed and impaired regeneration. Moreover, these grafts encounter several risks of failure after reperfusion, frequently leading to liver failure termed "small-for-size syndrome". In order to avoid this syndrome in living donor liver transplantation settings, immediate regeneration of the graft is essential to ensure hepatic mass recovery, and subsequent protective measures are required for maintaining liver function[1].

Stem cells responsible for self-repair and regeneration are found in various organs of the human body. Many

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recent reports have shown that MSCs have the capability to differentiate into hepatocyte-like cells[2-5], when exposed to specific conditions. There has also been much interest in their clinical potential for tissue repair and gene therapy, including the repair of the kidney[6], lung[7], and heart[8]. Stem cells have also been shown to be associated with liver regeneration after orthotopic liver transplantation[9]. In addition, bone marrow-derived MSCs have been a cell type of interest because of their accessibility and readiness for transfection with exogenous genes[10].

HGF has a potent cytoprotective action on hepatocyte, besides enhancing liver regeneration[11-14]. HGF also stimulates the migration and proliferation of activated hepatic stem cells into the liver parenchyma, where the cells differentiate into mature hepatocytes[15, 16]. And HGF plays a great role in inducing MSCs differentiating into hepatocytes *in vitro*.

Our research, by constructing MSCs with high expression of hHGF, aimes to provide a material basis for further *in vivo* exploration of application of HGF/MSCs in small-for-size liver transplantation of rats.

MATERIALS AND METHODS

Experimental Animals

Syngenic male Lewis rats; 8-12 weeks of age, weighing 250-340 g;(Vital River Experimental Animal Co, China) were used in all of the experiments to exclude immunologic interference. The animals were kept in the animal facilities at Nanjing Medical University, and the experiments were conducted in accordance with the guidelines approved by the China Association of Laboratory Animal Care.

Preparation of mesenchymal stem cells

Male Lewis rats were euthanized by CO_2 asphyxiation. Bone marrow MSCs were isolated by combining gradient density centrifugation with plastic adherence from both femurs and tibias, as previously described by Mizuguchi[17]. MSCs between the third and sixth passage were used in the experiments.

At least 2×10^5 MSCs were harvested and re-suspended in 0.1mL PBS containing 1% BSA (Sigma). The cell suspension was incubated with 0.2 μg FITC-or PE-conjugated primary antibody(1:100 dilution), mouse monoclonal anti-rat CD34(Santa Cruz Biotechnology, Inc,California, USA), anti-CD90, anti-11b and anti-CD29 (BioLegend, San Diego, USA), anti-CD45 and anti-CD54 (Catag Laboratories, Burlingame, USA) for 40 min at 4℃. The mouse IgG1 kappa antibody(Catag Laboratories, Burlingame, USA) was used as an isotype control. MSCs surface markers were analyzed by FACS (BD Calibur, USA).

Consruction of shuttle plasmid pDC316-HGF-IRES-EGFP

The HGF cDNA encoding the full-length hHGF gene was amplificated from the expression plasmid pCMV-HGF by PCR, using primers containing AgeI and NheI restriction enzyme sequence(HGF-AgeI-f:5′-TAAACTATACCGGTATGTGGGTGACCAAACTC-3′ and HGF-NheI-r:5′-TAAACTATGCTAGCCTATGACTGTGGTACCTT-3′, then digested with AgeI and NheI, and subcloned into the same site of the shuttle plasmid pDC316-IRES-eGFP vector(Vector Gene Technology Co., China), under the transcriptional control of the cytomegalovirus(CMV) immediate early enhancer/promoter. The recombinant shuttle plasmid was named pDC316-HGF-IRES-eGFP,and identificated with double digestion by restriction enzyme(AgeI+NheI) and sequencing.

Recombinant adenovirus production

Subsequently, we produced virus Ad-HGF by homologous recombination in human embryonic kidney cells, designated HEK293 cells. The recombinant shuttle plasmid was co-transfected with the skeleton plasmid pBHGlox_E1, 3Cre into HEK293 using AdMax packaging system. Recombinant adenoviruses were amplified and purified by two centrifugation steps on cesium chloride gradients. The number of viral particles was assessed by measurement of the optical density at 260 nm. Viruses were dialyzed against 10mM Tris HCl pH 8.0, 1mM $MgCl_2$, 10% glycerol and stored at −80℃ until use. Ad-GFP generated by the same procedure using the pDC316-IRES-eGFP vector was used for concurrent control.

Transfection of Ad-HGF to BM-MSCs

MSCs were harvested and inoculated into 6-well culture plates with 3×10^5 cells per well. After being cultured in incubator for 24 h, MSCs were infected with 100 μL Ad-HGF or Ad-HGF dissolved in 5mL medium, and cultured for 6h. After being added another 5mL fresh medium, they were cultured for another 24h. Seventy-two hours after transduction, they were passaged at the ratio of 1:3. In this way we generated HGF-producing MSCs, named HGF/MSCs. The GFP/MSCs were generated by the same procedure using the Ad-GFP.

Three groups were established in our experiment: group Ⅰ: no viruses for blank control; group Ⅱ: Ad-GFP vector for negative control; group Ⅲ: HGF/MSCs.

Measurement of transgenic efficiency

The efficiency of Ad-HGF transduction was assessed by FACS analysis using GFP gene expression.

Determination of hHGF level of cellular culture supernatant by ELISA

The cellular culture supernatants in each group were collected at day 0, 2, 4, 6, 8, 10, 12 and 14. The HGF concentrations in supernatants of HGF/MSCs were determined by ELISA using anti-human HGF monoclonal antibody.

Statistical analysis

All data were denoted as mean ±SD and performed with ANOVA test using SPSS 13.0. *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

Characteristics of MSCs

MSCs retained a fibroblastic morphology after repeated passages, and their identity was confirmed by FACS. Over 90% of the isolated MSCs expressed CD29, CD90, and CD54, but not CD34, CD11b/c and CD45 (***Fig 1***). These results were in accordance with well-established markers of bone marrow-derived MSCs[18-20]. These types of cells were stable and the homogeneity of the 1st and 2nd passages of amplified cells reached 95% and 98% respectively. Our results indicated that the isolated cells were of single type of MSCs, other than hematopoietic stem cell or fibroblast type.

Construction of Adenovirus vectors

After double digestion of pDC316-HGF-IRES-eGFP by AgeI＋NheI and agarose gel electrophoresis, a DNA segment of approximately 2.2 kb, the same in size as hHGF gene, was produced(***Fig 2A***), proving a successful cloning of target gene into shuttle plasmid pDC316-HGF-IRES-eGFP. PCR amplification of the virus specifically produced the bands with expected sizes, proving that the recombinant adenovirus contained the target segment(***Fig 2B***). Correctly identified plasmids were sent for sequencing and its HGF segment turned out to have a totally identical sequence with that of Genebank NM001010932 bases(***Fig 2C***).

Recombinant adenovirus production

Transfected with pDC316-HGF-IRES-eGFP, nearly all HEK293 cells expressed GFP protein(***Fig 3***).

Based on the fact that 1.0 A_{260nm}=1.1 × 10^{12} viral particles, the titer of purified virus was estimated to be 2.15 × 10^{12} particles/ml. A_{260nm}/A_{280nm} is an index for estimation of viral purity. When it is above 1.3, the viral purity is good. The value we measured was 1.35. The viral infection titer as examined by fast CPE was 7.15 × 10^{10}pfu/ml. Thus, the potency ratio(ratio between viral particles and viral infection titer) was 30.

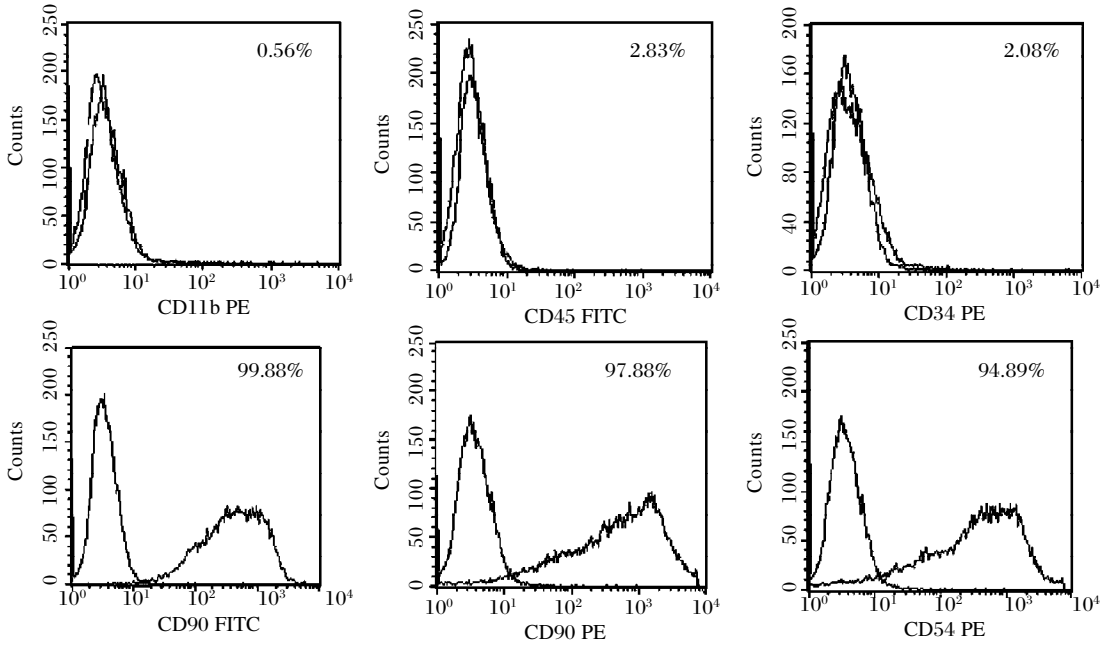
Transduction of Ad-HGF to MSCs

Transfection efficiency

Forty-eight hours after transduction, 96.89% of HGF/MSCs were GFP positive(***Fig 4***).

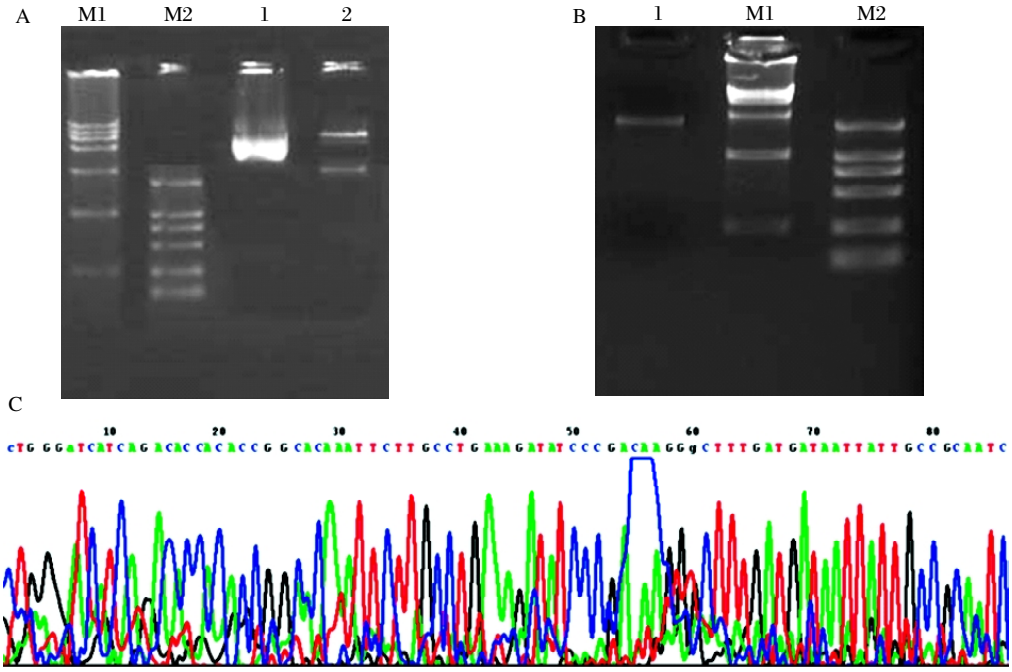
Measurement of hHGF level of cellular culture supernatant by ELISA

Peak concentration levels of hHGF(103 ng/mL) in the cultured supernatants were detected on day 2 post-transduction(***Fig 5A***). Adenovirus-mediated expression of HGF by MSC was maintained for at least 2 weeks *in vivo*(***Fig 5B***).



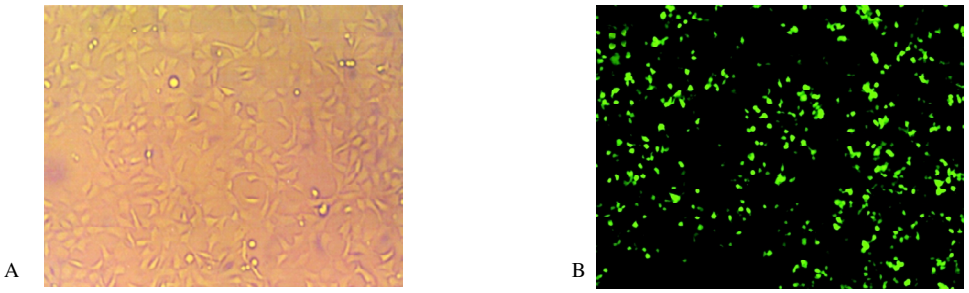
FACS analysis of rat MSCs. Numbers in the panels represented mean fluorescent intensity of the cells expressing each marker

Fig 1 Characterization of isolated MSCs



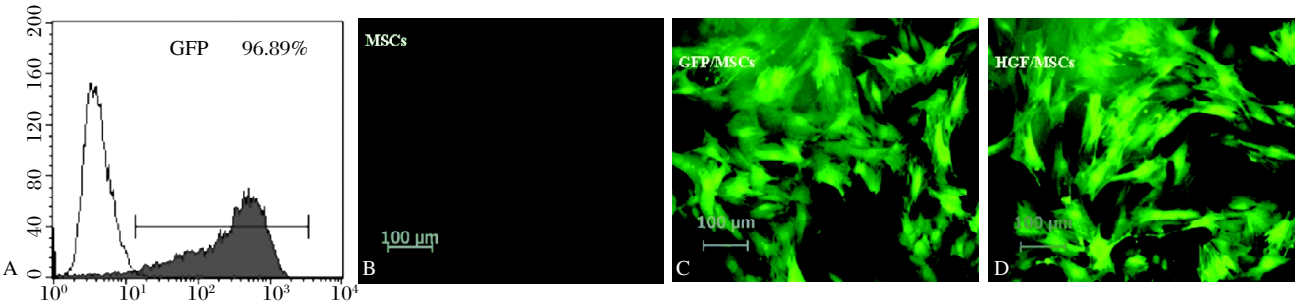
A:pDC316-HGF-IRES-eGFP was identificated with double digestion by AgeI + NheI restriction enzyme(M1:Marker DL15000; M2: Marker DL2000; 1:pDC316-HGF-IRES-eGFP; 2:pDC316-HGF-IRES-eGFP digested with AgeI+ NheI); B: the virus was identificated by PCR amplification(M1: Marker DL15000; M2:Marker DL2000; 1:HGF product); C:pDC316-HGF-IRES-eGFP was identificated with sequencing.

Fig 2 Construction and identification of pDC316-HGF-IRES-EGFP



A: HEK293 cells; B: HEK293 cells transfected with pDC316-HGF-IRES-eGFP

Fig 3 293 cells transfected with pDC316-HGF-IRES-eGFP

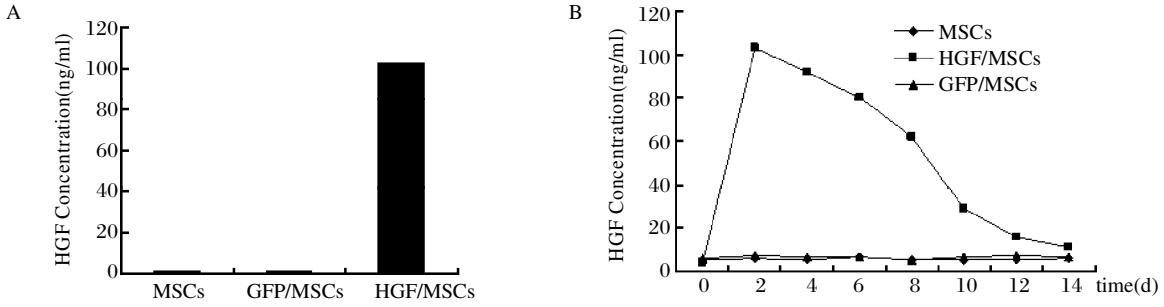


A: The transduction efficiency of MSCs was determined by flow cytometry using a GFP marker; B: MSCs; C: MSCs were transduced with Ad-GFP; D: MSCs were transduced with Ad-HGF.

Fig 4 MSCs transduced with Ad-HGF

DISCUSSION

Recent studies indicate that tissue damage after liver transplantation and liver resection caused increased serum levels of multiple cytokines, but found only I/R injuries associated with liver transplantation result in the mobilization of bone marrow stem/progenitor cells[10]. Additionally, studies[21] demonstrated that circulating bone marrow-derived stem cells migrated into the injured liver and contributed to liver regeneration. It has been suggested that stem cells might play a role in liver regen-



A: HGF concentrations in the supernatants of MSCs, GFP/MSCs, and HGF/MSCs; B:Adenovirus-mediated expression of HGF by MSCs over time.

Fig 5 Expression of hHGF by HGF/MSCs

eration after liver transplantation.

MSCs are of great interest in clinical application, due to their characteristics of having the potential to differentiate into hepatocytes and availability for patients and lack of rejection. Moreover, MSCs are suitable for transfection with exogenous genes. Thus, they can be expected to become a new method curing end-stage hepatic diseases.

HGF distributes widely in the human body and enjoys multiple biological features. It has a potent cytoprotective action on hepatocytes besides enhancing liver regeneration, and has been utilized for many experimental and clinical applications[22-24]. Upon binding to its receptor, c-met, HGF elicits multiple distinct actions on a number of target cells, and has been implicated in the control of expression of transcription factors related to progenitor pool maintenance, cell fate determination, cell growth and differentiation, and organ regeneration [25]. HGF influences the whole process of hepatic growth and regeneration[26]. Thus, we have chosen HGF as the key cytokine for our further study on the regeneration and protection of small-for-size liver transplant. Unfortunately, exogenous HGF is rapidly cleared by the liver from circulation and has a half-life of less than 15 minutes. So it is almost impossible to sustain a constant high level of exogenous HGF in circulation, even using repeated injections of HGF protein at short intervals. A logical way to overcome this problem is to develop a gene transfer strategy that would permit persistent expression of HGF protein *in vivo*.

The present study was designed to construct MSCs with high expression of hHGF, and aims to provide a material basis for further *in vivo* exploration of application of HGF/MSCs in a 30% small-for-size liver transplantation of rats.

Before this, the authors also constructed retrovirus expression vector plEGFP-HGF and recombinant plasmid vector pEGFP-HGF containing hHGF gene. But it is found in the research that the transfection of pEGFP-HGF is not efficient, and the hHGF contained in culture supernatant of plEGFP-HGF transduced MSCs is low in productivity. On the other hand, the adenovirus vector features high transfection efficiency and protein productivity.

Adenoviral vector is commonly used due to its easy preparation and its high-titer production of recombinant virus. AdMax packaging system enables stable and fast harvest of recombinant virus with high titer. After the transduction of adenovirus to host cells, the exogenous gene continues its expression for at least 2 weeks while not integrates into host cells genome, which is especially suitable for our next *in vivo* experiment.

In conclusion, we have achieved sustained, high levels of HGF gene expression in MSCs using adenovirus vectors. HGF/MSCs can be transplanted to models of liver transplantation or hepatic failure. HGF can be utilized to promote the regeneration of liver and protection of hepatocytes, and MSCs have the potential to differentiate into hepatocytes, taking effect to support and improve liver function. GFP is a bioluminescence reporting factor which is harmless to cells. It can be used for tracing and positioning within a human or animal body and for observing the settling, induction and differentiation of MSCs in organs. Thus, the HGF/MSCs provide great conveniences for further *in vivo* research.

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References

[1] Yu Y, Yao AH, Chen N, Pu LY, Fan Y, Lv L, *et al.* Mesenchymal Stem Cells Over-expressing Hepatocyte Growth Factor Improve Small-for-size Liver Grafts Regeneration. *Mol Ther* 2007;15:1382-9.

[2] Ong SY, Dai H, Leong KW. Inducing hepatic differentiation of human mesenchymal stem cells in pellet culture. *Biomaterials* 2006; 27:4087-97.

[3] Hong SH, Gang EJ, Jeong JA, Ahn C, Hwang SH, Yang IH, *et al.* In vitro differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocyte-like cells. *Biochem Biophys Res Commun* 2005;330:1153-61.

[4] Sato Y, Araki H, Kato J, Nakamura K, Kawano Y, Kobune M, *et al.* Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. *Blood* 2005;106:756-63.

[5] Lange C, Bruns H, Kluth D, Zander AR, Fiegel HC. Hepatocytic differentiation of mesenchymal stem cells in cocultures with fetal liver cells. *World J Gastroenterol* 2006;12:2394-7.

[6] Herrera MB, Bussolati B, Bruno S, Fonsato V, Romanazzi GM, Camussi G. Mesenchymal stem cells contribute to the renal repair of acute tubular epithelial injury. *Int J Mol Med* 2004;14:1035-41.

[7] Ortiz LA, Gambelli F, McBride C, Gaupp D, Baddoo M, Kaminski N, *et al.* Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A* 2003;100:8407-11.

[8] Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, *et al.* Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* 2003;9:1195-201.

[9] Lemoli RM, Catani L, Talarico S, Loggi E, Gramenzi A, Baccarani U, *et al.* Mobilization of Bone Marrow-Derived Hematopoietic and Endothelial Stem Cells after Orthotopic Liver Transplantation and Liver Resection. *Stem Cells* 2006;24:2817-25.

[10] Lee K, Majumdar MK, Buyaner D, Hendricks JK, Pittenger MF, Mosca JD. Human mesenchymal stem cells maintain transgene expression during expansion and differentiation. *Mol Ther* 2001;3: 857-66.

[11] Roos F, Terrell TG, Godowski PJ, Chamow SM, Schwall RH (1992). Reduction of alpha-naphthylisothiocyanate-induced hepatotoxicity by recombinant human hepatocyte growth factor. *Endocrinology*; 131: 2540-2544.

[12] Ishiki Y, Ohnishi H, Muto Y, Matsumoto K, Nakamura T. Direct evidence that hepatocyte growth factor is a hepatotrophic factor for liver regeneration and has a potent antihepatitis effect *in vivo*. *Hepatology* 1992;16:1227-35.

[13] Morita M, Watanabe Y, Akaike T. Protective effect of hepatocyte growth factor on interferon-gamma-induced cytotoxicity in mouse hepatocytes. *Hepatology* 1995;21:1585-93.

[14] Kosai K, Matsumoto K, Nagata S, Tsujimoto Y, Nakamura T. Abrogation of Fas-induced fulminant hepatic failure in mice by hepatocyte growth factor. *Biochem Biophys Res Commun* 1998;244: 683-90.

[15] Yao P, Zhan Y, Xu W, Li C, Yue P, Xu C, *et al.* Hepatocyte growth factor-induced proliferation of hepatic stem-like cells depends on activation of NF-kappaB. *J Hepatology* 2004;40:391-8.

[16] Nagy P, Bisgaard HC, Santoni-Rugiu E, Thorgeirsson SS. In vivo infusion of growth factors enhances the mitogenic response of rat hepatic ductal(oval) cells after administration of 2-acetylaminofluorene. *Hepatology* 1996;23:71-9.

[17] Mizuguchi T, Hui T, Palm K, Sugiyama N, Mitaka T, Demetriou AA, *et al.* Enhanced proliferation and differentiation of rat hepatocytes cultured with bone marrow stromal cells. *J Cell Physiol* 2001;189:106-19.

[18] Dezawa M, Kanno H, Hoshino M, Cho H, Matsumoto N, Itokazu Y, *et al.* Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J Clin Invest* 2004;113:1701-10.

[19] Wang PP, Wang JH, Yan ZP, Hu MY, Lau GK, Fan ST, *et al.* Expression of hepatocyte-like phenotypes in bone marrow stromal cells after HGF induction. *Biochem Biophys Res Commun* 2004;320: 712-6.

[20] Lee RH, Kim B, Choi I, Kim H, Choi H S, Suh K, *et al.* (2004). Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell Physiol Biochem*; 14: 311-324.

[21] Gehling UM, Willems M, Dandri M, Petersen J, Berna M, Thill M, *et al.* Partial hepatectomy induces mobilization of a unique population of haematopoietic progenitor cells in human healthy liver donors. *J Hepatol* 2005;43:845-53.

[22] Tashiro H, Fudaba Y, Itoh H, Mizunuma K, Ohdan H, Itamoto T, *et al.* Hepatocyte growth factor prevents chronic allograft dysfunction in liver-transplanted rats. *Transplantation* 2003;76:761-5.

[23] Fujiwara K, Nagoshi S, Ohno A, Hirata K, Ohta Y, Mochida S, *et al.* Stimulation of liver growth by exogenous human hepatocyte growth factor in normal and partially hepatectomized rats. *Hepatology* 1993; 18:1443-9.

[24] Ikegami T, Nishizaki T, Uchiyama H, Kakizoe S, Yanaga K, Sugimachi K. Deletion variant of hepatocyte growth factor prolongs allograft survival after liver transplantation in rats. *Surgery* 1999; 125:602-7.

[25] Liu Y, Centracchio JN, Lin L, Sun AM, Dworkin LD. Constitutive expression of HGF modulates renal epithelial cell phenotype and induces c-met and fibronectin expression. *Exp Cell Res* 1998;242: 174-85.

[26] Moriuchi A, Hirono S, Ido A, Ochiai T, Nakama T, Uto H, *et al.* Additive and inhibitory effects of simultaneous treatment with growth factors on DNA synthesis through MAPK pathway and G1 cyclins in rat hepatocytes. *Biochem Biophys Res Commun* 2001; 280:368-73.