

Hepatocyte Growth Factor is the Key Cytokine in Stimulating Potential Stem Cells in the Cord Blood into Hepatic Lineage Cells

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ABSTRACT

Background: This study was designed to investigate the role of the hepatocyte growth factor (HGF) with regards to differentiation of somatic stem cells originating from the human umbilical cord blood (UCB) into hepatic lineage cells *in vitro* culture system. **Methods:** Mononuclear cells from UCB were cultured with and without HGF based on the fibroblast growth factor (FGF)-1, FGF-2, and stem cell factor. The cultured cells were confirmed by immunofluorescent staining analysis with albumin (ALB), cyto-keratin-19 (CK-19), and proliferating cell nuclear antigen (PCNA) MoAb. ALB and CK-18 mRNA were also evaluated by reverse transcription-polymerase chain reaction. In order to observe changes in proliferating capacity with respect to the cultured period, CFSE with affinity to proliferating cells were tagged and later underwent flow cytometry. **Results:** In the HGF-treated group, cultured cells had a large oval shaped appearance with adherent, but easily detachable characteristics. In the HGF-non treated group, these cells were spindle-shaped with strong adherent characteristics. Expressions of ALB and CK-19 were evident in HGF-treated group compared to non-expression of those in to HGF-non treated group. Dual immunostaining analysis of the ALB producing cells showed presence of PCNA in their nuclei, and ALB and CK-18 mRNA were detected on the 21st day of cultured cells in the HGF-treated group. **Conclusion:** Our findings suggest that HGF has a pivotal role in differentiating somatic stem cells of human UCB into hepatic lineage cells *in vitro*. (*Immune Network* 2007;7(3):117-123)

Key Words: Hepatic growth factor, somatic stem cells, umbilical cord blood, differentiation, hepatic lineage cells

Introduction

The definite treatment for end stage liver disease is liver transplantation, although many patients are inaccessible to this option due to donor shortage. Liver transplantation has disadvantages in that even after a successful transplant; the patients need some form of immunosuppression and may suffer from graft re-

jection and infection. Recently, somatic stem cells have been introduced as a possible source for the many kinds of adult cells, including hepatocytes. If it becomes possible to use these stem cells as a source of transplantation to support liver function, it can be ground breaking hope for the patient with chronic liver disease (1).

Cells from various body parts have been suggested as the source of somatic stem cells. However, the bone marrow (BM) and umbilical cord blood (UCB) are the most plausible source for the supply of somatic stem cells (2,3). The BM contains various progenitor cells of somatic stem cells, including hematopoietic stem cells and mesenchymal stem cells. Hematopoietic stem

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cells are the precursor of all kinds of blood cells, and mesenchymal stem cells have the ability to differentiate into the mesodermal cells, such as osteocytes, adipocytes, neurons, brain cells and cardiac muscle cells (4,5). There are reports that BM contains the somatic stem cells that can differentiate into hepatocytes (1). We have reported that human UCB cells also contain somatic stem cells which can be differentiated into hepatic lineage cells like BM (3,6).

The precise mechanism of the differentiation process of somatic stem cells has not yet been fully characterized. Many investigators believe that the microenvironment surrounding the somatic stem cells affect the direction of differentiation to specific organic tissues (7,8). The microenvironment, comprised of cells on adjacent tissues, extracellular matrix molecules and cytokines, plays an important role in inducing the site-specific differentiation of somatic stem cells. Thus for example, the somatic stem cells introduced to the liver will differentiate into a hepatic lineage cells including hepatocytes and cholangiocytes by the influence of the microenvironment (9).

In the experimental setting, it is important to make the microenvironment optimal for somatic stem cells to differentiate into the purposed type of cells, such as hepatocytes. Various growth factors were presumed to affect the differentiation of somatic stem cells to hepatocytes. Among them, hepatocyte growth factor (HGF) may be the key cytokine. However, it is still not clear that HGF is mandatory in the differentiation into hepatocytes. It is evident that HGF affects differentiation to hepatocytes, however there has not been any literature that specifically targets the use or no use of solely this cytokine. In this study, we tried to find that HGF is absolutely essential in the differentiation of somatic stem cells to hepatocytes. We studied the role of HGF by evaluating of the differentiation of hepatocyte in the culture medium with various cytokines such as fibroblast growth factor-1 (FGF-1), FGF-2, and stem cell factor (SCF) with or without the HGF *in vitro*.

Materials and Methods

The study population. The human UCBs (n=5) were collected during the delivery of full-term pregnant women after obtaining the consent of the patient, (Ewha Womans University Mokdong Hospital, De-

partment of Obstetrics) Normal liver tissues as positive control were acquired from biopsies performed for metastasis evaluation for solid tumor resection (Seoul National University Bundang Hospital, Department of Surgery)

Methods.

Cell culture: MNCs were isolated from the UCB after performing Ficoll-hypaque (Amersham Biosciences, Wickstromes, Sweden) density gradient separation. The obtained MNCs were primarily cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, California, USA) that was supplemented with 15% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B and 300 mM monothioglycerol. The cytokines that we assessed included fibroblast growth factor (FGF)-1 (20 ng/ml) and FGF-2 (10 ng/ml), and these cytokines were purchased from Invitrogen (Carlsbad, CA, USA). Stem cell factor (SCF) (10 ng/ml) was kindly provided by the Kirin Brewery (Japan) and HGF was purchased from Chemicon (Temecula, California, USA). There were two groups for this experiment; HGF-treated group in which 10 ng/ml HGF was added and HGF-non treated group where HGF was not added.

The MNCs were plated onto 0.1% gelatin-coated tissue culture dishes (Corning Costar, Acton, Massachusetts, USA) and they were maintained at 37°C with 5% CO₂. The cells were observed during culture under a phase contrast microscope (Olympus, Tokyo, Japan).

Immunofluorescence staining analysis: The cultured cells were fixed with 80% acetone for 20 minutes at -20°C. After fixation, blocking was done with 10% normal human serum. The samples were washed three times with PBS and then incubated for 1 hour with the diluted primary antibodies at room temperature. Mouse anti-human ALB antibody and anti-human CK-19 monoclonal antibody (mAb; Dako, Produktionsvej, Glostrup, Denmark) and antiproliferating cell nuclear antigen (PCNA) mAb (Oncogene, Cambridge, MA, USA) were used. The PCNA mAb was overlaid onto the ALB pre-incubated sample. The samples were washed three times with PBS and then incubated for 30 minutes at room temperature with anti-mouse IgG-fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO, USA) or anti-mouse IgG-tetramethyl

rhodamine isothiocyanate (TRITC) (Sigma, St Louis, MO, USA). For each analysis, the negative control was also tested in parallel for each analysis by removal of the primary antibody from the protocol. The samples were analyzed using a Laser Scanning Microscope (Carl Zeiss, Oberkochen, Germany)

Flowcytometric analysis with Carboxyfluorescein diacetate, succinimidyl ester (CFSE): MNCs at 1×10^7 /ml in PBS/0.1% BSA were incubated at 37°C for 10 minutes with 20 (M of CFSE (Molecular Probes, Eugene, OR). Staining was terminated by adding ice-cold culture medium and the cells were washed twice in PBS. CFSE-labeled cells were resuspended with 300 μ l of PBS, and isolated for flow cytometric detection of

CFSE positive cells.

Reverse transcription-polymerase chain reaction (RT-PCR): Total mRNAs were obtained from normal control liver tissues and cultured cells at day 21, and the reverse transcription of mRNA was performed by a reverse transcription system (Promega, WI, USA). The complementary DNA was amplified by Tag DNA polymerase enzyme (Takara, Shiga, Japan). As with PCR, after the initial denaturation process at 94°C for 3 minutes, 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute were repeated, and after the elongation reaction at 72°C for 10 minutes, the reaction was terminated. For PCR, the primer of glyceraldehydes 3-phosphate dehydrogenase (GAPDH), ALB, and CK-18 were used (Table I).

The amplified products were separated by electrophoresis on 1% agarose gel and stained with 1.25 μ l ethidium bromide. Samples from both the HGF-treated group and HGF-non treated group were examined by the Gel Documentaion system (Biorad Co., CA, USA).

Table I. Primers used for reverse transcription-polymerase chain reaction

Primers	Sequences	Product
GAPDH	F: 5'-GTCTCCTCCACCATGG AGAAGGCT-3' R: 5'-CATGCCAGTGAGCTTC CCGTTCA-3'	395 bp
Albumin	F: 5'-TTGGAAAAATCCCACT GCAT-3' R: 5'-CTCCAAGCTGCTCAAA AAGC-3'	350 bp
CK-18	F: 5'-GAGATCGAGGCTCTCA AGGA-3' R: 5'-CAAGCTGGCCTTCAGAT TTC-3'	357 bp

bp, base pair; CK-18, cytokeratin 18; F, forward; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; R, reverse.

Results

HGF induced from MNCs into big oval cells. During the first 3 days, separated MNCs showed the same pattern of mixed cells in both HGF-treated group and HGF-non treated group. From the 4th day, dead cells were cleared, and the cells attached to the bottom of the flask began to grow. In the HGF-treated group, attached cells were amplified from the 10th day, and later became relatively big and oval, still attached to the bottom. They seemed to detach readily in response

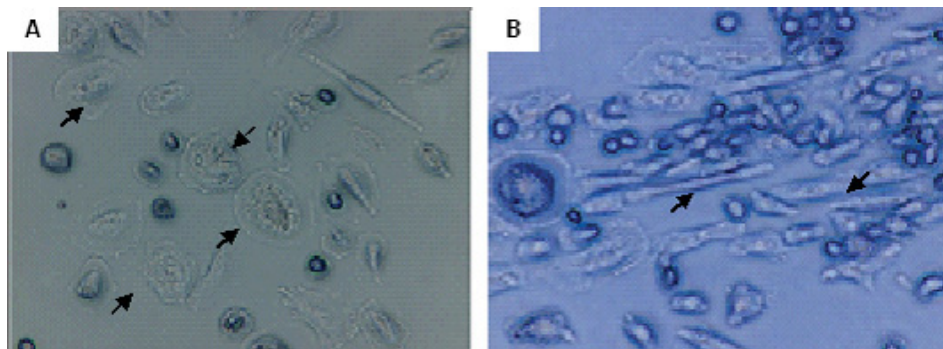


Figure 1. Morphology of cultured human UCB cells at 21 day with and without hepatic growth factors (HGF). UCB cells were cultured with growth/differentiation factors and morphology of cells was monitored by phase contrast microscope. (A) In HGF-treated group, cultured cells had a large oval-shaped appearance (arrows) with adherent characteristics but, easily detached. (B) In the HGF-non treated group, cultured cells were spindle-shaped (arrows) and had strong adherent characteristics (original magnification, $\times 400$).

to external stimuli, such as pipetting, when observed under phase contrast microscopy. And on the 21st day of culture, these cells were expanded and apparent (Fig. 1A). In contrast, in the HGF-non treated group, in which attached cells had a spindle shape, their adherence was so strong that they were not detached readily (Fig. 1B).

With HGF, MNCs expressed hepatic lineage markers. To verify the characteristic of cultured cells, the ex-

pression of the markers of hepatocytes and cholangiocytes by immunofluorescence microscope was examined.

In HGF-treated group, oval shaped cells were immunofluorescently stained with MoAb for ALB and CK-19. The oval-shaped cells detected by phase contrast microscope were found to be identical to the cells positive for ALB or CK-19 (Fig. 2A, B). Spindle-shaped cells were not stained. In contrast, the HGF-

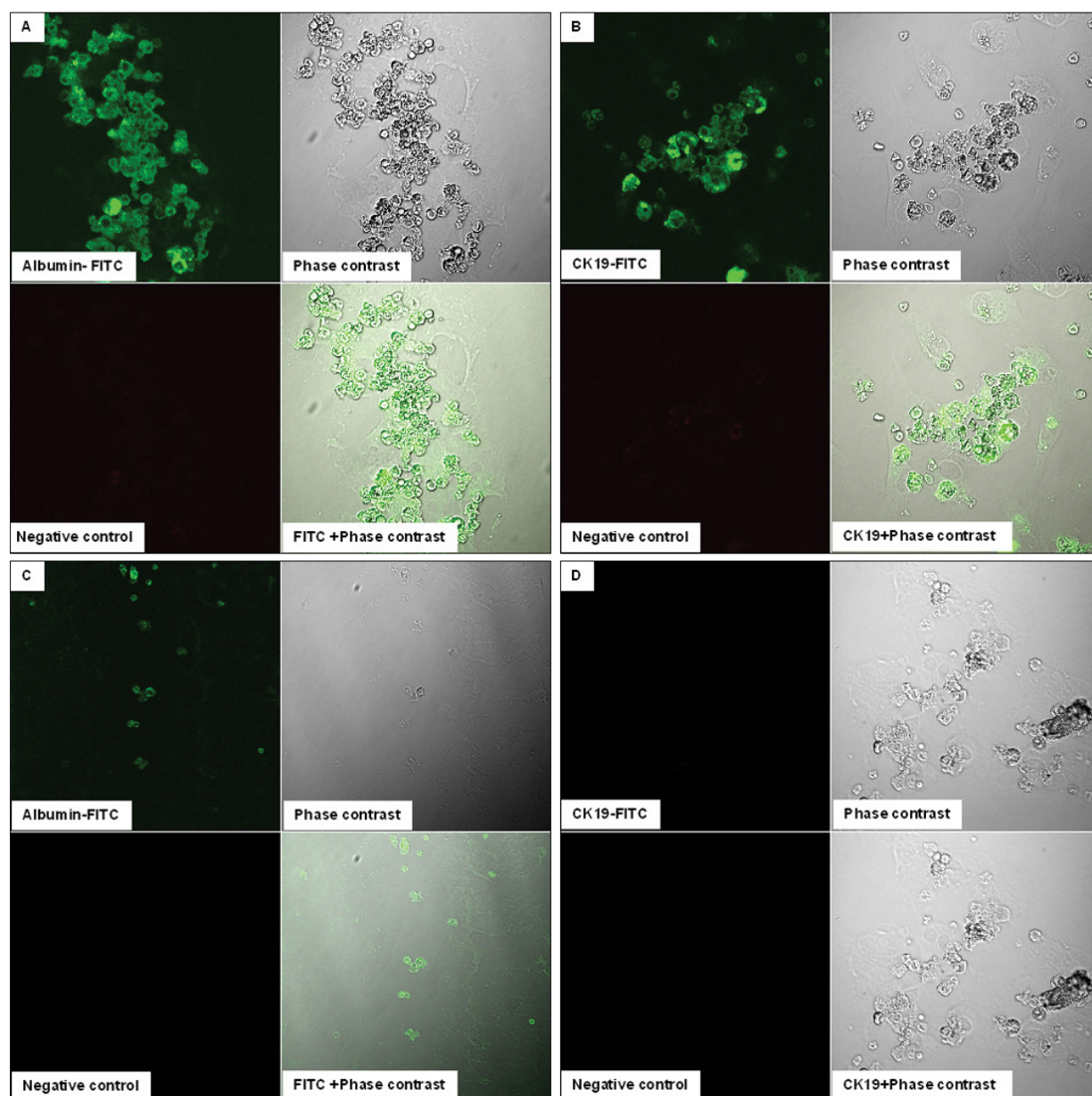


Figure 2. Immunofluorescent staining analysis of the cultured cells on the 21st day for hepatic lineage markers. Anti-human albumin (ALB) antibody and Anti-human cytokeratin-19 (CK-19) antibody were used for hepatocyte and cholangiocyte markers, individually. In HGF-treated group, the oval cells, but not the spindle-shaped cells, expressed ALB (A) and CK-19 (B) in the culture condition with HGF comparing to the group without HGF, respectively (C, D) (Original magnification, $\times 20$).

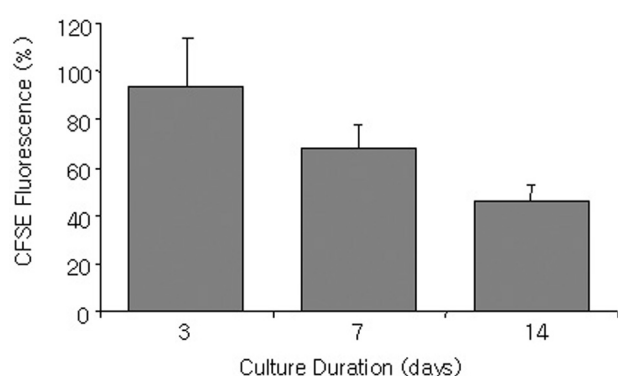


Figure 3. Carboxyfluorescein diacetate, succinimidyl ester (CFSE) fluorescence of cultures cells were analyzed by flow cytometry for evaluation of proliferation abilities. CFSE positivities decreased with time, but still remained until day 14 of culture.

non treated group, oval-shaped cells were rarely detected on phase contrast microscope, and cells stained positive for ALB or CK-19 were not detected.

Differentiated cells still maintained its proliferation abilities. To examine the proliferation activity of cells expressing ALB, simultaneous staining with ALB and PNCA MoAbs was examined. PNCA MoAb treated with TRITC was used for the analysis of proliferation activity. In the HGF-treated group, the cells showing positivity for ALB MoAb were confirmed to be the cells positive for PCNA MoAb (data not shown). Although, there were few cells positively stained to both MoAbs in the HGF-non treated group, their number was extremely small compared to the HGF-treated group.

To quantitatively examine the proliferation activity of the HGF-treated group, cultured cells were conjugated to CFSE that has affinity to proliferating cells, and the proliferation capacity with culture time was analyzed by flow cytometry. The positive expression rate for CFSE of cultured cells was 93.2 ± 15.6 on day 3. It decreased to 45.7 ± 7.3 on day 14. However, sufficient proliferation capacity was still maintained (Fig. 3).

HGF may affect stem cell differentiation into hepatocytes as well cholangiocytes lineage. Using RT-PCR, ALB and CK-18 mRNA were extracted to detect immune marker of hepatocytes and cholangiocytes, respectively. As positive control, normal liver tissues were used. On day 21 of culture, in the HGF-treated group, mRNA of ALB and CK-18 formed bands with intensity equivalent to the intensity shown by normal

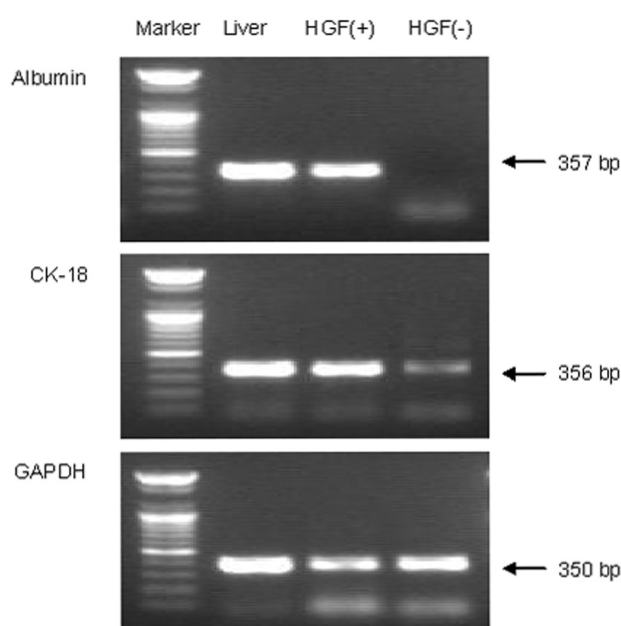


Figure 4. The hepatocyte lineage markers were examined to confirm that the cultured round cells derived from UCB. Albumin and CK-18 mRNA were detected from the day 21 cultured cells with HGF group, and normal liver tissue as a positive control. When comparing with or without HGF group, cells with HGF group produced more dense RT-PCR bands with the same dose of cells.

liver tissues. On the other hand, in the HGF-non treated group, ALB mRNA was not detected and CK-18 formed a weak band, which was the identical finding to the result of immunofluorescence staining (Fig. 4).

Discussion

Microenvironment of stem cells plays an important role in directing the differentiation of stem cells into specific organs. The microenvironment refers to pertinent growth factors, extracellular matrix molecules, and cell types surrounding the stem cells (10). For differentiation to hepatic lineage cells, the growth factors have been known to be more important rather than extracellular matrix molecules (11).

HGF is the important factor for the maturation and functional recovery of the liver (12). HGF is also the factor involved in the differentiation of the stem cells. FGF is a mitogen for fibroblasts and vascular endothelial cells and it has been known to affect the differentiation to hepatocytes (13,14). Likewise, both HGF and FGF have been reported to have the effect in augmenting the differentiation from mesenchymal cells to

hepatocytes (14).

SCF is known to play a role in the biological process of stem cells (15,16). But it is still unknown that their effects on the differentiation of stem cell act independently or synergistically. It is also not clear that HGF is absolutely essential for this process to occur.

The aim of this study was to examine whether adult stem cells could differentiate to hepatocytes and cholangiocytes that are required for each organ with the aid of various growth factors pertinent to the liver, and for this, the role of HGF was assessed.

In the HGF-treated group, relatively big oval-shaped cells that were presumed to be hepatic lineage cells began to appear from the early phase of culture. In contrast, in the HGF-non treated group, cells were mainly spindle-shaped with strong adherence ability, which is usually shown in hematopoietic cell culture (17). It is a well-known fact that the hepatic lineage cells are oval-shaped cells with a relatively big cell size (18). In this study, it was shown that supply of HGF is necessary to generate large oval cells, which seemed to be hepatic lineage cells.

To verify the characteristic of these cells, it is necessary to examine whether differentiated cells are truly hepatic lineage cells. Urea, ALB and (-fetoprotein (AFP) may be used as markers for the hepatocyte (19) but AFP is expressed on more immature hepatocytes in comparison with ALB. For cholangiocytes, their cellular differentiation could be assessed by the expression of CK-18 or CK-19.

In this study, MoAbs for CK-18, CK-19, and ALB were used as the methods to assess the hepatic lineage cells and PCNA MoAb was used to assess the proliferation activity of these cells simultaneously. Immunofluorescent staining in the HGF-non treated group showed that the number of cells detected under phase contrast microscope was small and most of these cells were not stained with ALB or CK-19 MoAb. In contrast, in the HGF-treated group, oval-shaped cells detected by phase contrast microscope were identical cells positive for ALB and CK-19. HGF with the aid of FGF-1, FGF-2 and SCF induced the differentiation to the hepatic lineage cells, and these cells were thought to be hepatoblasts, which can differentiate to hepatocytes and cholangiocytes.

The findings of RT-PCR were similar to the result of immunofluorescence staining. It was secondarily

proven that HGF has an absolute influence on the differentiation of the hepatic lineage cells.

This study was focused on the role of HGF, the presence of which revealed to be crucial in inducing hepatic lineage cells. The other experimental setting of combinations such as, HGF+FGF-1, HGF+FGF-2, HGF+SCF may be possible to see the role of SCF and FGF. But this study did not include those experiments. This is because that these two cytokines are obligatory for the culture even if it is not for the differentiation to hepatic lineage cells. SCF is necessary in the amplification in early stages culture and FGF-1 and FGF-2 are necessary for maintaining the culture (20).

The proliferation capacity of the differentiated cells can be an important factor that influences the fate of cells upon the introduction of the cells to the body (21). In this study, the proliferation capacity of differentiated cells was maintained up to day 14.

This study was undertaken to elucidate the optimal environment for the differentiation of stem cells to hepatic lineage cells and it was confirmed that the role of HGF is crucial. The studies on the role of extracellular matrix molecules besides growth factors and the *in vivo* reaction should be further investigated.

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