Low serum concentration facilitates the differentiation of hepatic progenitor cells

Yun He, MD, Wei-Yu Zhang, MD, Min Gong, MD, Jia-Yi Huang, MD, PhD, Ni Tang, MD, PhD, Tao Feng, MD, PhD, Guang-Hui Wei, MD, PhD, Tong-Chuan He, MD, PhD, Yang Bi, MD, PhD.

ABSTRACT

الأهداف: اختبار تأثير الأمصال المزروعة في ظروف مختلفة بالمختبر على تحفيز عمليتي تمايز وتكاثر خلايا الكبد الإنشائية.

الطريقة: أُجريت هذه الدراسة في معمل العلاج بالخلايا الجذعية بمستشفى الأطفال التابع لجامعة تشونغشينغ الطبية، تشونغشينغ، الصين وذلك خلال الفترة من أغسطس 2009م إلى يونيو 2010م. لقد تم تحفيز تمايز الخلايا الإنشائية بعقار الديكاميثازون، ومعامل نمو الخلايا الكبدية، ومعامل نمو الخلايا الليفية النشطة، ومن ثم تمت إضافة 10% أو 2% من مصل البروفين الجنيني، أو 2% من مصل الخيول. ولقد قمنا بالتأكد من تكون الألبومين الغير مباشر بواسطة احتبار لوسيفيراز غوسيا، في حين تم التأكد من حدوث التكاثر الخلوي بواسطة تعداد الخلايا. وبعد ذلك تمت الاستعانة بالتقنية النسخية العكسية من التفاعل التسلسلي المبلمر، وكذلك باختبار التصبغ المناعي من أجل تحديد مؤشرات الكبد التالية: DLK, CK18, TAT, ALB, AFP, UGT1A. وأخيراً تمت الاستعانة باختبار امتصاص صبغة السيانين، وكذلك اختبار تصبغ شيف الحامضي لتحديد مدى تحفيز الخلايا الكبدية.

النتائج: أشارت النتائج إلى أن تكاثر الخلايا الإنشائية قد تم تثبيطه في الأمصال التي كانت جرعتها 2%. وأظهرت الخلايا التي تم تحفيزها بما يعادل 2% من الأمصال نسباً عالية للمؤشرات الكبدية: ALB, CK18, UGT1A, TAT، ونسباً متدنية للمؤشرات: AFP, DLK وذلك عند المقارنة مع جرعة مصل البروفين الجنيني والتي كان مقدارها 10%. ولقد دُهشنا من تأثير الأمصال التي كان مقدارها 2% على امتصاص صبغة السيانين، إلا أنها قامت بتثبيط تصبغ شيف الحامضي للخلايا الإنشائية المُحفزة. وكان تأثير الجرعة 2% من مصل الخيول على تحفيز مؤشرات الكبد وتفاعل الخلايا الإنشائية أفضل من الجرعة نفسها من مصل البروفين الجنيني.

خاتمة: أثبتت الدراسة مدى فعالية مصل الخيول وخصوصاً الجرعة التي كان مقدارها 2% في تحفيز تمايز الخلايا الكبدية الإنشائية، ولم يكن لتصبغ شيف الحامضي ذو جدوى أثناء اختبار تحفيز الخلايا بواسطة الأمصال ذات التركيز المنخفض.

Objectives: To investigate the effect of serum culture conditions on the differentiation of hepatic progenitor cells (HPCs) in vitro.

Methods: This study was conducted at the Stem Cell Biology and Therapy Laboratory, The Children's Hospital of Chongqing Medical University, Chongqing, China from August 2009 to June 2010. Differentiation of HPCs was induced with dexamethasone, hepatic growth factor, and fibroblast growth factor-4 in 10% fetal bovine serum (FBS), 2% FBS, 2% horse serum (HS) conditions. First, we investigated the indirect synthesis of albumin by the albumin promoter-driven Gaussia luciferase assay, and cell proliferation by cell counting. Then, we performed reverse transcriptase-polymerase chain reaction and immunofluorescence to detect hepatic related markers Delta-like protein (DLK), cytokeratin 18 (CK18), tyrosine aminotransferase (TAT), albumin (ALB), α-fetoprotein (AFP), and glucuronosyltransferase 1A (UGT1A). Lastly, we used indocyanine green (ICG) uptake assay and periodic acid-Schiff (PAS) staining to determine the function of induced hepatocytes.

Results: The proliferation of HPCs was inhibited in 2% serum culture. Induced HPCs in 2% serum exhibited higher expression of ALB, CK18, UGT1A, and TAT, whereas lower expression of DLK and AFP, compared with 10% FBS. Interestingly, 2% serum culture improved ICG uptake function, but repressed PAS staining of induced HPCs. Generally, 2% HS was better at inducing mature hepatic markers and the function of HPCs than 2% FBS.

Conclusion: Two percent low serum concentration conditions, especially 2% HS, improve the hepatic differentiation of HPCs, and PAS staining may not be used in induction testing of low serum concentrations.

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From the Stem Cell Biology and Therapy Laboratory (He Y, Zhang, Gong, He T, Bi), The Children's Hospital of Chongqing Medical University, and the Key Laboratory of Developmental Diseases in Childhood (He Y, Zhang, Gong, Wei, Bi), Ministry of Education, Chongqing Medical University, Chongqing, China, and the Molecular Oncology Laboratory (He Y, Huang, Tang, Feng, He T, Bi), University of Chicago Medical Center, Chicago, Illinois, United States of America.

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Address correspondence and reprint request to: Dr. Yang Bi, Stem Cell Biology and Therapy Laboratory, The Children's Hospital of Chongqing Medical University, Chongqing 400014, China. Tel. +86 (23) 63633113. Fax. +86 (23) 63626904. E-mail: yangbi1981@yahoo.com

Orthotopic liver transplantation (OLT) plays an important role in the treatment of end-stage liver diseases.[1,2] Due to severe scarcity of donors and substantial complications of OLT,[3] increased research is moving towards taking advantage of the tremendous proliferative capacity of the differentiated hepatocyte.[4] Hepatocyte transplantation is a novel cell therapy and as an alternative to OLT, can be used to effectively treat liver failure, liver cancer, alcoholic and autoimmune liver diseases, especially genetic disorders.[5] It has been reported that extrahepatic stem cells can differentiate into hepatic lineages,[6] and be used in liver cell transplantation to enhance liver function.[7] However, these cells have low hepatic differentiation efficiency, and only perform limited hepatocyte phenotype and function.[8] So, as a major limitation of cell-based therapies for liver diseases, the availability of hepatocytes needs to be taken into consideration.[9] During liver development, the hepatocyte and cholangiocyte have been considered to be derived from a common hepatic progenitor cell (HPC), also known as hepatoblast.[10] The differentiation of various stem cells to mature hepatocytes must undergo this bio-potential progenitor state.[11] Therefore, experimental study on HPCs will help in interpreting the mechanism of liver development and stem cell differentiation, and thus promoting the clinical application of stem cells in liver diseases. In addition, the liver is the main organ of detoxification and purification, therefore, HPCs derived hepatocytes might also be helpful for in vitro drug testing. In most studies, hepatic differentiation of stem/progenitor cells was not efficient in producing adequate amounts of functional mature hepatocytes.[12,13] There are so many factors that contribute to differentiation efficacy, including variant cellular factors, and culture methods.[14] Serum is an essential cell culture condition, which contains a variety of cytokines, amino acids, trace elements, and so on,[15] and plays important roles in cell proliferation and differentiation. In this experiment, we mainly focused on the effect of different serum culture conditions on the differentiation of embryonic HPCs, for optimizing the robust and reliable methods of hepatic differentiation in vitro.

Methods. This study was conducted at the Stem Cell Biology and Therapy Laboratory, The Children's Hospital of Chongqing Medical University, Chongqing, China from August 2009 to June 2010. All experiments were approved by the Ethical Committee of the Children's Hospital of Chongqing Medical University.

Cell culture and chemicals. Immortalized mouse fetal HPCs expressing simian virus 40 large T antigen (SV40T) were obtained in our previous study.[16] Cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, Utah, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. For differentiation induction, HPCs were treated with 1μM Dexamethasone (Dex) / 10 ng/ml hepatic growth factor (HGF) / 20 ng/ml fibroblast growth factor-4 (FGF4) in different DMEM medium containing 10% FBS, 2% FBS, and 2% horse serum (HS, Hyclone, Logan, Utah, USA). Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell growth curve determination. Cell growth was determined by counting viable cells. Briefly, cells were trypsinized and suspended in phosphate buffer solution (PBS). In a small tube, 0.5 mL of cell suspension and 0.5 mL of 2× trypan blue buffer were mixed well. Then, the mixture was placed in the hemocytometer counting chamber. Viable cells which excluded trypan blue in each grid square were counted. The total number of cells per mL was expressed as: Cells/mL = the count per large square × dilution factor × 10^4.

Transfection of albumin promoter-driven Gaussia luciferase (ALB-Gluc) reporter and luciferase reporter assay. We first amplified the 2.5 kb mouse albumin (ALB) promoter gene with polymerase chain reaction (PCR), and subcloned it into a homemade pSEB-GLuc reporter vector to obtain a pSEB-ALB-GLuc vector, which drives the expression of the Gaussia luciferase protein.[16] Cells in the exponential growing process were implanted in 25 cm^2 cell culture flasks and transfected with 2 μg of pSEB-ALB-GLuc plasmid per flask using LipofectAMINE (Invitrogen, Carlsbad, California, USA). At 16 hours after transfection, cells were replanted into 24-well plates and treated with 1 μM Dex, 10 ng/ml HGF, and 20 ng/ml FGF4 in 10% FBS, 2% FBS, and 2% HS serum conditions. Gaussia luciferase, a natural secretory signal, can be driven by the ALB promoter and secreted into the cell medium. The medium of the treated cells was collected at the indicated time points for Gaussia Luciferase Assays by using the Gaussia Luciferase Assay Kit (New England Biolabs, Ipswich, Massachusetts, USA). Each assay condition was performed in triplicate. Reporter activity was expressed as mean ± SD.

Reverse transcription and semi-quantitative reverse transcriptase-PCR analysis. Reverse transcriptase-PCR (RT-PCR) was carried out as previously described.[17] The cDNA templates were generated from 10 micrograms of total RNA by reverse transcription with hexamer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, California, USA). As PCR templates, the first strand cDNA products were further diluted 5- to 10-fold.

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Table 1 - Reverse transcriptase-polymerase chain reaction primers.

Expressions	Forward	Reverse	Accesion NO.
GAPDH	GGCTGCCCAGAACATCAT	CGGACACATTGGGGGTAG	NM-008084
DLK	ACAATGGAACTTGCGTGGA	GGCAGGGAGAACCATTGAT	NM-010052
CK18	CTGGGCTCTGTGCGAACT	ACAGAGCCACCCCAGACA	NM-010664
TAT	ACCTTCAATCCCATCCGA	TCCCGACTGGATAGGTAG	NM-146214

GAPDH - glyceraldehyde phosphate dehydrogenase, DLK - Delta-like protein, CK18 - cytokeratin 18, TAT - tyrosine aminotransferase

All 18-20mers PCR primers were designed using the Primer3 program to amplify the 3'-end (approximately 120-150 bps) of the gene of interest (**Table 1**). The RT-PCR reaction was performed by using a touchdown protocol: 94°C × 20", 65°C × 20", 72°C × 20" for 10 cycles, with 1°C degree decrease per cycle, followed by 25-30 cycles at 94°C × 20", 56°C × 20", 72°C × 20". The RT-PCR products were resolved on 1.5% agarose gels. Endogenous glyceraldehyde phosphate dehydrogenase was used to normalize all samples.

Immunofluorescence staining. Immunofluorescence staining was performed as previously described.[17] At day 9 after induction, cells were fixed with methanol at -20°C for 15 minutes. After PBS washing, fixed cells were permeabilized with 1% NP-40 for 10 minutes, and blocked with 10% bovine serum ALB for one hour, then followed by incubating with ALB, α-fetoprotein (AFP), or glucuronosyltransferase 1A (UGT1A) antibody (Santa Cruz Biotechnology, Carlsbad, California, USA) for 60 minutes and incubated with DyLight 488-labelled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) for 30 minutes. The pictures of stains were captured under a fluorescence microscope. Stains with control IgG were used as negative control. Except for fixation, all steps were performed at room temperature.

Indocyanine green (ICG) uptake and release. Cells were cultured in 24 well plates with 9 days treatment, then washed with PBS and incubated in complete DMEM medium containing one mg/ml fresh prepared ICG at 37°C for one hour. Green-stained cells were captured under the microscope. Afterward, culture was changed into complete medium and incubated at 37°C for more than 6 hours, and then checked again under the microscope. Ten un-overlapped images were analyzed.

Periodic acid-Schiff (PAS) staining. Cells were fixed with 4% paraformaldehyde for 10 minutes, treated with 0.5% PAS solution for 5 minutes, then incubated with the Schiff's reagent for 15 minutes, and counterstained

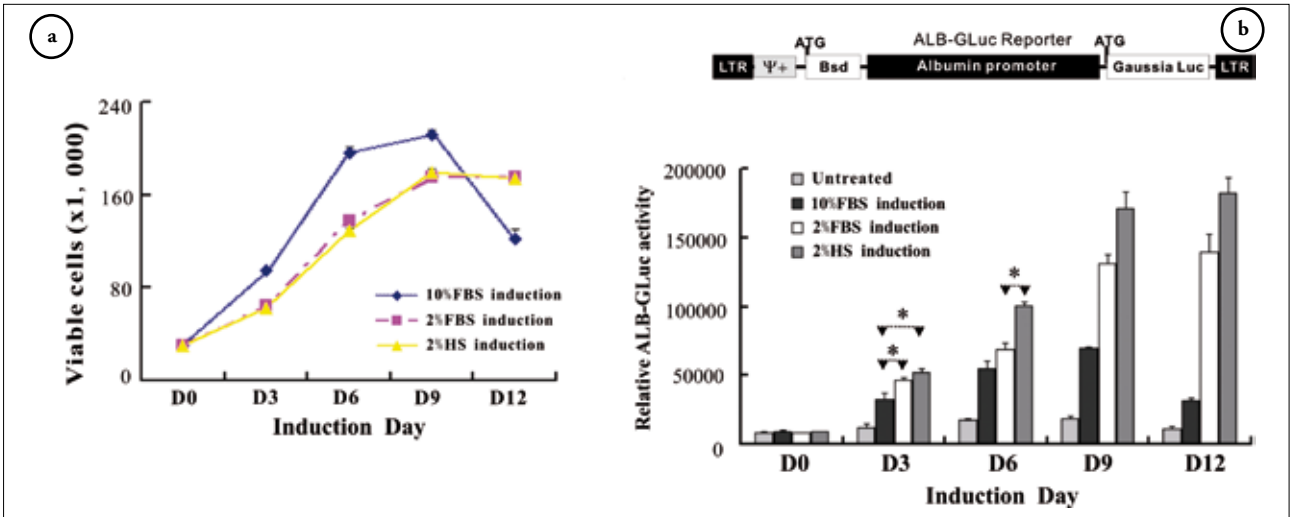

Figure 1 - Low serum concentration inhibited cell growth but improved ALB hepatic differentiation of HPCs. **a**) Cell growth curves were analyzed to detect induced HPCs proliferation in different serum cultures. (p=0.0135 10% FBS versus 2% FBS and 2% HS; p=0.9686 2% FBS versus 2% HS). **b**) An albumin promoter-driven gaussia luciferase reporter (Alb-GLuc) is schematically shown. Gaussia luciferase assay was carried out in triplicate to check indirect ALB expression of pSEB-ALB-GLuc vector transfected HPCs in different serum induction mediums. The data are shown as mean ± SD (p=0.0316 10% FBS versus 2% FBS at D3, p=0.03005 10% FBS versus 2% HS at D3, p=0.01084 2% FBS versus 2% FBS at D6). ALB - albumin, HPCs - hematopoietic progenitor cell, FBS - fetal bovine serum, HS - horse serum, LTR - long terminal repeat, Bsd - Blasticidin.

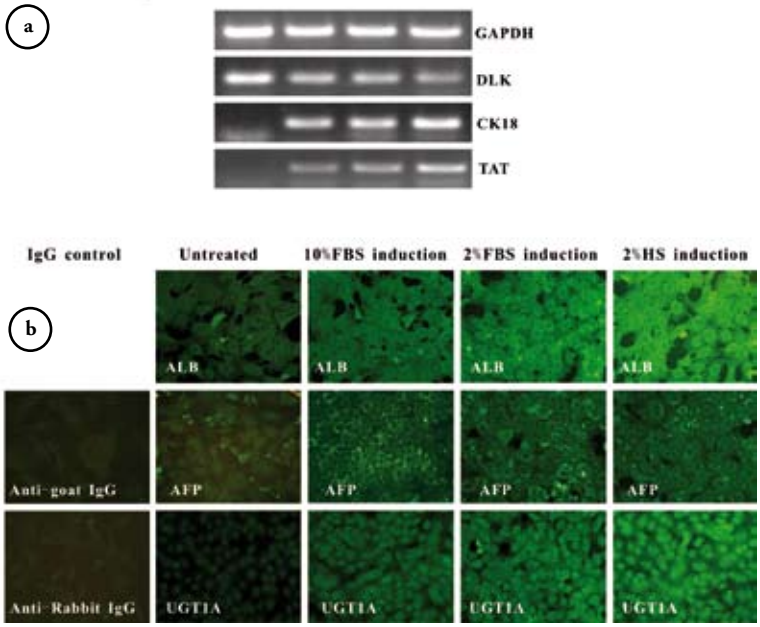

Figure 2 - Low serum conditions improved the expression of hepatic related markers of induced HPCs. **a)** the RT-PCR analysis of hepatic marker gene DLK and the liver late marker CK18 and tyrosine aminotransferase, TAT of HPCs in different serum culture at day 9 of induction. All samples were normalized with GAPDH. The RT-PCR results were confirmed in at least 3 batches of independent experiments, and representative results are shown. **b)** Immunofluorescence staining of hepatoblast marker (alpha-fetoprotein, AFP) and mature hepatocyte markers (Albumin, ALB, and glucuronosyhransferase 1 A, UGT1A) of induced HPCs at day 9 as the set of cells. Magnification, 200×. RT-PCR - reverse transcriptase-polymerase chain reaction, TAT - tyrosine aminotransferase, HPCs - hematopoietic progenitor cell, AFP - α-fetoprotein, ALB - albumin, DLK - Delta-like protein, CK18 - cytokeratin 18, GAPDH- glyceraldehyde phosphate dehydrogenase, HS -horse serum.

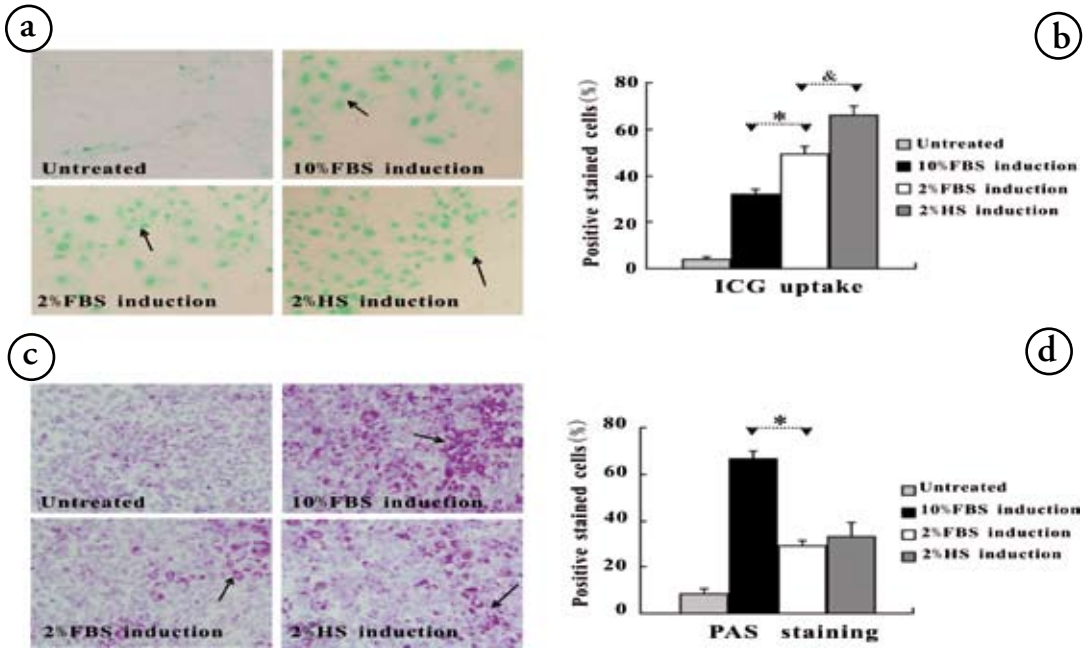

Figure 3 - Serum conditions effected the hepatic function of induced HPCs. The HPCs were treated as described in **Figure 2**. The staining results were confirmed by at least 3 batches of independent experiments, and representative results are shown. **a)** Indocyanine green uptake was carried out to detect transport and metabolism function of induced HPCs. Cells were cultured in fresh prepared indocyanine green medium for 4 hours. Green-stained cells are indicated by arrows. **b)** The ratio of ICG positive stained cells are shown as mean ± SD (*p=1.42E-06 2%FBS versus 10%FBS, $^{\&}p$=1.92E-05 2%HS versus 2%FBS). **c)** The PAS staining was performed to check glycogen storage function of induced HPCs. Cells were fixed and subjected to PAS staining. Positive stains (purple red) are indicated by arrows. **d)** The ratio of PAS positive stained cells are shown as mean ± SD (*p=9.93E-10 2% FBS versus 10%FBS, p=0.1613 2%HS versus 2%FBS). HPCs - hematopoietic progenitor cell, PAS -Periodic acid-schiff, ICG - indocyanine green, HS - horse serum, FBS - fetal bovine serum

with hematoxylin solution for 2 minutes. All steps were performed at room temperature, and cells were washed with tap water after each step. Positive stained cells (purple red color in cytoplasm) of 10 un-overlapped vision fields were recorded using a microscope.

Statistical analysis. The standard deviations and statistically significant differences between samples were calculated with the Microsoft Excel program (Microsoft, Office Professional 2003) using the two-tailed Student t test. A $p<0.05$ was considered to be significant.

Results. ***Low serum concentration inhibited cell growth.*** First, we sought to detect the cell growth status in different serum cultures. We plated cells in 6-well plates at 20% cell density and counted viable cells at different induced time-points. The cell number continuously increased as time went by, and reached the highest level on day 9 in 10%, and on day 12 in 2% serum culture. The number of viable cells in the presence of 10% serum was significantly higher than 2% serum from induction day 3 to 9 (p=0.0135), but deceased sharply at day 12 because of limited growth area. No difference in the viable cell number was found between 2, 2% serum culture groups (**Figure 1a**). The results indicate that 2% serum may inhibit the proliferation of HPCs.[18]

Low concentration of serum conditions improved hepatic differentiation of HPCs. To quantitatively assess the hepatic differentiation, we constructed an ALB promoter-driven Gaussia luciferase reporter (pSEB-ALB-GLuc).[16] The HPCs were transfected with pSEB-ALB-GLuc reporter plasmid and induced with Dex/HGF/FGF4 in different serum conditions. Relative Gaussia luciferase activity was assayed at day 0, 3, 6, 9, and 12 after treatment. We found that the relative ALB-GLuc activity was significantly increased in 10% FBS induced cells at day 3, and sharply decreased at day 12 of induction, while the ALB-GLuc activity of 2% serum induced HPCs was still at high levels on day 12. The HPCs in 2% serum medium had significantly higher ALB-GLuc activity than in 10% FBS medium from day 3 after hepatic induction (p=0.0316 10% FBS versus 2% FBS at D3; p=0.03005 10% FBS versus 2% HS at D3). No difference was found on day 3 of induction between 2, 2% serum groups, however, the induced effect of HS was statistically higher (p=0.01084) than FBS in the same 2% concentration on day 6 of induction (**Figure 1b**).

Next, we sought to determine the differentiation of induced HPCs by detecting hepatic related markers at day 9 of induction. As shown in **Figure 2a**, the HPCs expressed a high level of Delta-like protein (DLK), a stem cell marker gene,[19] whereas the expression of cytokeratin 18 (CK18) and tyrosine aminotransferase (TAT), mature hepatic genes,[20] were undetectable in primary HPCs. The expression of DLK decreased, while the expression of CK18 and TAT increased obviously, especially in the 2% HS induced group. The immunofluorescence results showed that the ALB located in cytoplasm, UGT1A, existed in the nucleus and cytoplasm, while the AFP was expressed in the cytoplasm near the nucleus. The expression of those 3 markers was much higher in the induced group than the control. The expression of ALB and UGT1A was highest in 2% HS, followed by 2% FBS, and lowest in 10% FBS culture medium, whereas AFP exhibited an opposite expression tendency in the 3 induced groups (**Figure 2b**).

Low serum concentration culture promotes ICG uptake, but attenuates glycogen storage of hepatocytes. The ICG is a nontoxic organic anion that is eliminated exclusively by mature hepatocytes and is used clinically to evaluate liver function.[21] We detected ICG uptake and release of induced hepatocytes on day 9 of induction. As shown in **Figure 3a and 3b**, only less than 4% of HPCs exhibited the green-stain in the nucleus, while 31.7% of ICG positive cells were obtained in 10% FBS, 49.1% in 2% FBS, and 65.8% in 2% HS induced medium (p=1.42E-06 2% FBS versus 10% FBS, p=1.92E-05 2% HS versus 2% FBS). Almost all the cells released ICG after 6 hours in complete medium with the withdrawal of ICG. The functional hepatocyte can synthesize and accumulate glycogen granules, which can be stained by Schiff's reagent to a purple color.[22] The PAS-positive stained cells were significantly increased in the treated group after 9 days of induction. The above results suggest that low concentration of serum can improve the differentiation and hepatic metabolic function of HPCs. However, fewer red positive cells were found in 2% FBS and 2% HS induced groups compared with that in the 10% FBS induced group (p=9.93E-10) (**Figures 3c & 3d**).

Discussion. The liver has an astounding regeneration capacity in the face of insults, such as partial hepatectomy, chemical injury, and liver fibrosis. Activation and expansion of liver stem cells may play important roles in liver regeneration and functional recovery.[7,23] Liver stem cells are widely considered to be isolated from intrahepatic or extrahepatic tissues, and are capable of giving rise to hepatic lineages.[24,25] Embryonic stem cells, hematopoietic stem cells, and bone marrow-derived mesenchymal stem cells are defined as extrahepatic sources of liver stem cells, and can differentiate into hepatocyte-like cells with the expression of hepatic specific marker genes and partial function in vitro, but the differentiation efficiency and recovery function in vivo are much lower than hepatic progenitor cells from liver origin.[26,27] During embryonic development, the major live constituent is hepatoblasts, which are known as the bipotential precursors hepatocytes as well as cholangiocytes. Hepatoblasts begin to differentiate

into hepatocytes and cholangiocytes at embryonic day 14 (E14).[28] In this report, we used immortalized HPCs isolated from E14.5 mouse liver. This intermediate state of hepatic precursors is an essential and inevitable process of diverse stem cells differentiating into mature liver cells.[11]

Various cell factors are involved in the different stages of hepatocyte development. Studies have revealed that Dex, HGF, and FGF4 are widely used in inducing hepatocyte-like differentiation from embryonic stem cells and mesenchymal stem cells, respectively or cooperatively.[29-31] Serum-free medium is combined with chemical or cellular factors in many cell differentiation induction methods.[32,33] However, cells could not survive in serum-free culture medium after 3 days incubation in our experiment. Serum is an essential component in cell culture, which contains abundant nutrients, hormones, growth factors, trace elements, and ions, providing necessities for cell proliferation and metabolism.[15,34] The FBS is suitable for culturing most mammalian cells, but sometimes other serum is applied appropriately in specific cells culture.[35] Some studies have reported that HS can promote differentiation of the embryonic stem cells into the specific cell type with induction reagents. The HS is better at inducing differentiation of myoblasts and cardiomyocytes, and 2% concentration is commonly used in most research.[36,37] So far, few studies have reported the effect of HS on hepatic differentiation. Interestingly, in this study, we discovered that 2% serum concentration, especially HS could induce higher expression of hepatic specific markers, such as ALB, CK18, UGT1A, and TAT, compared with that of 10% FBS culture condition. However, the expression of DLK, a sensitive hepatoblast marker, and AFP, an early marker of hepatocyte, was at the lowest level in the 2% HS group after 9 days induction. Therefore, our results demonstrated that hepatocyte differentiation could be improved in a 2% HS culture environment. In addition, ALB-Luc activity increased after 3 days with our induction method in all serum conditions. The start of ALB expression of induced extrahepatic stem cells was later than that of HPCs.[38,39] The possible reason maybe that HPCs are intrahepatic progenitor cells from embryo, and more sensitive to induced factors to differentiate into mature hepatocytes.

The liver is a major organ of detoxification and metabolism. Mature hepatocytes have the function of up-taking ICG, a nontoxic tricarbocyanine dye, to form green color in the nucleus, and then excreting it.[21,40] More ICG-positive cells were induced in 2% serum culture compared with 10% FBS medium, indicating that low serum conditions improve maturation of HPCs differentiation. Moreover, green stained cells in the 2% HS group were statistically higher than the 2% FBS group. Another important function of the liver is glycogen synthesis and storage. Periodic acid-Schiff is a histochemical reaction used to detect glycogen in tissues or cells.[22] The PAS stained purple cells, which represented functional hepatocyte, were significantly increased in all treated group at day 9. Though, most of our above data reveals that 2% serum culture promoted hepatic differentiation, the PAS staining results showed us that glycogen storage in low concentration serum was lower than that in higher concentration serum. We believe that glycogen synthesis needs materials, factors, and their interactions, which are mostly supplied by the serum.[41] Fang et al[42] reported that serum-free starved human embryonic kidney 293 cells decreased the background phosphorylation level of glycogen synthase kinase 3, then inactivating its activity. Even though induced hepatocytes in 2% serum have the ability to synthesis glycogen, low serum concentrations could not provide enough material to synthesize glycogen granules in the cell cytoplasm. However, carbohydrates, one of the most important glyco-materials was similar in a low and high serum environment. This need to be further investigated. Nonetheless, 2% HS could induce more purple-stained cells than that of the 2% FBS group, at least supporting that HS is better to be used in hepatic differentiation in vitro.

In summary, we have demonstrated that low serum culture conditions, especially 2% HS can facilitate mature and functional differentiation of HPCs. Also, our results indicated that the PAS staining method may not be used to detect the function of hepatocytes cultured in low serum concentrations. Our study has several limitations, we chose to use immortalized HPCs but not fresh isolated mouse fetal liver cells, although the SV40T antigen has demonstrated no effect on HPC differentiation in our previous study. We did not investigate the molecular causes behind the conversed phenomenon of more mature hepatocytes with less PAS-stained cells in low serum condition. Nonetheless, this report affirmed an important effect of serum culture conditions in hepatic differentiation, expanding our knowledge of hepatocyte induction in vitro.

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