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• BASIC RESEARCH •

Selection, proliferation and differentiation of bone marrow-derived liver stem cells with a culture system containing cholestatic serum *in vitro*

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Abstract

AIM: To explore the feasibility of direct separation, selective proliferation and differentiation of the bone marrow-derived liver stem cells (BDLSC) from bone marrow cells with a culture system containing cholestatic serum *in vitro*.

METHODS: Whole bone marrow cells of rats cultured in routine medium were replaced with conditioning selection media containing 20 mL/L, 50 mL/L, 70 mL/L, and 100 mL/L cholestatic sera, respectively, after they attached to the plates. The optimal concentration of cholestatic serum was determined according to the outcome of the selected cultures. Then the selected BDLSC were induced to proliferate and differentiate with the addition of hepatocyte growth factor (HGF). The morphology and phenotypic markers of BDLSC were characterized using immunohistochemistry, RT-PCR and electron microscopy. The metabolic functions of differentiated cells were also determined by glycogen staining and urea assay.

RESULTS: Bone marrow cells formed fibroblast-like but not hepatocyte-like colonies in the presence of 20 mL/L cholestatic serum. In 70 mL/L cholestatic serum, BDLSC colonies could be selected but could not maintain good growth status. In 100 mL/L cholestatic serum, all of the bone marrow cells were unable to survive. A 50 mL/L cholestatic serum was the optimal concentration for the selection of BDLSC at which BDLSC could survive while the other populations of the bone marrow cells could not. The selected BDLSC proliferated and differentiated after HGF was added. Hepatocyte-like colony-forming units (H-CFU) then were formed. H-CFU expressed markers of embryonic hepatocytes (AFP, albumin and cytokeratin 8/18), biliary cells (cytokeratin 19), hepatocyte functional proteins (transthyretin and cytochrome P450-2b1), and hepatocyte nuclear factors (HNF-1α and HNF-3β). They also had glycogen storage and urea synthesis functions, two of the critical features of hepatocytes.

CONCLUSION: The selected medium containing cholestatic serum can select BDLSC from whole bone marrow cells. It will be a new way to provide a readily available alternate source of cells for clinical hepatocyte therapy.

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INTRODUCTION

Several recent reports have highlighted the broad developmental potential of bone marrow-derived stem cells and the term "stem cell plasticity" has been coined[1]. Bone marrow-derived stem cells have been reported to produce not only all of the blood lineages, but also skeletal muscle[2,3], neurons[4,5], cardiac muscle[6,7], pulmonary epithelium[8], and liver epithelium[9]. The transdifferention of bone marrow-derived cells into hepatic cells was described in rats[9], mice[10] and humans[11,12]. This has brought a new hope for cell therapy using autologous bone marrow cells which have few ethical problems and applied to severe liver disease[13]. However, bone marrow contains hematopoietic[14,15], mesenchymal stem cells[16,17] and multipotent adult progenitor cells[18]. The characteristic surface markers of these specific bone marrow-derived liver stem cells (BDLSC) are still obscure. It is difficult to identify and sort these particular cells by immunological methods, such as fluorescent activated cell sorting (FACS)[14] and magnetic activated cell sorting (MACS)[19]. Although various bone marrow stem cells were found to proliferate injured liver cells, previous attempts at isolation of liver stem cells resulted in a mixture of hemotopoietic cells and potential hepatocyte progenitors because they all shared common cell surface receptors and antigens, including hematopoietic stem cell markers CD34, Thy-1, c-Kit, and flt-3[20-22]. It is necessary to find a new way to isolate and purify BDLSC. According to the principle that cells in culture can survive only when they accommodate to the existing environment, we assumed to develop a culture system that can select BDLSC from whole bone marrow cells directly. Within such a system, only BDLSC can survive while the other bone marrow cells cannot. This particular culture system must contain factors that can activate the proliferation of liver stem cells and ingredients inhibiting the growth of other cells. Studies of the relative mechanisms of liver regeneration and liver stem cells corresponding to liver injury[23-26] indicated that the pathological serum after severe liver injury might provide the above conditions. We developed the culture system using cholestatic serum obtained after ligation of common bile duct to induce liver lesions, to select and proliferate BDLSC from whole bone marrow cells *in vitro*. We named this medium system as "pathological microenvironment" selecting medium.

MATERIALS AND METHODS

Preparation of conditional selection medium

Preparation of cholestatic serum Sprague-Dawley (SD) rats weighing 200-250 g were performed common bile duct ligation and transection under general (ether) anesthesia to induce cholestasis. After 10 d, whole blood of each rat was collected and serum was separated. The serum was then subjected to liver function test, and inactivated and asepticized for culture use.

Ingredients of conditional selection medium Twenty mL/L, 50 mL/L, 70 mL/L, and 100 mL/L cholestatic serum were added into DMEM (Gibco) containing 20 mmol/L HEPES (Sigma), 10^{-7} mol/L dexamethasone (Sigma), and antibiotics to act as the conditional selection medium.

Selection, proliferation and differentiation of BDLSC

Culture of whole bone marrow cells Rat bone marrow cells were obtained by flushing femurs. The femurs were accessed through laparotomy to avoid contamination and to increase the cell yield. Bone marrow cells were suspended in DMEM and plated at the density of 1×10^9 cells/L onto culture dishes. DMEM enriched with 100 mL/L fetal bovine serum (FBS, Hyclone), 20 mmol/L HEPES, 10^{-7} mol/L dexamethasone, and antibiotics was used. Dishes were placed in a humidified incubator containing 50 mL/L CO_2-950 mL/L O_2 at 37 ℃.

Selection, proliferation and differentiation of liver stem cells Three days after the culture, the medium and suspended cells were discarded and replaced by conditional selection medium with various concentrations of cholestatic serum. Cells cultured in medium containing FBS were used as control. After 4 d, the media were enriched with 25 μg/L hepatocyte growth factor (HGF, Peprotech EC). Medium was replaced every 3 d and the cultures were maintained for about 2 wk.

Morphological and phenotypic markers of differentiated cells

Immunohistochemistry Bone marrow cells were cultured and selected with the conditional selection medium on 6-well dishes with cover glasses. Twelve days after replacement of the conditional selection medium, cover glasses and the above differentiated BDLSC were taken out and fixed with 40 g/L paraformaldehyde (Sigma) for 30 min at room temperature. The primary antibodies were goat anti-rat albumin, alpha-fetoprotein (AFP), and cytokeratin -8/18 (CK8/18) polyclonal antibodies. The slides were incubated with various primary antibodies at room temperature for 2 h in a humidified chamber. Blocking serum and biotinylated secondary antibody were matched with the primary antibody. Immunoperoxidase was stained and counterstained with DAB and Gill-I hematoxylin.

Electron microscopy Culture dishes were washed with PBS (pH 7.4), and fixed in cold 25 g/L glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 48 h. After fixation, cells were curetted and centrifuged to form aggregates. After postfixed in 10 g/L osmium tetroxide in 0.1 mol/L sodium cacodylate (pH7.4), cells were dehydrated in graded alcohols and embedded in low viscosity epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed under electron microscope.

Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) Total RNA was extracted from 1×10^6 differentiated cells by using Trizol (Promega). cDNA was prepared from 2 μg of total RNA in a buffer containing MMLV reverse transcriptase (Promega) and 0.5 μg random primer (Takara). The RNA was incubated at 70 ℃ for 5 min, at 37 ℃ for 1 h, and at 95 ℃ for 5 min. Samples of cDNA corresponding to the input RNA were amplified in PCR reaction buffers containing primers and LA Taq DNA polymerase (Takara). Primers used for amplification were hepatocyte nuclear factor-1α (HNF-1α), hepatocyte nuclear factor-3β (HNF-3β), CK18, CK19, albumin, AFP, transthyretin (TTR), and cytochrome P450-2b1 (CYP2b1). The sequences are listed in Table 1. After initial denaturation (at 94 ℃ for 5 min), 30 cycles of PCR were performed (at 94 ℃ for 30 s, annealing at the optimal temperature for each pair of primers for 30 s, at 72 ℃ for 30 s), and extension at 72 ℃ for 7 min. Amplified products were subjected to electrophoresis in 20 g/L agarose gels and stained with ethidium bromide. All the procedures were performed according to the manufacturer's instructions.

Function tests of differentiated cells

Periodic acid-Schiff staining for glycogen Differentiated cells were fixed in 950 mL/L ethanol for 10min, rinsed in dH_2O. Afterwards, cells were oxidized in 10 g/L periodic acid for 15 min and rinsed three times in dH_2O, then treated with Schiff's reagent for 30 min and rinsed in dH_2O for 10 min, stained with Mayer's hematoxylin for 1 min and rinsed in dH_2O.

Urea assay Urea concentrations were determined by colorimetric assay (640-A, Sigma). Differentiated cells were plated on 6-well dishes. Each well contained 1×10^6 cells. Urea concentrations were detected every day for 4 d according to the manufacture's instructions. Culture medium and blank tube were used as negative controls.

Table 1 Sequence of primers and length of fragments

Gene	Primer	Fragment (bp)
HNF-1α	S:5'-AGCTGCTCCTCCATCATCAGA-3'	138
	A:5'-TGTTCCAAGCATTAAGTTTTCTATTCTAA-3'	
HNF-3β	S:5'-CCTACTCGTACATCTCGCTCATCA-3'	68
	A:5'-CGCTCAGCGTCAGCATCTT-3'	
CK-18	S:5'-GCCCTGGACTCCAGCAACT-3'	70
	A:5'-ACTTTGCCATCCACGACCTT-3'	
CK-19	S:5'-ACCATGCAGAACCTGAACGAT-3'	83
	A:5'-CACCTCCAGCTCGCCATTAG-3'	
Albumin	S:5'-CTGGGAGTGTGCAGATATCAGAGT-3'	141
	A:5'-GAGAAGGTCACCAAGTGCTGTAGT-3'	
AFP	S:5'-GTCCTTTCTTCCTCCTGGAGAT-3'	145
	A:5'-CTGTCACTGCTGATTTCTCTGG-3'	
TTR	S:5'-CAGCAGTGGTGCTGTAGGAGTA-3'	152
	A:5'-GGGTAGAACTGGACACCAAATC-3'	
CYP2b1	S:5'-ACTTTCCTGGTGCCCACA-3'	157
	A:5'-TCCTTCTCCATGCGCAGA-3'	

RESULTS

Preparation of the conditioning selection medium

Ten days after the ligation of common bile duct, TBIL of the cholestatic rats was 107±48.2 μmol/L. The conditional selection media containing 20 mL/L, 50 mL/L, 70 mL/L, and 10 mL/L cholestatic sera had TBIL concentrations of 2.1 μmol/L, 5.3 μmol/L, 7.4 μmol/L and 10.8 μmol/L accordingly.

Bone marrow cells cultured in different concentrations of cholestatic serum

In routine culture condition, part of the bone marrow cells attached to the plate in about 3 d, and then became elongated and fibroblast-like, conforming to the mesodermal characteristics of their origin. After replaced by the conditional selection medium containing 20 mL/L cholestatic serum, the attached cells still survived while the rapid growth was hindered. Seven days after cultured in the medium, colonies were mainly composed of fibroblast-like cells. Only a few colonies acquired hepatocyte-like morphology, although HGF was added. In the presence of 50 mL/L cholestatic serum, a large number of the attached cells exfoliated within 3 d, while many small colonies appeared (Figure 1). These colonies were composed of small, undifferentiated cells in the center, and epithelioid cells at the periphery. The cells enriched with HGF proliferated rapidly. In 70 mL/L cholestatic serum condition, most of the cells exfoliated, fewer colonies appeared, and HGF could not stimulate their proliferation. When the concentration increased to 100 mL/L, all of the cells exfoliated and became apoptosis.

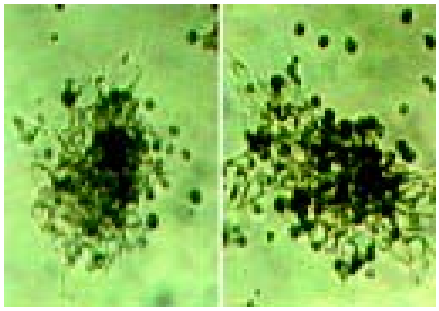

Figure 1 Cell colonies 3 d after selection. Polygonal surrounding cells could be seen.

Morphological evidence of BDLSC differentiation

During the first 3 d many colonies appeared in conditional selection culture containing 50 mL/L cholestatic serum. After enriched with HGF, the colonies enlarged, and the cells proliferated rapidly. Large hepatocyte-like colony-forming units (H-CFU) came into being in about 12 d. The H-CFU were composed of small, undifferentiated cells in the center, and large cells with regular multilateral contours, low nuclear-to-cytoplasmic ratio, and single round nuclei at the periphery. The differentiated cells formed cords or trabeculae resembling the hepatocyte cords in hepatic lobules (Figure 2). The largest colonies contained 1×10^6 cells and could be observed macroscopically, whereas the small colonies were about 30-50 cells. The central undifferentiated cells could still form H-CFU when they were picked out and cultured in the selection medium containing 50 mL/L cholestatic serum and HGF in about 1 wk.

Ultrastructurally, the differentiated cells were rich in endoplasmic reticulum and ribosomes and contained abundant ellipsoid mitochondria (Figure 3), which were different to the control cells. These ultrastructural features are typical of adult hepatocytes.

Phenotypic markers of differentiated cells

The identification of hepatocyte-like cells in 12-day-old selection medium cultures prompted us to analyze these cells for biochemical evidence of hepatocytic differentiation. Immunohistochemistry was performed in cells grew on the cover glasses for the existence of albumin (Figure 4), AFP and CK8/18, the characteristic proteins expressed during hepatocyte development. Immunoperoxidase staining for these proteins revealed diffuse cytoplasmic staining of hepatocte-like cells. In contrast, the fibroblast-like cells grew in the routine cultures did not express these proteins. RT-PCR further convinced the hepatocytic characteristics of differentiated cells as the results showed that there were mRNA transcripts of HNF-1α, HNF-3β, albumin, AFP, CK-18, CK-19, TTR, and CYP2b1, all of which were hepatic specific (Figure 5).

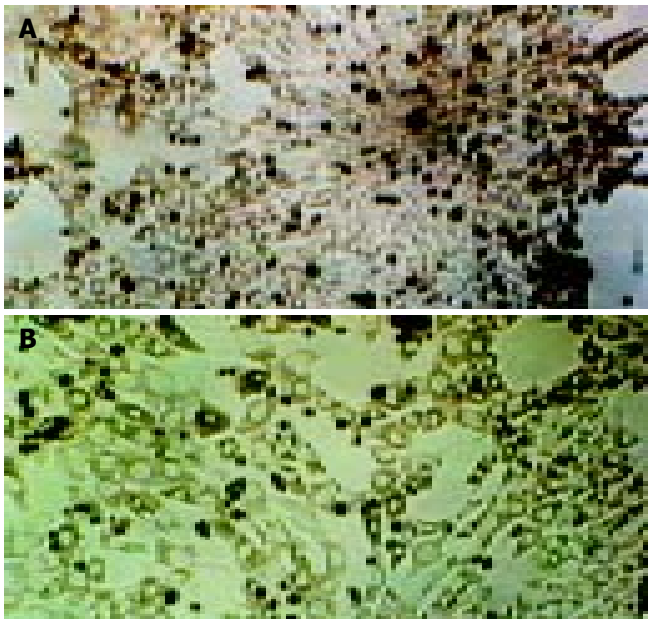

Figure 2 Appearance of hepatocyte-like colony forming units (H-CFU) 12 d after selection A: H-CFU, undifferentiated round cells in the center, surrounded by polygonal hepatocyte-like cells B: Regular arrangement of surrounding hepatocyte-like cells similar to the cords of hepatocytes.

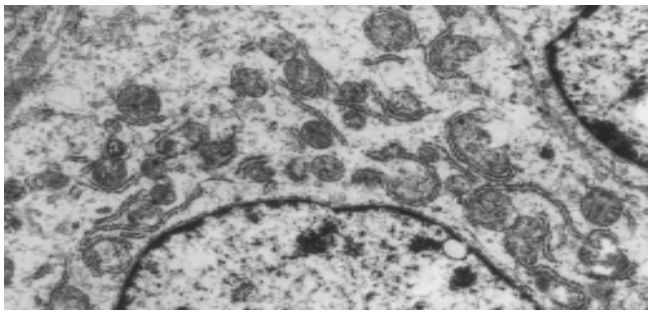

Figure 3 Ultrastructure of hepatocyte-like cells. 9 000×.

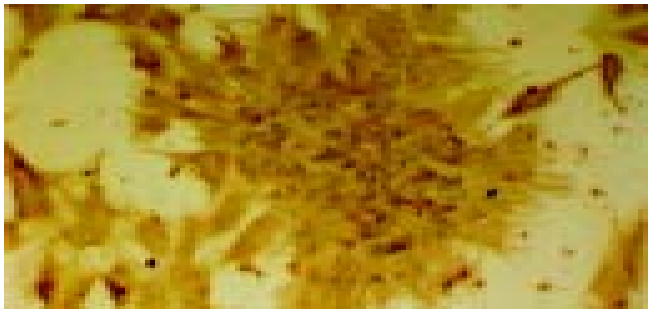

Figure 4 Positive staining of albumin immunohistochemistry 12 d after selection. ABC staining 200×.

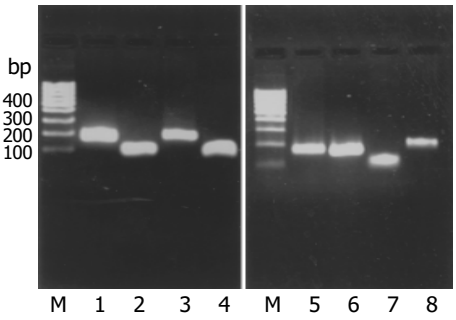

Figure 5 RT-PCR results M: marker, 1: AFP, 2: CK-18, 3: CYP2b1, 4: HNF-3β, 5: Albumin, 6: TTR, 7: CK-19, 8: HNF-1α.

Function tests of differentiated cells

We analyzed the levels of glycogen storage by periodic acid-Schiff (PAS) staining. Glycogen storage was seen as accumulation of magenta staining in the cytoplasms of hepatocyte-like differentiated cells (Figure 6). The control cells did not show similar staining. Urea production and secretion by hepatocyte-like cells were measured at various time points after the differentiated morphology appeared. Bone marrow cells and fibroblast-like cells did not produce urea. Urea assay revealed that urea concentration in the medium increased in a time-dependent manner (Figure 7).

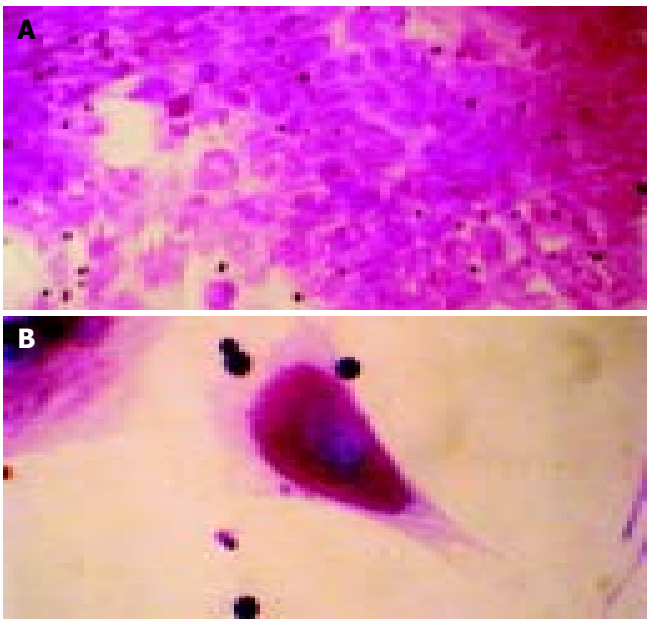

Figure 6 PAS staining of hepatocyte-like differentiated cells. The cells were positive in the cytoplasm (A) 200×, (B) 400×.

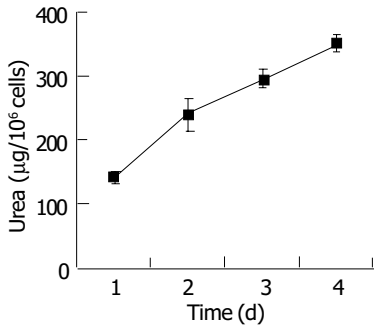

Figure 7 Urea synthetic function of bone marrow-derived liver stem cells (mean±SD, n = 4).

DISCUSSION

Since the results of cross-sex and cross-strain bone marrow and whole liver transplantation experiments indicated that the bone marrow might be a source of hepatocyte progenitors, great interests have been aroused in identification and isolation of liver stem cells from bone marrow cells. Until recently, several subsets of bone marrow cells have been found to have the potential to differentiate into hepatocytes, such as KTLS cells (c-kit^{high}Thy^{low}Lin$^-$Sca-1$^+$)[20], β_2-m$^-$Thy-1$^+$ cells[27,28], multipotent adult progenitor cells (MAPC, CD44$^-$CD45$^-$HLA$^-$c-kit$^-$)[18], and C1qR$_p^+$ Lin$^-$CD45$^+$CD38$^-$CD34$^{+/-}$ cells[29]. These stem cells could express different surface markers, and might represent different stages or different branches along the differentiation and developmental processes of bone marrow stem cells. So far, the characteristic surface markers of BDLSC and their pedigrees in the derivation of bone marrow stem cells remain obscure. Any subset sorting methods based on phenotypes would certainly lose other stem cells that have the ability to differentiate into hepatocytes. Furthermore, the sorting manipulations of the above stem cells with complicated surface markers are very difficult.

Studies on liver regeneration showed that liver stem cells could correspond to and repair liver injury only under certain severe pathological circumstances such as extensive liver necrosis due to chemical injury or hepatocytes treated with chemicals that could block their proliferation[24]. Based on this mechanism, we assumed to imitate the *in vivo* pathological microenvironment of liver injury in culture of whole bone marrow cells, in order to provide specific proliferation signals for BDLSC, and to avoid resource loss of liver stem cells and complicated manipulation due to subset sorting. The key of this method is to develop an optimal "pathological microenvironment". Olynyk *et al*. demonstrated that in the cholestatic model of common bile duct ligation, intrahepatic liver stem cells showed great proliferation[25]. Avital *et al*. recently reported that β_2-m$^-$Thy-1$^+$ cells in bone marrow could differentiate into cells with hepatocyte-like phenotypes when co-cultured with adult hepatocyes in a medium containing cholestatic serum[27], suggesting that there are stimulating factors in cholestatic serum. In our experiments, we used pathologically conditional selection medium containing cholestaic serum to culture whole bone marrow cells. Results showed that in the selection medium, a large number of the bone marrow cells exfoliated, only part of the remaining cells presented colonal growth during the first 3 d. When enriched with HGF on d 4, rapid proliferation and differentiation emerged, and H-CFU with various size appeared in 2 wk. The morphology and phenotypic markers manifested that the cells of H-CFU were similar to embryonic hepatoblasts. They expressed markers of embryonic hepatocytes (AFP, albumin and CK18), biliary cells (CK19), hepatocyte functional proteins (TTR and CYP2b1), and hepatocyte nuclear factors (HNF-1α and HNF-3β). HNF-1α is a transcription factor required for subsequent hepatocyte differentiation, and transcription factor HNF-3β was known to be important in endoderm specification. These results proved that H-CFU were colonies of liver stem cells.

In the culture system containing cholestatic serum, the concentration of "pathological microenvironment" needs to be considered. We found that 100 mL/L cholestatic serum was fatal to all of the bone marrow cells, 70 mL/L cholestatic serum prevented the colonies from growth and proliferation, 20 mL/L cholestatic serum could not inhibit the growth of other cells, while only 50 mL/L cholestatic serum acquired pure H-CFU. The differences can be explained by the toxic contents of cholestatic serum, which are the metabolic products cumulated under the circumstances of common bile duct obstruction and hepatic insufficiency, including bilirubin, bile acid, endotoxin, and ammonia, *etc*. When cholestatic serum was confined to the optimal concentration, BDLSC, possessing functions of bilirubin conjugation and detoxification[30], could metabolize these toxic

products and survive, and selectively proliferate responding to the signal of liver injury. On the other hand, non-BDLSC could not adapt to the environment and resulted in apoptosis. To confirm that if the H-CFU had functional characteristics of hepatocytes, we tested the glycogen and urea synthesis functions of the cells and proved that the selected cells possessed hepatocyte-like functions. Therefore, the "pathological microenvironment" selecting medium based on cholestatic serum can select BDLSC in two ways, providing selective proliferation signals for BDLSC and eliminating non-BDLSC, so as to purify BDLSC.

We demonstrated that a "pathological microenvironment" selecting medium could select, proliferate and differentiate BDLSC from whole bone marrow cells *in vitro*. This method can not only provide a new and effective way for the isolation and purification of extrahepatic liver stem cells, but also supply a clue to obtain a readily available alternate source of cells for clinical hepatocyte therapy.

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