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The existence of epithelial-to-mesenchymal cells with the ability to support hematopoiesis in human fetal liver

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Abstract

The fetal liver is the major hematopoietic organ during mid-gestation, and it is also a source of stem cells that exist in a complex environment. In this study, we isolated a population of actively replicating cells with the characteristic of the epithelial-to-mesenchymal transition (EMT) from fetal liver. These cells were identified with the epithelial markers, including α-fetoprotein (AFP), albumin (ALB), cytokeratins (CK) 7, and CK18, as well as the mesenchymal markers, such as α-smooth muscle actin (ASMA), CD29, CD44, CD49, CD54, collagen I and osteopontin (OPN). Furthermore, they also expressed some hematopoiesis-related genes. In addition, the cell population had the ability to retain hematopoietic stem cells (HSCs) in an undifferentiated state in vitro during cytokine-stimulated proliferation. These results provide an insight about early human liver development and may also help to understand hematopoiesis in the fetal liver.

Keywords: Human fetal liver; Identification; Epithelial-to-mesenchymal transition; Hematopoietic supportive ability

1. Introduction

In mice, the liver develops from the ventral foregut endoderm at embryonic day 8 (ED8). Hepatic cells are directly descended from the endoderm and the precardiac tissue, and the septum transversum mesenchyme plays a crucial role in hepatic specification (Zaret, 2000; Jung et al., 1999). Hepatic cells, known as hepatoblasts during early liver development, are bipotential stem cells that give rise to both hepatocytic and biliary lineages. Hepatic oval cells that occur after liver damage in adult rats are also bipotential stem cells and they are similar to hepatoblasts (He et al., 2003). Hepatoblasts express AFP, ALB and some special ribosomal proteins. Although mature hepatocytes don't express some hepatoblast markers, such as AFP, during liver development, they still express CK18 and ALB. Recent research has demonstrated that the bone marrow-derived hematopoietic stem cells can be recruited and induced to differentiate into hepatocytes and contribute to restoring the damaged liver (Petersen et al., 1999; Alison et al., 2000).

During the 5th week of human gestation, HSCs migrate from the yolk sac to the fetal liver, which becomes the major hematopoietic site through mid-gestation (Timens and Kamps, 1997). The HSC population undergoes a period of intensive expansion and generates sufficient stem cells for blood cell production throughout life (Orkin, 1996). Hepatic progenitors and other cell lineages constitute the hematopoietic niche (Hata et al.,

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Nomenclature

AFP	α-fetoprotein
ALB	albumin
ASMA	α-smooth muscle actin
CK	cytokeratins
OPN	osteopontin
HSC	hematopoietic stem cell
FITC	fluorescein isothiocyanate
RT-PCR	reverse transcriptase-polymerase chain reaction
FACS	fluorescence-activated cell sorting
EMT	epithelial-to-mesenchymal transition
UCB	umbilical cord blood

1993). Using AFT024, Moore et al. (1997) demonstrated that stromal cells derived from mouse fetal liver were able to support HSC growth. They also studied the molecular profile of AFT024 to further elucidate the stem cell niche (Hackney et al., 2002). However, little is known about the human fetal liver microenvironment.

We have isolated fibroblast-like cells from human fetal liver by enzymatic digestion, characterized their phenotypes by flow cytometric analysis, and examined the expression of several genes related to the support of hematopoiesis using the reverse transcription polymerase chain reaction (RT-PCR). Interestingly, we found that human fetal liver contains some cells that have an epithelial-to-mesenchymal transition (EMT) phenotype and the ability to support hematopoiesis.

2. Materials and methods

2.1. Materials

Human blood and fetal tissue collections for research was approved by the Research Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences. Experiments complied with national guidelines regulating the use of fetal tissues for research.

Monoclonal antibodies against human albumin and AFP were obtained from Sigma. Antibodies against human CK18 and CK7 were purchased from DAKO, and fluorescein isothiocyanate (FITC)-labeled secondary goat anti-mouse or goat anti-rat antibodies were from Dako (Carpinteria, CA, USA). Anti-FITC-labeled α-smooth muscle actin was from Sigma. Antibodies against human antigens, including CD14, CD29, CD31, CD34, CD44, CD45, CD49d, CD54 and HLA-DR, and isotype, were purchased from Becton Dickinson.

2.2. Isolation of fibroblast-like cells from human fetal liver

Human fetal liver tissue was minced in Hank's balanced salt solution (HBSS) (Gibco BRL, Grand Island, NY, USA) and passed through a wide bore syringe. Tissue was digested with 0.10% (w/v) collagenase IV (Gibco BRL, Grand Island, NY, USA) for 30 min at 37 °C in a shaking bath. The dissociating cells were collected and centrifuged at $500 \times g$ for 5 min at 4 °C. The pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA), 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 10^{-7} M dexamethasone, 5 μg/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were seeded on culture dishes coated with collagen. At 80% confluence they were digested with 0.25% trypsin containing 0.01% EDTA for 2 or 3 min at 37 °C and cultured in 25 cm^2 flasks.

2.3. Flow cytometric analysis of cultured fibroblast-like cells

For analysis of intracellular antigens, cells grown in flasks were treated with trypsin and a total of 1×10^5 cells were fixed in acetone for 2–5 min at 4 °C. After centrifugation at 1000 rpm for 5 min at 4 °C, the pellets were suspended in 100 μl PBS and incubated with primary antibodies for 60 min at 37 °C. Centrifugation was performed at 1000 rpm for 10 min and followed by extensive washes with PBS. The pellets were resuspended in 100 μl PBS to prepare for cell suspension. Binding of primary antibody was detected using an FITC-labeled secondary antibody. For analysis of membrane antigens, cell suspension was stained with fluorescein-conjugated antibodies for 45 min at 37 °C. Cells were acquired by flow cytometry (Becton Dickinson, San Jose, Ca, USA) with the record of 10,000 events for each analysis, and the data were analyzed with Cell-Quest software (Becton Dickinson, San Jose, Ca, USA).

2.4. RNA extraction and RT-PCR

Total RNA was extracted from cells after culture for 7 days using TRIzol (Life Technologies, Inc. Galthersburg, MD, USA) according to the manufacturer's protocol. The concentration and purity of total RNA were evaluated by measuring the 260/280 nm ratios. Total RNA was reverse-transcribed into the first strand cDNA in a reaction primed by oligo(dT) primer. The RT reaction was performed for 45 min at 55 °C and stopped by heat inactivation for 5 min at 85 °C. cDNA samples were subjected to PCR amplification with specific primers under linear conditions. The primers used in the present study were shown in Table 1. After the amplification, each

Table 1
PCR primer base pairs used in this study

Gene name	Sequence
SDF	Forward: 5′-AACGCCAAGGTCGTGGTCGTGCTG-3′
	Reverse: 5′-CACATCTTGAACCTCTTGTTTAAAAGC-3′
VEGF	Forward: 5′-TCGGGCCTCCGAAACCATGA-3′
	Reverse: 5′-CCTGGTGAGAGATCTGGTTC-3′
KL	Forward: 5′-GACAGCTTGACTGATCTTCTGGAC-3′
	Reverse: 5′-ACTGCTGTCATTCCTAAGGGAGCT-3′
FL	Forward: 5′-AACAACCTATCTCCTCCTGCT-3′
	Reverse: 5′-GGCACATTTGGTGACAAAGTG-3′
TPO	Forward: 5′-TGCTCCGAGGAAAGGTGCGTT-3′
	Reverse: 5′-GGGAAGAGCGTATACTGTCCA-3′
Jagged 1	Forward: 5′-GATCCTGTCCATGCAGAACG-3′
	Reverse: 5′-GGATCTGATACTCAAAGTGG-3′
Dlk-1	Forward: 5′-CGGGATCCCTCCACACAGATTCTCCTG-3′
	Reverse: 5′-CGGAATTCTTAGATCGGCTCTGTGCAGTAG-3′
Shh	Forward: 5′-ACTGGGTGTACTACGAGTCCAAGG-3′
	Reverse: 5′-AAAGTGAGGAAGTCGCTGTAGAGC-3′
Wnt-5a	Forward: 5′-ACACCTCTTTCCAAACAGGCC-3′
	Reverse: 5′-GGAATTGTTAAACTCAACTCTC-3′
Collagen I	Forward: 5′-AGGGCTCCAACGAGATCGAGATCCG-3′
	Reverse: 5′-TACAGGAAGCAGACAGGGCCAACGTCG-3′
Collagen II	Forward: 5′-ATGATTCGCCTCGGGGCTCC-3′
	Reverse: 5′-CATTACTCCCAACTGGGCGC-3′
OPN	Forward: 5′-TTGCTTTTGCCTCCTAGGCA-3′
	Reverse: 5′-GTGAAAACTTCGGTTGCTGG-3′
β-actin	Forward: 5′-TCATGTTTGAGACCTTCAA-3′
	Reverse: 5′-GTCTTTGCGGATGTCCACG-3′

reaction mixture was analyzed by 1.0% agarose gel electrophoresis and visualized by ethidium bromide staining. β-actin served as a positive control.

2.5. Co-culture of fetal liver cells and $CD34^+$ hematopoietic cells from human umbilical cord blood

Fetal liver cells were seeded in culture dishes coated with 1% gelatin and irradiated with 80 Gray when they became confluent. $CD34^+$ hematopoietic cells were isolated from human umbilical cord blood by MACS. The cells were expanded for 7 days in medium supplemented with 10 ng/ml stem cell factor (SCF), 10 ng/ml IL-6, 10 ng/ml TPO and 10 ng/ml FL, with or without the irradiated fetal liver cells. The cells were fed every 3 days and harvested on day 7. Total cell numbers were calculated and the $CD34^+$ and $CD34^+CD38^-$ subpopulation were analyzed by flow cytometry.

3. Results

3.1. Morphological characteristics of the isolated liver cells

Mononuclear cells (0.6×10^7–3×10^8 from each fetal liver sample) were isolated with a viability of up to 95% as estimated by Trypan blue exclusion. The initial cell population consisted of hematopoietic cells and epithelial cells. These cells were allowed to adhere overnight and the non-adherent cells were removed when the medium was changed. The adherent cells were seen to be fibroblast-like (Fig. 1).

3.2. Phenotypic properties of the isolated fibroblast-like cells

As shown in Fig. 2, most of the fibroblast-like cells expressed AFP and ALB, indicating that they possess epithelial characteristics. To further clarify their characteristic phenotypes, we analyzed the expression of other epithelial markers (CK18 and CK7) and the mesenchymal marker (ASMA). Interestingly, we found that about 52% of the cells expressed both CK7 and ASMA (Fig. 3b), and more than 40% of the cells expressed both CK18 and ASMA (Fig. 3c).

The mRNA expression of mesenchymal markers, such as OPN, collagen I, and collagen II was determined in fibroblast-like cells by RT-PCR. Fig. 4 shows high levels of expression of OPN and collagen I mRNA, but collagen II mRNA expression was undetectable. Thus, the fibroblast-like cells expressed both epithelial and mesenchymal markers.

We next analyzed the phenotypic characteristics of second or third passage cells to confirm their identity. Flow cytometric assays revealed that these cells were

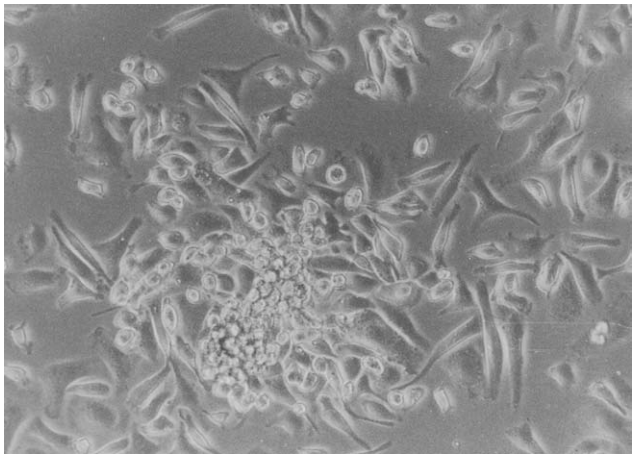

Fig. 1. Phase-contrast micrographs of the primary culture of fibroblast-like cells derived from human fetal liver. Magnification: ×100.

negative for CD34 and CD45, indicating that they were non-hematopoietic origin, so hematopoietic cells had been eliminated during cultivation (Fig. 5). The cells were also negative for CD14, CD31 (PECAM-1), and HLA-DR (Fig. 5); but positive for integrins CD29 (integrin-β1), CD49d (integrin-α4), the hyaluronate receptor CD44, and CD54, an intercellular adhesion molecule. This was consistent with the immunophenotype of mesenchymal stem cells (MSCs) derived from mouse fetal liver, bone marrow, and umbilical cord blood (Campagnoli et al., 2001; Lee et al., 2003).

3.3. Fetal liver fibroblast-like cells support hematopoiesis

The $CD34^+$ cells became apoptotic when cultured in medium without cytokines (data not shown), but survived and proliferated when cytokines were added to the medium. In the presence of cytokines alone, the cell number increased 21.7-fold, but in the presence of both feeder cells and cytokines the cell increase was only 9.3-fold (Table 2). Flow cytometric analysis showed that $CD34^+$ progenitors represented about 27.5% of the total cell population after 7 days of culture with feeder cells and cytokines; in the presence of cytokines alone they represented only 11.36% of cells. The absolute numbers of $CD34^+$ progenitor cells were not significantly different between these two conditions. The $CD34^+CD38^-$ populations represented, on an average, 21.31% and 7.39% of the total cell numbers in culture without and with feeder cells, respectively (Table 2).

3.4. Gene expression analysis of fetal liver fibroblast-like cells

Human fetal liver cells expressed wnt5A and Dlk-1, which are members of the Wnt and Notch signal pathways, respectively, but did not express jagged 1 or Shh as detected by RT-PCR. We also examined the expression of genes that regulate the development of HSCs: as shown in Fig. 6, the cells expressed VEGF, KL, FL, SDF-1 but not TPO.

4. Discussion

The adult liver is the center of metabolism and has important functions, including the synthesis of serum proteins and homeostasis. However, the fetal liver has little such activity. Transient hematopoiesis occurs in the

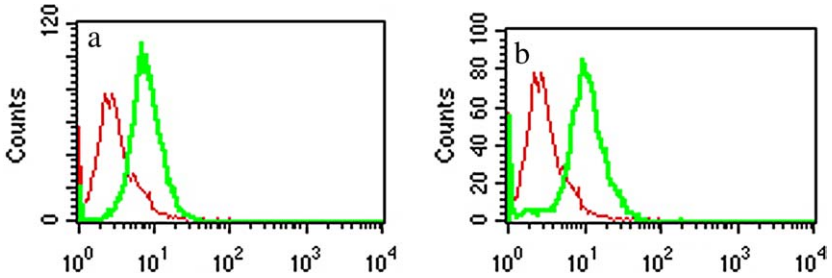

Fig. 2. Phenotype analysis of fibroblast-like cells from human fetal liver. FACS analysis showed expression of epithelial markers AFP (a) and ALB (b) in freshly isolated or third passage cells. Red line indicates control immunoglobulin; green line denotes specific antibody.

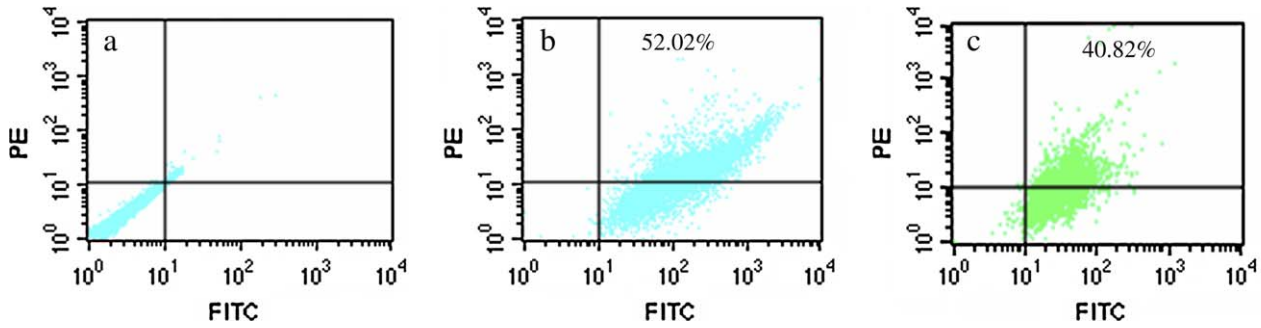

Fig. 3. The expression of mesenchymal markers in fetal liver cells. FACS analysis showed that 50.02% of the cells expressed both ASMA and CK7 (b), and up to 40.82% expressed both ASMA and CK18 (c). Replacement of the primary antibodies with PE-labeled and FITC-labeled IgG served as a negative control (a).

fetal liver, a principal site of stem cell expansion, to yield the entire complements necessary for a lifetime. Hematopoiesis decreases during the liver development. Hepatic and other cell types make up the niche in which hematopoietic stem cells develop. The initial event in liver development is the commitment of the foregut endoderm to the hepatic lineage, which is characterized by two liver specific epithelial markers, ALB and AFP (Zaret, 2000). In the present study, we isolated fibroblast-like cells with unique phenotypic characteristics from human fetal liver. The expression of AFP and ALB by these cells indicated an epithelial character (Alison, 1998), but they also expressed the mesenchymal markers, namely ASMA, OPN, and collagen I (Charbord et al., 2002), and the epithelial markers, including CK18 and CK7. Epithelial-to-mesenchymal transition is a highly conserved process based on morphogenesis in multicellular organisms (Thiery, 2002). Thus, these results indicated that human hematopoietic fetal liver contains the EMT cell population seen in 11.5-dpc murine liver (Chagraoui et al., 2003).

In view of their spatial relationship to the hematopoietic cells, epithelial cells (developing hepatocytes) were first considered as supporting cells (Medlock and Haar, 1983). Murine-stable hepatocyte cell lines (MMH) expressed hematopoietic cytokines and support in vitro

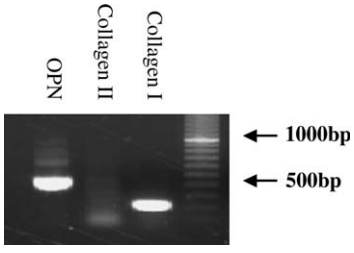

Fig. 4. RT-PCR analysis of OPN and collagen I gene expression in human fetal liver cells. RNA was extracted from second or third passage cells. PCR products, as described in Section 2, were separated by electrophoresis on a 1.0% agarose gel and stained with ethidium bromide. M, 100 bp ladder.

hematopoiesis (Aiuti et al., 1998). Vascular smooth muscle-like mesenchymal cells also constitute a microenvironment in the fetal liver, and a hematopoietic-supporting stromal cell line from mouse fetal liver has been established (Moore et al., 1997). Flow cytometric analysis demonstrated that the cells isolated in the present study are positive for various integrins and matrix receptors such as CD29, CD44, CD49 and CD54, which is consistent with the immunophenotype of mesenchymal cells from other hematopoietic sites. Therefore, we proposed that the EMT cells derived from human fetal liver might be involved in constituting the microenvironment in which HSCs develop. For these reasons, we used human fetal liver cells as a feeder layer during standard cytokine-based in vitro UCB expansion. We found that these cultured fetal liver cells can somehow slow down the proliferation of hematopoietic progenitor cells stimulated by cytokines but do not affect the self-renewal properties of hematopoietic progenitors.

It has been shown that signals from the microenvironment such as soluble factors and adhesion/matrix proteins play a vital role in the division of stem cells. The bone marrow is the principal location of stem cells in the adult and provides cytokines and signals for the determination of stem cell fates. Several groups have studied the expansion of hematopoietic progenitors on the feeder layers in vitro with various cytokines (Moore et al., 1997; Punzel et al., 2003). Recently, Zhang et al. (2003); and Calvi et al. (2003) provided insights into the nature of HSC niches in adult animals and confirmed that osteoblasts, the cells that reside in the bone marrow, have a crucial role in HSC regulation. There is much evidences that the Wnt signaling pathway is critical for the self-renewal of HSC in vitro and in vivo (Reya et al., 2003; Murdoch et al., 2003). Bhardwaj et al. (2001) have demonstrated that sonic hedgehog (Shh) treatment induced the expansion of human pluripotent repopulating cells in immunodeficient mice and their functions depended on the signal downstream of BMP. Several studies also supported a broad physiological role

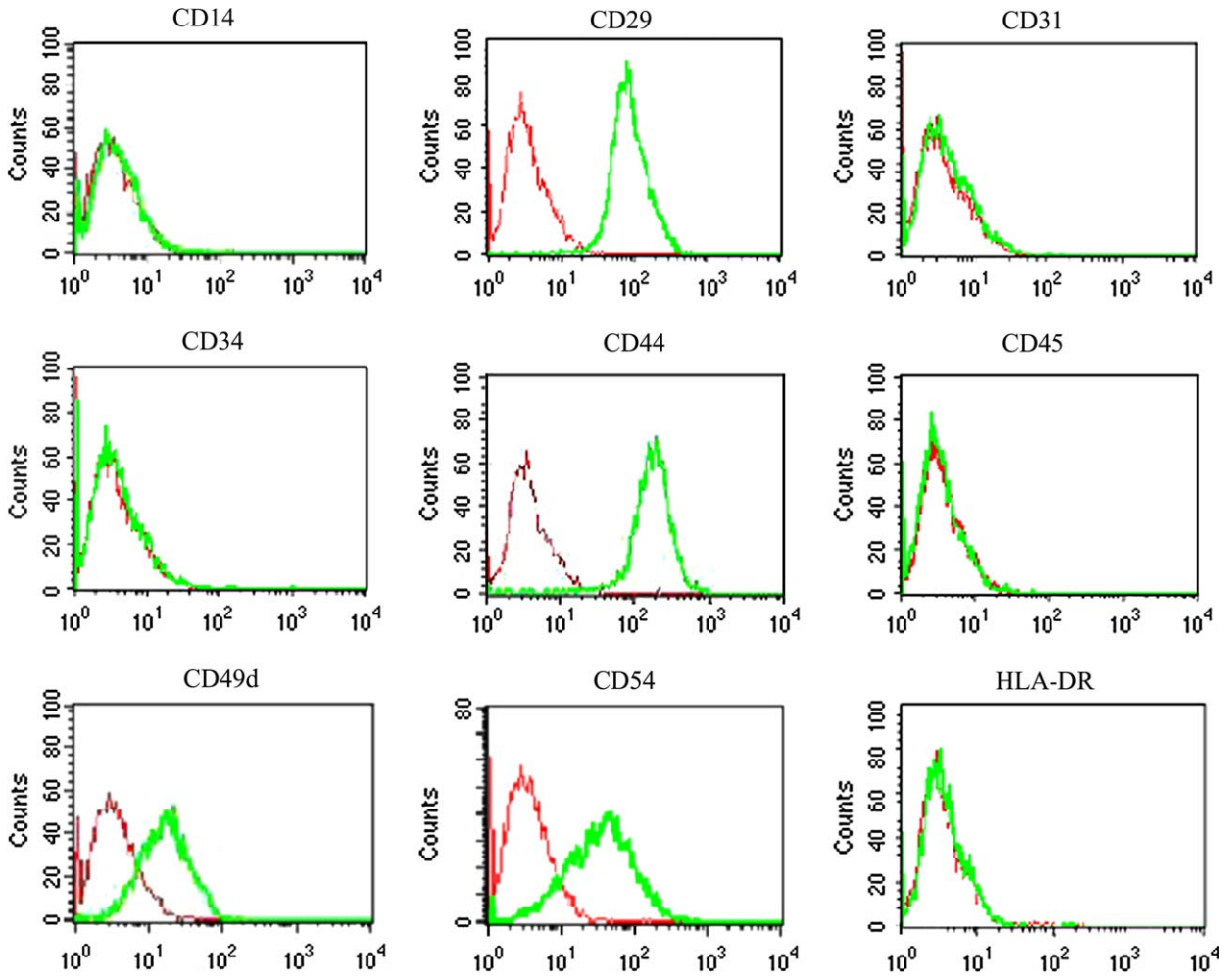

Fig. 5. Immunofluorescence detection of the fibroblast-like cells (third passage). The cells were labeled with antibodies against CD14, CD29, CD31, CD34, CD44, CD45, CD49d, CD54 and HLA-DR and were analyzed using an FACS-Calibur. Red line indicates control immunoglobulin; green line denotes specific antibody.

for the Notch signal in regulating hematopoiesis, favoring self-renewal over differentiation (Varnum-Finney et al., 2000). The human fetal liver cells also expressed some factors such as Wnt5A and DLK, a Notch ligand. Surprisingly, these fetal liver cells do not promote self-renewal of hematopoietic progenitor cells, shown by the fact that providing cultured fetal liver cells as feeder cells does not increase the hematopoietic stem cell number. However, the EMT cells that we obtained from human fetal liver expressed the junction molecule, SDF-1, indicated that they may play a pivotal role in recruiting HSCs and retaining them in a primitive state (Juarez and Bendall, 2004).

This study revealed a cell population with a complex phenotype, expressing both epithelial and mesenchymal markers, which could be related to the functional switch of fetal liver from hematopoietic site to metabolic organ.

Table 2
The effect of cytokines and EMT cells on the growth of nucleated cells, $CD34^+$ cells and $CD34^+CD38^-$ cells

Culture condition	Nucleated cells (fold)	$CD34^+$ cells (%)	$CD34^+CD38^-$ cells (%)
Cytokines	21.7 ± 14.69	11.36 ± 1.98	7.39 ± 1.81
Cytokines ± EMT cells	9.3 ± 6.58	27.49 ± 2.23	21.31 ± 1.99

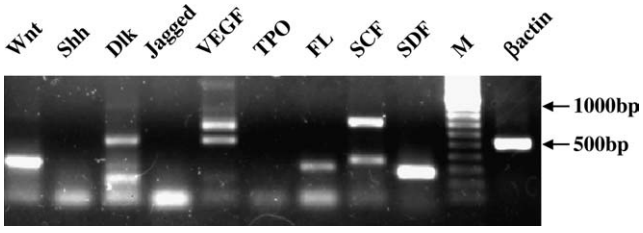

Fig. 6. RT-PCR analysis of expression of hematopoiesis-related genes in the fibroblast-like cells from human fetal liver. PCR products, as described in Section 2, were separated by electrophoresis on a 1.0% agarose gel and stained with ethidium bromide. M, 100 bp ladder.

This could offer an insight into the development of the human fetal liver.

Acknowledgements

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