Research Article

Differentiation of Human Umbilical Cord Lining Membrane-Derived Mesenchymal Stem Cells into Hepatocyte-Like Cells

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Background. Mesenchymal stem cells (MSCs), isolated from bone marrow, adipose tissue, and umbilical cord tissue, have been known to differentiate into hepatocyte-like cells. MSCs can also be easily obtained from umbilical cord lining membrane (CLMSCs). CLMSCs are more primitive MSCs than those isolated from other tissue sources. Objectives. The aim of this study was to investigate the in vitro differentiation of CLMSCs into hepatocyte lineage. Materials and Methods. In this study, CLMSCs were isolated through a tissue attachment method. Cells were characterized for expression of MSC-specific markers and differentiation potency. CLMSCs were induced to differentiate into hepatocytes by a simple two-step protocol. Differentiated cells were examined for the expression of hepatocyte-specific markers and hepatocyte functions. Results. CLMSCs expressed MSC-specific markers and differentiated into adipocytes and osteoblasts. RT-PCR, real-time qRT-PCR, Western blot, and immunocytochemistry analyses demonstrated that differentiated CLMSCs, having hepatocyte-like morphology, expressed several liver-specific markers, such as ALB, AFP, CK18, and CK19, at both mRNA and protein levels following hepatocyte differentiation. Furthermore, periodic acid-Schiff staining and low-density lipoprotein (LDL) uptake assay showed that differentiated cells could store glycogen and uptake LDL. Conclusion. This study demonstrated that CLMSCs can differentiate into functional hepatocyte-like cells. CLMSCs can serve as a favorable cell source for tissue engineering in the treatment of liver disease.

1. Introduction

Mesenchymal stem cells (MSCs) are referred to as highly proliferating and adherent fibroblastic cells featuring a unique expression profile of cell surface molecules. Like any other stem cells, they own capacity of self-renewal and multilineage differentiation [1]. MSCs can be obtained from various tissue sources, such as bone marrow (BM), adipose tissue (AD), and umbilical cord (UC) [2, 3]. BM is the main source for MSC isolation and BM-derived MSC (BMMSCs) have been extensively studied. However, the disadvantages of autologous BMMSCs, such as a highly invasive procedure for the donors and a significant decrease in both quantity and differentiation potential of cells with age, have restricted their use in tissue engineering and organogenesis [4]. Hence, it has accelerated the search for alternative sources of stem
cells. In recent years, many research groups have focused on various types of umbilical cord-derived MSCs (UCMSCs), including UC matrix stem cells, UC perivascular stem cells, UC stroma cells, Wharton’s jelly MSCs, and cord lining membrane MSCs (CLMSCs) because of many advantages [5]. They were more primitive MSCs than those isolated from other tissue sources and possessed immunosuppressive properties, which have been widely used for transplantation. Furthermore, they also had their higher proliferation potential and differentiation capacity, noninvasive accessibility, and lack of ethical controversy [6, 7]. Therefore, these advantages enable UCMSCs to be an attractive cell source for not only stem cell-based therapeutics but also tissue engineering.

Even though liver is a highly regenerative organ, liver diseases which are caused by several factors (e.g., infectious agents, autoimmune attack, malignant transformation, and inborn genetic deficiencies) can damage regenerative potential of mature hepatocytes or hepatic progenitor cells and cause hepatocyte dysfunction leading to eventual liver failure [8]. It is well known that whole liver transplantation (WLT) or hepatocyte transplantation (HT) are the main therapeutic procedures for patients with acute or chronic end stage liver disease. Despite technical advances, significant morbidity and mortality rates remain. WLT is severely limited by the shortage of donor organs and complications associated with rejection and immunosuppression, increasing mortality of patients [9]. Meanwhile, HT is a mean of temporary liver support in cases of acute liver failure or metabolic liver disease because of its low availability and transplantation-inconsistent liver cell sources (steatosis, cirrhosis, fibrosis, and other reasons) [10]. The replacement of diseased hepatocytes and the regeneration of functional hepatocytes by the stimulation of endogenous or exogenous stem cells is the main alternative and promising therapy for the treatment of liver diseases [11].

Following this report, many studies have been published about the potential of MSCs, including bone marrow-derived MSCs, adipose-derived MSCs, and umbilical cord tissues-derived MSCs [8, 12–14] to differentiate into hepatocyte-like cells in vitrunder the appropriate culture conditions. Most commonly used growth factors and cytokines in hepatic differentiation are hepatocyte growth factor (HGF), fibroblast growth factor-4 (FGF-4), and oncostatin M (OSM). However, these growth factors, cytokines, and biochemicals have been treated at the same time in many published reports. More recently, some researchers have tried the sequential differentiation which is composed of induction and maturation steps because the growth factors, cytokines, and biochemicals play different roles in the liver development and regeneration as previously described [12, 13, 15]. In addition, in vivo studies have also demonstrated that MSCs preserve liver of damages by engraftment [16] or indirect effects [17]. In this study, cord lining membrane-derived MSCs (CLMSCs) were also induced to differentiate into hepatocyte-like cells by a simple two-step protocol with initiation and maturation media. Differentiated cells were examined for the expression of hepatocyte-specific markers and some hepatocyte-specific functions.

2. Materials and Methods

2.1. Isolation and Culture of CLMSCs. Umbilical cord (UC) samples were collected from pregnant women who were informed with a written consent form at Hung Vuong Hospital, Ho Chi Minh City. These samples were negative for HBV, HCV, and HIV. Residual blood from UC was removed and the UC was cut into 2–4 cm long pieces. The epithelium was separated from individual pieces to expose the underlying Wharton’s jelly. After removal of the UC veins and arteries, the residual tissue pieces were incubated at 37°C, 5% CO2 with DMEM (GIBCO Invitrogen, USA) for approximately 4–6 hours. The Wharton’s jelly absorbed DMEM; therefore, it can be distinguished from the membrane of umbilical cord. The Wharton’s jelly was eliminated using razor blades; the pieces of cord lining membrane were rinsed and chopped into 2-3 mm3 explants. Then, they were transferred to culture dishes premoistened with 5 mL DMEM/F12 (GIBCO Invitrogen, USA) supplemented with 15% FBS, 10 ng/mL bFGF, 10 ng/mL EGF, IX ITS, and 2 mM L-glutamine (all bought from Sigma-Aldrich, USA) [18]. The explants were removed after 10–12 days. When reaching about 80% confluency, cells were subcultured for propagation.

2.2. Immunophenotypical Analysis of CLMSCs. The markers of MSC candidates were determined by flow cytometry techniques. Cells were trypsinized, washed, and resuspended in PBS at a concentration of 106 cells/mL. They were then stained for 20 minutes at 4°C in the dark with FITC or PE-conjugated antibodies as follows: anti-CD14-PE, anti-CD19-FITC, anti-CD34-FITC, anti-CD45-FITC, anti-CD73-PE, anti-CD90-PE, anti-CD105-PE, and anti-HLA-DR-FITC (BD Biosciences, USA). Cells were washed two times with FACSflow to remove excess antibodies and resuspended in 500 μL FACSflow. The fluorescence intensity was evaluated by flow cytometer (FACSCalibur, BD Biosciences, USA). Using forward and side scatter profile, debris and dead cells were gated out. All data were analyzed by using Cell Quest Pro software (BD Biosciences, USA).

2.3. Differentiation of CLMSCs into Adipocytes and Osteoblasts. Cells were plated at 1 × 104 cells per well in 6-well plates. At 70%–80% confluence, for differentiation into adipogenic cells, cells were cultured for 2 weeks in the previously well-known medium [2]. Adipogenic differentiation was evaluated by observing lipid droplets in cells under microscope and staining with Oil Red solution (Sigma-Aldrich, USA). For differentiation into osteogenic cells, cells were also induced for 2 weeks in the previously published medium [2]. Osteogenic differentiation was confirmed by RT-PCR for gene expression of osteocalcin and osteopontin. Noninduced cells were served as control groups.

2.4. In Vitro Differentiation of CLMSCs into the Hepatocyte Lineage. Prior to hepatic differentiation, the third passage cells were plated at a density of 1 × 105 cells/cm2 in culture medium and grown to 70% confluency. For initiation, the cells were incubated for 2 days in low glucose-DMEM
supplemented with 20 ng/mL EGF and 10 ng/mL bFGF (conditioning step) to stop cell proliferation, prior to induction of differentiation toward a hepatic phenotype. Then a 2-step differentiation protocol was performed, followed by a sequential addition of growth factors, cytokines, and hormones: step-1 (initiation step) differentiation medium, consisting of DMEM supplemented with 50 ng/mL HGF and 10 ng/mL FGF-4 (all bought from Sigma-Aldrich, USA) for 7 days, followed by step-2 (maturation step) differentiation medium, consisting of DMEM supplemented with 20 ng/mL OMS, 1 μmol/L dexamethasone, 4.9 mmol/L nicotinamide, and 10 μg/mL ITS + premix (final concentration: 100 μg/mL insulin, 6.25 μg/mL transferrin, 3.6 μmol/L selenious acid, 1.25 mg/mL BSA, and 190 μg/mL linoleic acid) (all bought from Sigma-Aldrich, USA) to achieve cell maturation up to day 21. Media were changed twice weekly and hepatic differentiation was assessed by RT-PCR, real-time qRT-PCR, Western blot, and immunocytochemistry for liver-associated genes and markers.

2.5. RT-PCR and Real-Time qRT-PCR. Total RNA was extracted using RNeasy Mini Kit (Qiagen, USA). RT-PCR was performed from total RNA using AccessQuick RT-PCR kit (Promega, USA), in accordance with the protocols of the manufacturer under the following condition: initial reverse transcription at 45° C for 45 min and 95° C for 2 min followed by 35 cycles of denaturing for 30 s at 94° C, annealing for 30 s at 55–60° C, and extension for 45 s at 72° C. After completing the last cycle, all samples were incubated for 10 min at 72° C. The PCR products were analyzed by electrophoresis with 2% agarose gel, visualized with EtBr staining, and photographed by bioimaging system (UVP, USA).

The gene expression of ALB, ALP, CK18, and CK19 at different time points was evaluated by real-time qRT-PCR using the Superscript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, USA). Internal calibration curves were generated by the real time software. A melting curve analysis was carried out between 60° and 95° C with a plate read every 0.5° C after holding the temperature for 20 s. The threshold cycle number (Ct) at which the signals crossed a threshold set within the logarithmic phase and the peak of melting curves were recorded. The relative quantitation of gene expression in terms of fold change was calculated using the 2 −ΔΔCt method [9]. All calculations were normalized using the GAPDH gene as a reference control.

Primers sequences for study include the following. osteocalcin F: CGCAGGCCACCGAGACACCAT, R: GGGCAA-GGGCGAGGGAAGA (405 bp), osteopontin F: CTAGCGCATCACGTGCGCATAACC, R: CTATCTTAGACT- CTTGACGACTGTA (330 bp), Albumin F: TGCTTGAAT- GTGCTGATGACAGGG, R: CATAGCGAGCAGCCCAAAGAA- GGA (315 bp), CK19-F: ATGGCCGAGCAGACCCGGAA, R: CGATGAGCTGCTGACGCTC (382 bp), CK18-F: GAGCTCAGCGCTCAGCTAAGGA, R: CAAGCTGGCCTT- CAGATTTC (400 bp), GAPDH F: ACGACCAATCCGGTACTC (94 bp).

2.6. Immunocytochemistry Analysis. After hepatocyte differentiation, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.25% Triton X-100 for 40 min at room temperature. Then, they were blocked with a blocking solution containing 1% BSA and incubated overnight with mouse monoclonal Ab against human α-fetoprotein (AFP), albumin (ALB), and cytokeratin 18 (CK18), cytokeratin 19 (CK19) (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were washed with PBS and incubated for 1h with PE- or FITC-conjugated goat anti-mouse IgG (1:400; Santa Cruz Biotechnology, USA). In all immunocytochemistry assays, negative staining controls were carried out by omitting the primary antibody. After washing, cell nuclei were stained with Hoechst 33342 (Sigma-Aldrich, USA) for 10 min. Images were captured using a fluorescent microscope (Olympus, Japan).

2.7. Western Blot Analysis. After washing with cold PBS, the cells were lysed by a lysis buffer containing 0.01 M Tris, pH 7.5, 0.1 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, with added protease inhibitors. Total proteins in cell lysates were separated by electrophoresis and transferred to membranes. About 15 μg of proteins was subjected to 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidine fluoride (PVDF) blotting membrane (Sigma-Aldrich). The membranes were blocked in blocking solution and incubated with mouse monoclonal Ab against AFP, ALB, CK19, and CK18 (Santa Cruz Biotechnology, USA) for 1 h at room temperature. After washing, the membranes were incubated for 45 min with horseradish peroxidase (HRP) linked goat anti-mouse IgG (Santa Cruz Biotechnology, USA). The protein bands were visualized by enhanced chemiluminescence (Amersham Biosciences, USA). Mouse monoclonal Ab against β-actin (1:100) was used as a housekeeping gene control.

2.8. Low-Density Lipoprotein (LDL) Uptake Assay. The undifferentiated and differentiated cells were washed with PBS and incubated in medium containing 10 μg/mL 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine labeled acetylated low-density lipoprotein (DiI-Ac-LDL; Invitrogen, USA) for 24 h at 37° C. Cells were fixed with 4% formaldehyde and, after washing, counterstained with Hoechst 33342 (Sigma-Aldrich, USA). Incorporation of fluorochrome-labeled LDL and Hoechst 33342 into cells was observed under a fluorescent microscope (Olympus, Japan).

2.9. Periodic Acid-Schiff Staining for Glycogen Storage. After hepatocyte differentiation for 21 days, cells were harvested with trypsin and cultured at 1 × 10^5 cells/cm^2 in a 24-well plate overnight. Cells were washed with PBS, fixed for 1 min in formalin-ethanol fixative solution. They were incubated with periodic acid solution (Sigma-Aldrich, USA) for 10 min at room temperature. After rinsing, cells were immersed in Schiff’s reagent (Sigma-Aldrich, USA) for 20 min at room temperature and rinsed in deionized dH2O for 5 min. Cells were counterstained in hematoxylin solution for 90 s and
2.10. Statistical Analysis. Data are expressed as mean ± standard error of the mean. Statistical comparisons were performed using the Student’s t-test (Excel, Microsoft, USA). \( P \) values < 0.05 were considered statistically significant (\( n = 3 \)).

3. Results

3.1. Morphology and Proliferation Ability of CLMSCs. Using a tissue attachment culture method, scattered, long, and spindle-shaped cells were observed in the gaps between the tissue blocks under an inverted microscope after 7 days. After 10–12 days in culture, the cell number in each small colony reached up to a few hundred (Figure 1(a)). At that time, the tissue blocks were removed. The cell shape gradually changed to a uniform spindle shape. The cells reached about 80% confluency after approximately 3 weeks in culture. After three passaging processes, the cells reached homologous fibroblast-like cell population and arranged in parallel arrangement (Figure 1(b)). The observed morphologies of CLMSCs were similar to those of bone marrow-derived MSCs or umbilical cord blood-derived MSCs [2, 3, 12].

3.2. Immunophenotypical Characteristics of CLMSCs. To confirm whether the CL-derived cell is one of the MSC populations, FACS analysis was performed. The third subcultured cells were negative for markers CD19, CD34, CD45, and HLA-DR. In addition, these cells were found to be positive for the markers CD73, CD90, and CD105. Interestingly, they also expressed CD14 (Figure 2).

3.3. Osteogenic and Adipogenic Differentiation of CLMSCs. In adipogenic medium, MSCs began changing into ovoid morphology and accumulating intracellular lipid droplets which could be observed from day 7. At the beginning of the two weeks, the lipid droplets enlarged and invaded the entire cytoplasm. They were red in color with Oil Red staining (Figure 1(d)). In control group, cells did not differentiate (Figure 1(c)). When induced to differentiate under serum-free osteogenic conditions, the spindle shape of these cells flattened and broadened with increasing time of induction. The osteoblastic phenotype of differentiated cells was also detected the expression of osteoblast-specific genes, such as osteopontin and osteocalcin after 2 weeks, but not in undifferentiated cells (control group) (Figure 3(b)).

3.4. Morphological Change Analysis of CLMSCs Derived Hepatocyte-Like Cells. Sequential differentiation mimicking liver development induced gradually morphological change from spindle fibroblast-like shape to oval and round epithelial-like shapes. After exposure to the initiation medium containing HGF and FGF-4 (initiation step), the cells gradually lost their sharp edges and were progressively shrunk away, resulting in the transition from fibroblastic bipolar morphology to round or oval shapes. The change of
Figure 2: Flow cytometric analysis of CLMSCs with fluorescein FITC or PE-conjugated antibodies against the indicated markers. CD14-PE, CD19-FITC, CD34-FITC, CD44-PE, CD45-FITC, CD73-PE, CD90-PE, CD105-PE, and HLA-DR-FITC. All experiments were repeated in triplicate.

Figure 3: RT-PCR analysis of gene expression. In the hepatogenic medium, cells expressed ALB, AFP, CK18, and CK19 after 21 days (a). (Lane 1: noninduced MSCs-negative control, lane 2: differentiated cells; lane 3: HepG2-positive control.) In the osteogenic medium, induced cells expressed osteopontin and osteocalcin after 14 days (lane 1: 0 day, lane 2: 7 days; lane 3: 14 days) (b). GAPDH serves as an internal control.
morphology was detected as early as on day 7 in the region of high cell density and propagated into the area of low cell density, so that approximately 70% of the treated cells became oval and round in shape by the end of the initiation step. The cytoplasmic contraction was further progressed during the maturation step, and most of the incubated cells turned into small round cells that were highly similar to oval cells in appearance (Figure 1(f)). However, cells did not change their morphology in control group (Figure 1(e)).

3.5. Detection of Hepatocyte-Related Gene Expression by RT-PCR and Real-Time qRT-PCR. The transcription of hepatocyte-specific genes, including ALB, AFP, CK18, and CK19, was detected in differentiated cells by RT-PCR (Figure 3(a), lane 2). The results were the same as those of gene expression of HepG2 cells (lane 3). CLMSCs weakly express ALB gene (lane 1).

Real-time qRT-PCR analysis was performed to determine the mRNA expression level of ALB, AFP, CK18, and CK19 at different time points. Among them, ALB and CK18, CK19 had a similar mRNA expression pattern. Their expression levels were not much altered during the initiation step (day 0–day 7) but began to substantially increase thereafter when the cells were incubated into maturation medium (day 7–day 21). On the contrary, the mRNA expression level of AFP strongly increased on day 7 and day 14 and decreased on day 21 following hepatocyte induction (Figure 5(a)).

3.6. Detection of Hepatocyte-Specific Marker Expression by Immunocytochemistry and Western Blot. After CLMSCs were incubated in hepatocyte differentiation medium for 21 days, they became positive for the hepatocyte-specific markers ALB, AFP, CK18, and CK19. The percentage of positive cells following hepatocyte differentiation was 92 ± 5% for ALB, 94 ± 4% for AFP, 93 ± 5% for CK18, and 91 ± 3% for CK19 (n = 3) (Figures 4(b), 4(d), 4(f), and 4(h)). Cells cultured in growth medium as negative controls did not show any positive signals for AFP, CK18, and CK19 but had a low signal for ALB (Figures 4(a), 4(c), 4(e), and 4(g)).

Western blot analysis was performed to confirm further the expression of ALB, AFP, CK18, and CK19 at the protein level. Expression of ALB was gradually increased on day 7, day 14, and day 21 following hepatocyte differentiation. Expression of CK18 was also detected on day 14 and increased on day 21. Expression of AFP was detected on day 7 and day 14 at a similar level and decreased on day 21. Expression of CK-19 was detected on day 7 and day 14 at a similar level and increased on day 21. However, the expression of the four hepatocyte-specific genes was not detected in the control group (day 0). The protein expression of four genes detected by Western blot analysis was relatively similar to mRNA expression by real-time qRT-PCR analysis (Figure 5(b)).

3.7. Detection of Glycogen Storage and LDL Uptaking by Periodic Acid-Schiff Staining Assay and LDL-Uptake Assay. After hepatocyte differentiation for 21 days, cells were analyzed for hepatocyte differentiation by periodic acid-Schiff staining for glycogen accumulation and LDL-uptake assay for LDL uptake. Cells were positively stained by periodic acid-Schiff treatment (Figure 6(d)). Cells could also uptake LDL (Figure 6(b)). The cells were cultured in growth medium as negative controls; they were negative for both assays (Figures 6(a) and 6(c)).
**Figure 5**: Real-time qRT-PCR analysis of hepatocyte-specific gene expression and Western blot analysis of hepatocyte-specific marker expression. Total RNA was extracted from CLMSC on days 0, 7, 14, and 21 following hepatocyte induction. The mRNA expression of hepatocyte-specific genes, such as ALB, AFP, CK19, and CK18 was analyzed by real-time RT-PCR and normalized with GAPDH gene. $P < 0.05$ relative to day 0 ($n = 3$) (a). Total cellular protein was extracted from CLMSCs on days 0, 7, 14, and 21 following hepatocyte induction. After electrophoresis and electo transfer to membranes, the membranes were incubated with mouse Ab against the hepatocyte-specific markers ALB, AFP, CK19 and CK18 and then with HRP-linked goat anti-mouse IgG. $\beta$-Actin was used as a housekeeping gene control. The size of each protein is indicated (b).

### 4. Discussion

Stem cell-based therapy and transplantation are of potential value in tissue and organ replacement and regeneration approaches. Among various types of human stem cells, MSCs have emerged as one of the most promising cell therapeutics. The present study showed that CLMSCs isolated by attached tissue culture might attain cell homogeneity and might share most of the characteristics with bone marrow-derived MSCs [2]. The cells could differentiate into osteoblast and adipocytes under *in vitro* conditions. They were negative for CD19 (B lymphocyte antigen), CD34 (hematopoietic stem cell antigen), CD45 (leukocyte common antigen), and HLA-DR indicating no hematopoietic stem cell lineage in the analyzed cell populations. In addition, they also expressed a set of marker proteins on their surface, CD73 (SH3, SH4), CD90 (Thy-1), and CD105 (SH2 or endoglin). Currently, CD73, CD90, and CD105 are considered important protein markers to identify human mesenchymal stem cells from many sources such as umbilical cord, umbilical cord blood, adipose tissue, and bone marrow [2, 3, 18]. Surprisingly, in this study, we also observed the CD14 expression on CLMSCs that is different from immunophenotypic characteristics for defining human MSCs [20]. However, this result was consistent with the study of Kita, 2010. It is possible that the cells might acquire CD14 expression after the isolation from a tissue. The CD14 expression might be a unique property to distinguish CLMSCs from other adult tissue-derived MSCs. In addition, the CD14 function on MSCs might explain how MSCs can home to injured or infected sites [18].

The MSCs isolated from different tissue sources have been examined for their hepatocyte differentiation potential although the stem cell transdifferentiation mechanism by stimulating with suitable factors or by genetic reprogramming *in vitro* remains unclear. It is known that liver development from the endodermal layer is known to proceed via several distinct steps that involve extracellular signals induced by growth factors and cytokines. However, the choice of exogenous factors and the time course to induce hepatogenic transdifferentiation is important to lead induction efficiency. Consequently, this study examined the hepatogenic differentiation potential of CLMSCs using a two-step protocol. The results showed that the naive CLMSCs weakly expressed ALB for mRNA and protein (shown by RT-PCR and immunocytochemistry). And they expressed ALB, AFP, CK18, and CK19 at mRNA and protein levels following hepatogenic induction (RT-PCR, immunocytochemistry, and...
Western blot analysis gave similar results). It has been reported that albumin, the abundant protein synthesized by mature hepatocytes, starts in early fetal hepatocytes and reaches the maximal level in adult hepatocytes [12]. AFP is produced by immature hepatocytes or hepatoblasts [21]. CK19 and CK-18 are regarded as the markers of epithelial cells [12].

To confirm hepatocyte differentiation process, we chose to determine expression changes of some hepatocyte-specific genes that play important roles in the physiologic processes of hepatogenic differentiation or maturation. The results of real-time qRT-PCR and Western blot showed that cells expressed ALB and CK19 and strongly exhibited AFP following induction with HGF and FGF-4. HGF and FGF-4 played a critical role in the early stages of hepatogenesis [21, 22]. By cooperation of HGF and FGF-4, the differentiation of MSCs might be triggered and MSCs developed into hepatocytes. Expression of ALB, CK18, and CK19 substantially increased, but AFP strongly decreased when cells were incubated into maturation medium containing oncostatin M, dexamethasone, and nicotinamide. The results showed the importance of the sequential addition of liver-specific factors in a time-dependent manner in hepatogenesis. Oncostatin M, a member of the interleukin 6 family cytokines, played an important role in the progression of hepatocytic development toward maturation. Although OSM alone had very weak effects on hepatocyte functions, albumin secretion was greatly enhanced when combined with nicotinamide. Nicotinamide significantly enhances the in vitro maturation of fetal liver cells [23]. In addition, ITS has been shown to be effective in promoting the proliferation and survival of primary hepatocytes [12]. Dexamethasone has been reported to promote the expression of a hepatocyte phenotype through suppression of cell division [24]. The results also indicated there were some similar points of gene expression between in vivo and in vitro hepatogenic differentiation. That was supported by a recent study of Schmelzer. The gene expression profile of in vivo hepatic cells ranging from the stem cell stage to the mature functional stage was examined. They showed that hepatic stem cells expressed high levels of CK19, low levels of ALB, and no AFP or adult liver-specific proteins. Mature hepatocytes lacked expression of AFP and CK19, and they have acquired the well-known adult-specific expression profile that includes high levels of ALB [25]. In this study, the naive CLMSCs also expressed ALB to some extent, strongly expressed AFP after step 1 and strongly exhibited ALB after step 2. This observation implies that hepatic differentiation by two-step
Hepatocyte differentiation was further evaluated by functional assays. Periodic acid-Schiff staining and LDL uptake assay are typical assays for the determination of hepatocyte functions [12, 13]. This study has shown that CLMSCs become positive for periodic acid-Schiff staining for glycogen storage and can uptake LDL, which are two of typical functions of mature hepatocytes on day 21 following differentiation, whereas undifferentiated CLMSCs did not possess these hepatogenic characteristics. These data demonstrated that cells cultured in hepatocyte differentiation medium not only express hepatocyte-specific markers but also the characteristics of functional hepatocytes. However, whether hepatocyte-like cells derived from CLMSCs can engraft in liver tissue after transplantation, produce growth factors or cytokines that inhibit liver injuries (i.e., CCL4-induced liver fibrosis), and improve liver functions needs to be demonstrated in order to establish a novel therapeutic approach for liver disease. Therefore, the studies of cell transplantation in a mouse model of liver injuries are required to assess their potential therapeutic roles in the following experiment.

5. Conclusion

We provide in vitro experimental data to support hepatic differentiation potential of CL-derived MSCs. The cells were characterized at mRNA and protein levels and exhibited hepatocellular synthesis and metabolic functions specific to hepatocytes. Since these cells have more clinical and practical advantages than BM-derived cells, our findings here raise hope that CLMSCs can be a good cell source for the liver-directed cell therapy and tissue engineering.

List of Abbreviations

- AFP: α-Fetoprotein
- ALB: Albumin
- bFGF: Basic fibroblast growth factor
- BM-MSCs: Bone marrow-derived MSCs
- CK18: Cytokeratin 18
- CK19: Cytokeratin 19
- CLMSCs: Umbilical cord lining membrane-derived MSCs
- EGFR: Epidermal growth factor
- FGF-4: Fibroblast growth factor-4
- HGF: Hepatocyte growth factor
- ITS: Insulin-transferrin-selenium
- LDL: Low-density lipoprotein
- MSCs: Mesenchymal stem cells
- OSM: Oncostatin M
- UC: Umbilical cord.

Conflict of Interests

The authors declare that they have no competing interests.

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