

Derivation of Hepatocytes from Human Umbilical Cord Lining Epithelial Stem Cells

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Introduction

Multipotent stem cells have recently been isolated from various compartments of the human umbilical cord and placenta,¹ over and above its established role as a source of cord blood from which hematopoietic and mesenchymal stem cells can be obtained.^{2–4} Anatomically, the umbilical cord is the long soft tissue conduit between fetus and placenta. It encloses two umbilical arteries through which fetal blood is transported to the placenta, where the exchange of oxygen, nutrients and fetal excretory products with the maternal circulation takes place before it is returned to the fetus via the umbilical vein. The amniotic membrane forms the outermost layer of the umbilical cord. Embryologically, the epiblast layer gives rise to all fetal embryonic germ layers, including the amniotic membrane which lines the interior of the gestational sac, covering both the placenta and the umbilical cord. Morphologically, the amnion transitions from a fine membrane covering the chorion and the placenta to a thicker

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membrane covering the umbilical cord, and becomes skin when the fetal stage is reached.

The attraction of the placenta or umbilical cord complex lies in its disposability after infant birth. The fact that stem or progenitor cells can be derived from this “medical waste” without morbidity to mother or fetus/infant has considerable scientific, clinical and social implications. To date, mesenchymal stem cells have been isolated from the placenta and its membranes,^{5–7} from Wharton’s jelly^{8,9} as well as the umbilical cord perivascular support tissue and amniotic fluid.^{10,11} Epithelial cells with stem cell characteristics have also recently been isolated from the placental amniotic membrane¹² and amniotic fluid.¹³

The stem cell sources above share the problem of a limited cell harvest and thus *in vitro* expansion while maintaining multipotency of the cells is required for most experimental applications. The requirement for even more cells, for the seeding of synthetic scaffolds for translational tissue replacement, would mean subjecting the cells to an increased number of expansion cycles.¹⁴ Repeated expansion *in vitro* has been shown to induce cell senescence and genetic instability, affecting cell multipotency, and ultimately, its intended utility.^{15,16}

In this chapter, we report the amniotic membrane of the umbilical cord to be a high-yielding primary source of epithelial cells with stem or progenitor cell features. We have termed them “cord lining epithelial cells” (CLECs) to differentiate them from the other sources above. In addition, we demonstrate that CLECs express hepatic stem cell markers and possess the capacity to differentiate into hepatic-like cells *in vitro*. These findings demonstrate the amniotic membrane of the human umbilical cord to be an easily available, high-yielding source of epithelial stem cells for differentiation along hepatocyte cell lineages for liver regeneration and therapy.

Umbilical Cord Tissue Can Be Easily Collected and Decontaminated

Umbilical cords from both sexes were obtained at the time of delivery by both vagina and Caesarian section from healthy women with uncomplicated pregnancies. Signed informed consent after reading the provided Patient Information Sheet was taken by the patient’s obstetrician prior to

the time of delivery. The consent form was written following guidelines derived from the Singapore Bioethics Advisory Committee Report on Human Tissue Research 2003 (<http://www.bioethics-singapore.org/resources/articles.html>).

Upon delivery, the umbilical cord was excised and immediately transferred into a 500 ml sterile glass bottle containing a culture transport medium [L-15 medium supplemented with 50 IU/ml penicillin, 50 μ g/ml streptomycin, 250 μ g/ml fungizone and 50 μ g/ml gentamicin (Invitrogen)]. The cord was kept in a cool environment of about 10°C in the time taken for transport to the laboratory within 24–48 h. In the laboratory, cell extraction was conducted in a laminar flow hood under sterile conditions. The cord was first placed in a sterile stainless steel tray, where cord vessels were cannulated and all remaining blood elements removed by repeated flushing with a syringe containing warm phosphate-buffered saline (PBS) supplemented with 5 IU/ml heparin (Sigma) to loosen clotted blood. Plain PBS without heparin was used in the final washes. The umbilical cord tissue was then cut into 2 cm segments and transferred into 10-cm-diameter cell culture dishes, where further washing and sterilization was performed with 70% ethanol, followed by multiple washes using PBS containing an antibiotic mixture until the solution became clear.

A clear advantage of using cord lining membrane as a source of stem cells is that the process of derivation is consistent and uncomplicated by virtue of its smooth surface, which is easily decontaminated by rinsing in the initial steps of processing. The umbilical cord amniotic membrane appears to be a robust structure which under normal sterile conditions is resistant to contamination and easily handled. This characteristic remains regardless of whether the birth is a Caesarian section or a vaginal delivery, unlike placental amniotic membrane, which is more easily obtained through Caesarian section, while vaginal delivery literally shreds this gossamer membrane in the delivery process. As the umbilical cord can be consistently obtained at birth, the ability to process the cord amniotic membrane for CLEC harvesting becomes a distinct possibility. Banking of CLECs simply becomes an extrapolation of cord blood collection and banking, which is a viable commercial proposition as it affords the storage of epithelial, mesenchymal and hematopoietic stem or progenitor cells for the patient: providing in essence all stem cell types for future clinical applications should they be required.

Stem Cells of High Yield Can Be Isolated and Cultivated from the Umbilical Cord Lining Membrane

The umbilical cord amniotic membrane was first dissected free from other umbilical cord contents. The isolated amniotic membrane was then divided into small squares of approximately 0.5 cm² for cell isolation. Explant cultures were performed by placing the squares of the umbilical cord amniotic membrane on tissue culture dishes under specific cell culture conditions for isolation of epithelial cells (CLECs). Briefly, explant tissue samples were placed on cell culture plastic surfaces and submerged in 5 ml Medium-171 (Cascade Biologics, Oregon) before returning to the CO₂ incubator at 37°C. The medium was changed every two or three days. Cell outgrowths from tissue culture explants were monitored under light microscopy. Outgrowing cells were harvested by trypsinization (0.0125% trypsin in 0.05% EDTA) in Medium-171. Cell pellets were then resuspended in Medium-171 supplemented with 50 µg/ml insulin-like growth factor 1 (IGF-1), 50 µg/ml platelet-derived growth factor BB (PDGF-BB), 5 µg/ml transforming growth factor β1 (TGF-β1) and 5 µg/ml insulin (all obtained from R&D Systems), at a density of 1 × 10⁶ cells/dish. After 24 h, attached cells were gently washed with warm PBS and the medium was replaced with supplement-added Medium-171. The medium was changed every two or three days. Cell growth and expanding clonal formation were monitored under light microscopy. At a confluence of about 70%, cells were subcultured by trypsinization (0.0125% trypsin in 0.05% EDTA) for further expansion and/or cryopreservation.

The significant aspect of CLECs is the tremendous number of cells that can be derived from each umbilical cord. Based on average cord dimensions, a potential total cell harvest of approximately 4–8 billion cells, each of CLEC strains, is totally feasible. Translating this into clinical applications, cell expansion requirements will thus be diminished for direct cell application or integration into scaffolds for clinical transplantation. We have managed to subculture the cells to passage 40 for both CLECs in our laboratories with no change in the multipotency of the cell strains, which continue to form colonies as efficiently as from primary explant strains. Further expansion experiments will be performed to establish the passage number beyond which mutations or early senescence is noted. Realistically, however, such expansion should not be necessary as this large harvest of cells would likely be sufficient for translational purposes in the first place.

Cord Lining Epithelial Cells Express Hepatic Stem Cell Characteristics

Hepatic differentiation of CLECs

The CLECs were plated and cultured in an expansion medium on 10 cm culture dishes until 80% confluence. They were then washed with 1X PBS three times before a hepatocyte culture medium (HCM) supplemented with HCM SingleQuot containing ascorbic acid, BSA-FAF, transferrin, insulin, hEGF and GA-1000 (Cambrex, UK) was added. The medium was collected on day 7 for analysis and the plates refilled with a new HCM medium with HCM SingleQuot for another seven days. After 14 days of differentiation, the medium was collected and replaced with hepatocyte maintenance medium (HMM) supplemented with HMM SingleQuot containing insulin, dexamethasone GA-1000) (Cambrex, UK). On day 21 of hepatic differentiation, the medium was again collected and replaced with a new HMM with HMM SingleQuot. On day 28, the medium and cells were collected for analysis.

Undifferentiated CLECs cultured in basal medium PTTe1 were observed as cuboidal epithelial morphology on day 0. After culturing in the HCM supplemented with HCM SingleQuot, cell morphology started to change on day 7, demonstrating early rounding on day 14 (Fig. 1).

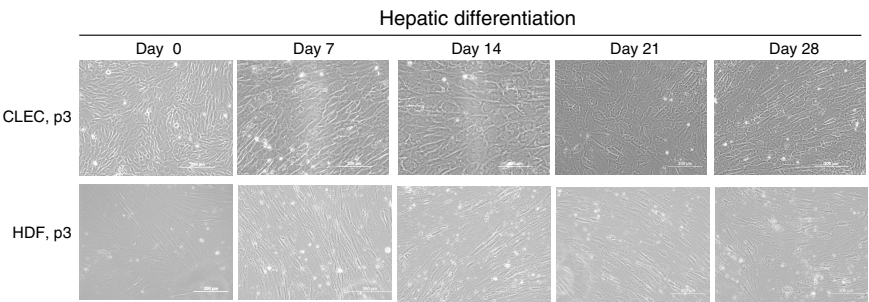


Figure 1. Morphological change during hepatic differentiation of CLECs. Undifferentiated CLECs were cultured in basal medium PTTe1. The morphology of these cells is cuboidal or epithelial on day 0. CLECs were cultured for seven days in a hepatocyte culture medium (HCM) supplemented with HCM SingleQuot, and for 21 and 28 days in a hepatic differentiation medium comprising a hepatocyte maintenance medium (HMM) supplemented with HMM SingleQuot (Cambrex). These cells have round or oval morphology, similar to primary human hepatocytes. In contrast, there was no change in the morphology of skin fibroblasts during hepatic differentiation. The scale bars represent 200 μ m.

At this time point, we cultured the CLECs in the HMM supplemented with HMM SingleQuot for two more weeks. After 21 and 28 days, all cells demonstrated round or oval morphology, similar to primary human hepatocytes. In contrast, no change in the morphology of human dermal fibroblast controls was observed during hepatic differentiation.

CLECs express hepatic stem cell markers that are altered during the course of differentiation into mature hepatic cells

RNA was extracted from CLECs using RNEasy (Quiagen, California) as per the manufacturer's instructions. One microgram of the messenger RNA was treated with DNase I (Fermentas, USA) and reverse-transcribed by Superscript II reverse transcriptase (Invitrogen, California). The complementary DNA was amplified using SupperArray's ReactionReady™ Hotstart "sweet" PCR master mix with the RT² PCR primer sets for alpha fetoprotein (AFP); albumin (ALB); tyrosine aminotransferase (TAT); cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4); cyto-keratin 18 (CK18); cytokeratin 19 (CK19); hepatocyte nuclear factor 4; alpha (HNF4A); hepatocyte nuclear factor 1, beta (HNF1B); and factor VIII (F-VIII) and GAPD (SuperArray, USA). Amplification reactions were performed on a thermal cycler at 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec for 40 cycles. The PCR products were then separated by electrophoresis on 2% agarose gels.

To further analyze the cytoplasmic expression of albumin and AFP in CLECs previously assayed by the RT-PCR technique, cultured CLECs were lysed in RIPA buffer and subjected to Western blotting. Twenty microgram total protein extracts were separated by 14% SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose membrane. Blots were incubated with the specific antibodies against human albumin (Dako, UK) and AFP (Santa Cruze Biotechnology Inc., USA) with horse-radish-peroxidase-conjugated rabbit antimouse as a secondary antibody (Abcam, UK). They were visualized with a chemiluminescence-based photoblot system (Amersham Biosciences, USA).

Expression of albumin, AFP, CK18 and CK19 in CLECs was further confirmed by immunocytochemistry. To perform these experiments, CLECs were seeded onto 10-cm-diameter tissue culture dishes at a density of 200 cells per dish and maintained in growth media for 12 days. Colony formation was monitored with light microscopy. The colonies were fixed by incubation in 4% (v/v) paraformaldehyde in PBS for 20 min at room

temperature. They were then washed twice with PBS. The fixed cells were permeabilized by exposure to 0.1% Triton X-100 in PBS for 15 min at room temperature. The cells were then rinsed with PBS and immunocytochemical analysis was performed using the R.T.U. Vectastain® ABC kit (Vector Laboratories). After treatment with albumin, AFP, CK-18 and CK-19 antibody solutions (Santa Cruz Biotechnology and Chemicon Inc., USA) in a 1.5% blocking serum for 1 h at room temperature followed by a universal secondary antibody for 15 min provided in the R.T.U. Vectastain ABC kit, the cells were incubated with the streptavidin peroxidase complex reagent provided in the kit. This was followed by treatment with DAB (Dako) until the desired stain intensity developed. The cells were then counterstained with hematoxylin, observed, and photographed through the light microscope.

RT-PCR analysis reveals a temporal expression pattern of hepatocyte-specific markers of differentiated CLECs

RT-PCR analysis showed that undifferentiated CLECs express AFP, albumin, CK18, CK19 and F-VIII but did not express TAT, CYP3A4, HNF4A, and HNF1B (Fig. 2). Expression of AFP, albumin, CK18 and CK19 was detected at all time points of hepatic differentiation with no significant difference. Increased expression of CYP3A4 and F-VIII was increased with the duration of differentiation. Expression of TAT, a late hepatocyte marker gene, was observed on day 7, increased until day 21 and was lost on day 28 of hepatic differentiation. The expression of CYP3A4, a transcription factor largely expressed in the adult liver, was only detected on day 28 of hepatic differentiation.

CLEC differentiation into hepatic cells in vitro was reconfirmed by immunocytochemical staining and Western blot experiments

Immunocytochemical results demonstrated that AFP expression was found in undifferentiated CLECs but was decreased on day 7 and lost from day 14 of hepatic differentiation [Fig. 3(A)]. The results correlated with Western blot analysis [Fig. 3(B)]. In contrast, albumin expression was found in undifferentiated CLECs and noted to be increased after 7 and 14 days of hepatic differentiation. This albumin expression was lost after 21 days of hepatic differentiation. CK18, expressed in undifferentiated CLECs, peaked on day 14 of hepatic differentiation and declined after that

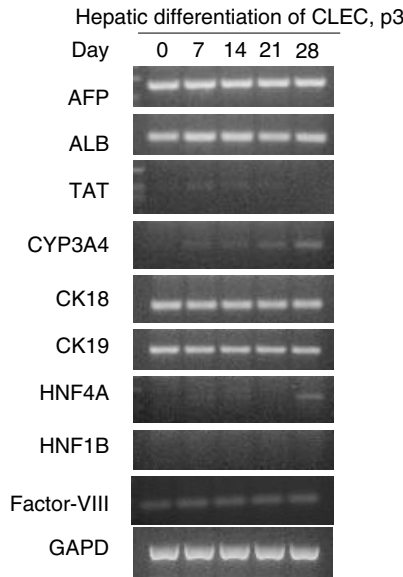


Figure 2. Temporal expression of hepatic-specific genes during hepatic differentiation of CLECs. RNA was isolated from undifferentiated CLECs (day 0), and from CLECs, during hepatic differentiation on days 7, 14, 21 and 28. RT-PCR was performed to evaluate the expression of hepatic-specific genes such as alpha fetoprotein (AFP); albumin (ALB); tyrosine amino-transferase (TAT); cytochrome P450; family 3, subfamily A, polypeptide 4 (CYP3A4); cytokeratin 18 (CK18); cytokeratin 19 (CK19); hepatocyte nuclear factor 4, alpha (HNF4A); hepatocyte nuclear factor 1, beta (HNF1B); and factor VIII. GAPD was used as internal control. No significant difference was observed in the expression of AFP, ALB, CK18 and CK19 between undifferentiated and differentiated CLECs. There was an increase in the expression of CYP3A4, HNF4A and factor VIII in differentiated CLECs compared to undifferentiated cells in a time-dependent manner. However, both undifferentiated and differentiated CLECs did not express TAT and HNF1B.

time. CK19 expression progressively decreased during the time of hepatic differentiation.

Hepatocyte-like Cells Differentiated From CLECs Show Physiological Function *In Vitro*

To prove physiological function of CLECs differentiated into hepatic-like cells, four established assays were employed: (i) albumin secretion; (ii) urea production; (iii) glycogen storage; (iv) uptake of low-density lipoprotein.

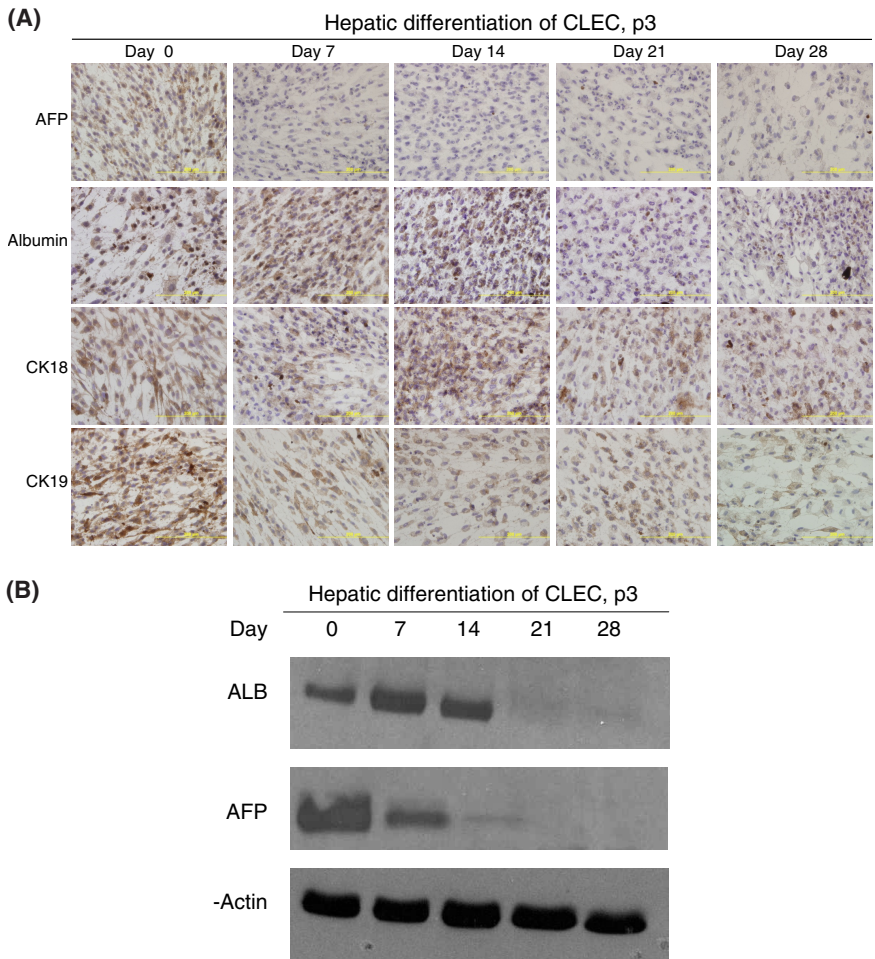


Figure 3. Temporal expression of hepatic-specific proteins during hepatic differentiation of CLECs. **(A)** Immunocytochemical staining of undifferentiated and differentiated CLECs for 7, 14, 21 and 28 days with anti-AFP, antialbumin, anti-CK18 and anti-CK19 antibody. **(B)** Western blot analysis indicated that these hepatocyte-specific markers were actively synthesized during hepatic differentiation.

The conditioned medium of undifferentiated and differentiated CLECs on days 7, 14, 21 and 28 were collected and subjected to the Elisa assay to assess albumin secretion; the QuantiChrom™ urea assay (DIUR-500) for determination of urea production; periodic acid-Schiff (PAS) staining for

glycogen storage in cells; and Dil-Ac-LDL staining (Biomedical Technologies Inc., Stoughton, USA) for uptake of low-density lipoprotein by CLECs, undifferentiated and differentiated.

In the albumin production assay, we found that CLECs cultured in a hepatic differentiation medium for 21 days secreted significantly higher quantities of albumin compared to the control [Fig. 4(A)]. Surprisingly, albumin secretion ceased on day 28 of hepatic differentiation. The urea

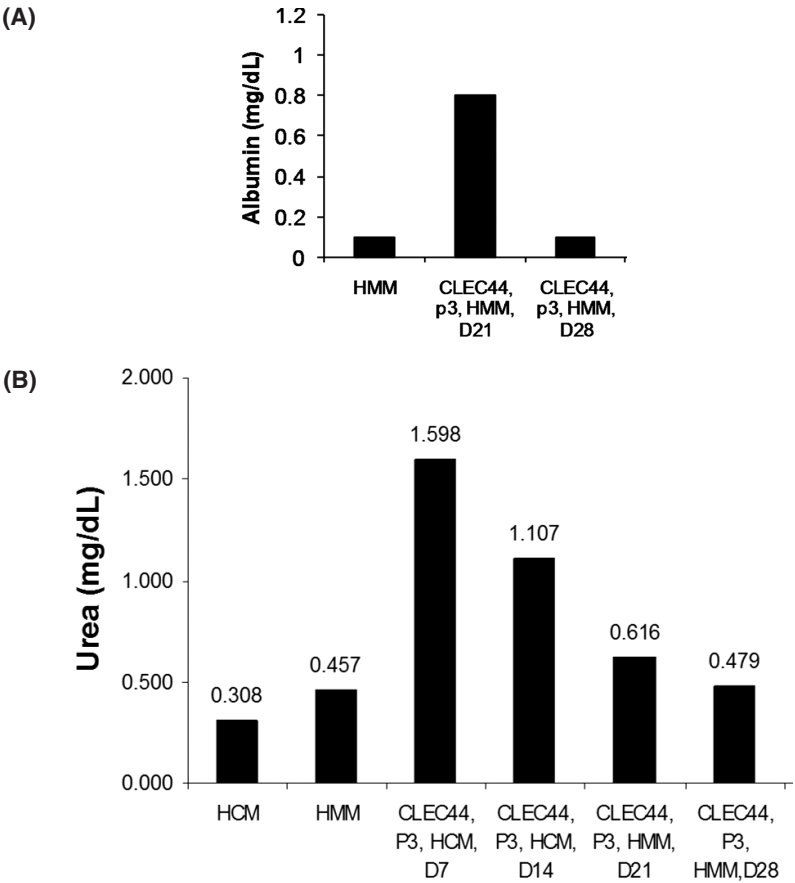


Figure 4. *In vitro* Functional characterization of hepatocytes differentiated from CLECs, albumin secretion (A), urea secretion (B), glycogen storage (C) and LDL uptake (D) of undifferentiated and differentiated CLECs at 7, 14, 21 and 28 days.

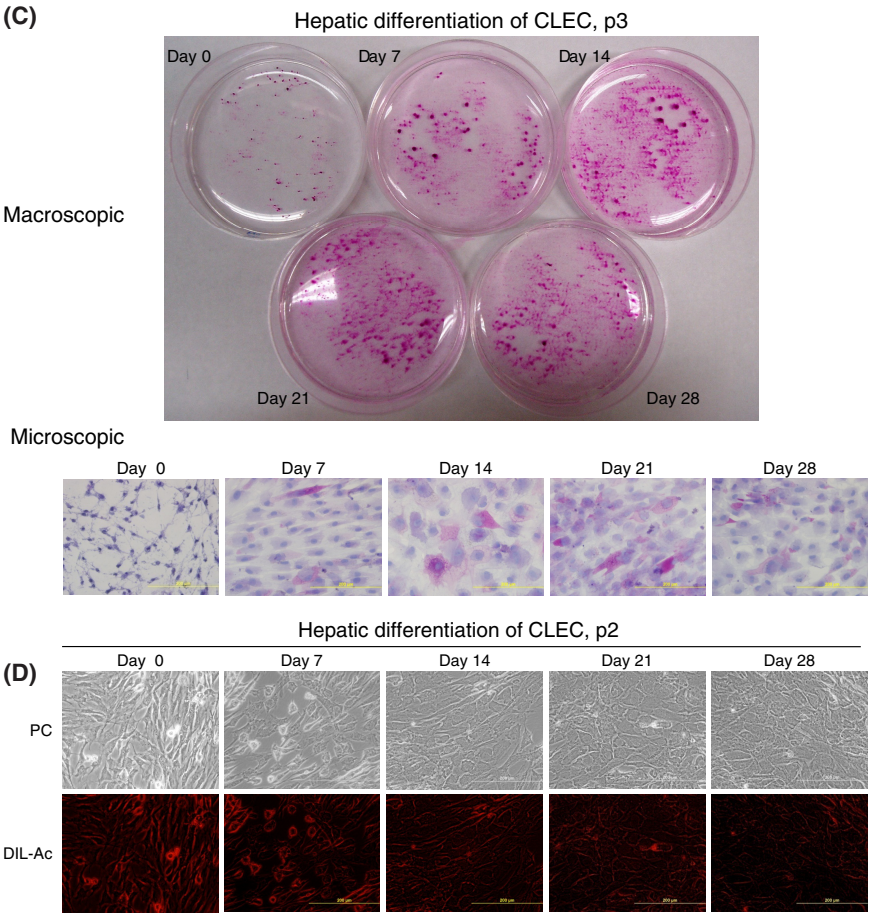


Figure 4. (Continued)

assay showed the highest levels of urea secretion in CLECs after exposure to differentiation media for seven days. This decreased with further hepatic differentiation [Fig 4(B)]. Undifferentiated CLECs did not show any positive glycogen storage, whereas CLECs exposed to differentiation media showed increased glycogen storage from day 7 to 21, followed by a decrease on day 28 of exposure [Fig. 4(C)]. A similar pattern was observed with LDL uptake [Fig. 4(D)].

Discussion

Transplantation of hepatocytes has been shown to be easier, more efficient and safer than whole organ transplantation in the treatment of patients suffering from end-stage liver diseases.²⁰ The maintenance of differentiated hepatocytes *in vitro* is difficult if not nearly impossible. Therefore, the generation of stem-cell-derived hepatocytes holds considerable promise for future clinical applications. Ideally, these pluripotent stem cells should come from a source which is easily expanded and be derived from sources with minimal or preferably no donor morbidity.¹⁷ In terms of ethical acceptability, the placenta or umbilical cord complex has proven highly suitable as a zero-donor-morbidity source of stem cells for both the lay and medical communities. To date, there have only been a few studies demonstrating the hepatogenic potential of epithelial stem cells from two major sources: amniotic fluid¹³ and amniotic membrane.^{18,19}

The umbilical cord amniotic membrane is embryologically the epiblastic continuation between the membranous, avascular gestational sac or placental amnion and the fetal skin. Although the term “amniotic membrane” may seem to unify these structures, umbilical cord amniotic membrane appears to have characteristics different from those of placental amniotic membrane. Umbilical cord amniotic membrane has an increased thickness compared with placental amniotic membrane; the latter is also characterized by its proximity to metabolically active placental cotyledons, which are likely to exert paracrine influences upon it and its contained cells. In contrast, umbilical cord amniotic membrane is adjacent to relatively quiescent Wharton’s jelly.

In the study, our results demonstrated that fresh isolated CLECs expressed a subset of hepatocyte-related genes such as albumin, AFP, CK18, CK19 and F-VIII. However, undifferentiated CLECs did not express HNF-4 α , which is essential for proper liver morphogenesis and function; HNF-1 β , which contributes to the early stages of liver development; TAT, which is responsible for amino acid metabolism, and CYP3A4, which is a drug-metabolism-related gene. In addition CLECs did not secrete albumin and urea, store glycogen or demonstrate LDL uptake. Taken together, undifferentiated CLECs may possess some characteristics of early hepatocyte-like cells but not functionality.

In order to induce hepatic differentiation of CELCs, we used HCM supplemented with ascorbic acid, BSA, FAF, transferrin, insulin, hEGF and GA-1000. Our results showed that CLECs started to transform into

hepatocyte-like morphology and express TAT and CYP3A4 but not HNF-4 α and HNF-1 β . Decreased CK19 expression, increased expression of albumin and CK18, as well as loss of AFP expression, were observed during hepatic differentiation of CLECs. Functional assays further demonstrated that these differentiated CLECs could secrete urea, store glycogen and uptake LDL, indicating that they had been successfully differentiated into functional hepatocyte-like cells.

In order to maintain the differentiated hepatic state of the CLECs, we subsequently cultured them in an HMM supplemented with insulin, dexamethasone and GA-1000. We observed significant reduction in albumin and urea secretion; however, these cells still possessed the ability to store glycogen and uptake LDL. Interestingly, the differentiated CLECs expressed CYP3A4, suggesting that they were now capable of drug metabolism. We speculated that these differentiated cells were prone to apoptosis, which would explain some loss of hepatic function. It appears that there is a two-week window of time where they are most functionally hepatocyte-like between 14 and 28 days after initiation of differentiation.

In conclusion, we have demonstrated for the first time that the umbilical cord amniotic membrane is a realistic and viable source of stem or progenitor cells for hepatocyte differentiation, highlighted by the following points:

- (1) The umbilical cord tissue is disposed of after delivery, making it an ideal stem cell source with no maternal or infant morbidity.
- (2) Epithelial stem cells or progenitor cells can be isolated from the umbilical cord amniotic membrane, and they possess some hepatocyte characteristics.
- (3) CLECs have been successfully differentiated into functional hepatocyte-like cells *in vitro*.
- (4) A high yield of CLECs is possible from umbilical cord amniotic membrane, and raises the potential of banking these cells for future applications.

Summary and Conclusion

We have successfully isolated both epithelial and mesenchymal stem cells from the outer amniotic lining of the umbilical cord, with a significant high yield. Interestingly, these multipotent cells also possess limited

embryonic stem cell-like characteristics, and have been differentiated into specific cells of the skin, bone and fat. In this chapter, we examined the differentiation of umbilical cord lining-derived epithelial stem cells (CLECs) into hepatocytes *in vitro*. These cells were cultured in a two-step protocol with the use of a hepatocyte culture medium and a hepatocyte maturation medium. Expression of a variety of hepatic markers was analyzed by RT-PCR, Western blot and immunocytochemistry. The functionality of differentiated cells was assessed by their ability to produce albumin, secrete urea, store glycogen and uptake low-density lipoprotein. After seven days of induction, the cells were morphologically transformed into hepatocyte-like cells with increased mRNA expression of most hepatic markers. Moreover, increased protein expression of albumin and cytokeratin-18, and a decreased protein expression of α -fetoprotein and cytokeratin-19, were observed. Differentiated cells further demonstrated *in vitro* behavior of liver cells, including albumin production, glycogen storage, urea secretion and uptake of low-density lipoprotein. In conclusion, CLECs are a good cell source for differentiation into hepatocyte lineages. Such differentiated cells will have potential uses in transplantation for the treatment of metabolic disorders or the construction of a bioartificial liver system.

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