Stage specific differentiation of human embryonic stem cells into hepatocyte-like cells using conditioned medium from a human hepatoma cell line

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Abstract

Hepatocytes derived from human embryonic stem cells (hESC) promise to be an inexhaustible source of functional cells for use in biomedical research, drug discovery and treatment of liver diseases. We have developed a unique strategy to efficiently differentiate hESC into functional hepatocyte-like cells (HLC) *in vitro*. The robustness of our protocol was assessed by duplicating the process of differentiation in two of our in house derived hESC lines, Relicell®hES1 and Relicell®hES2, and in the well studied BG01 cell line. To induce early hepatic commitment, undifferentiated hESC were initially primed with conditioned medium from HepG2, a human hepatoma cell line, which resulted in an enriched population of definitive endoderm. We have also attempted to recapitulate the hepatogenetic events occurring *in vivo* by sequential application of growth factors involved in liver development, such as aFGF, HGF, oncostatin, dexamethasone and EGF. Our differentiation process yielded a homogenous population of HLC exhibiting the typical polygonal morphology of hepatocytes. This population expressed hepatic lineage markers including HNF4α, AFP and ALBUMIN, and drug metabolizing enzymes such as CYP3A4 (Phase I) and GSTA1 (Phase II). Flow cytometric analysis showed that over 70% of the differentiated cells expressed albumin and CK8/18. The differentiated HLC exhibited hepatic characteristics such as glycogen storage and production of albumin and urea. Our results indicate that functional HLC generated by this method can be utilized in regenerative medicine and as a screening platform in the discovery and development of new drugs.

Introduction

Until recently, the only treatment modality for liver damage was orthotopic liver transplantation (OLTx). The major limitations of OLTx are the non-availability of donor liver, requirement of a major surgical procedure, high cost and long-term immunosuppression.[1] Primary hepatocyte transplantation is emerging as an attractive method for the treatment of liver damage because of its technical simplicity and easy availability of cells. Besides this, hepatocytes can also serve as an *in vitro* model system for the discovery of new pharmaceutical drugs and cytotoxicity assays since liver is the main detoxification organ of the body.[2] Existing assays have been developed using primary hepatocytes, but these cells have limited replicative capability and do not maintain their differentiated characteristics *in vitro*.[3] In addition, available hepatic cell lines contain very low levels of metabolizing enzymes and proteins differing substantially from the native hepatocytes.[4]

Human embryonic stem cells (hESC) derived from discarded human embryos have demonstrated their versatility in *in vitro* differentiation into functional cells from all the three lineages.[5,6] Because of their unlimited capacity for self renewal and differentiation, hESC have been proposed for use in a wide range of applications, including cell therapy, *in vitro* toxicology, tissue engineering and basic developmental biology study. Several groups have examined the differentiation potential of mouse and human ESC into hepatocyte-like cells[7-9] and also the utility of these differentiated cells in assessing *in vitro* toxicity of compounds.[10] Most of these existing protocols for hepatocyte generation are hindered by insufficient differentiation and maturation, leading to low yield and heterogeneous cell populations in cultures. Basma *et al.* isolated a homogenous population of hepatocytes from hESC by sorting with a surface marker, asialoglycoprotein receptor. However, these enriched cells were found to retain immature fetal liver characteristics.[11]

Here we have developed a novel method to generate hepatocyte-like cells (HLC) from hESC by stimulating the undifferentiated hESC with cytokine cocktails to mimic the main phases of embryonic and fetal development resulting in the sequential fating of hESC to definitive endoderm (DE), hepatoblasts and hepatocytes. This simple differentiation strategy was used to evaluate and compare the efficiency of hepatocyte differentiation potential in three different cell lines, BG01,[12] Relicell®hES1,[13] and Relicell®hES2,[14] using conditioned medium from HepG2 cells (HepG2-CM). HepG2 is an established human hepatocellular carcinoma cell line in routine use for various hepatic functional studies. It has previously been demonstrated that conditioned medium derived from HepG2 cells is efficacious for neuronal,[15,16] osteogenic[17] and hematopoietic[18] differentiation of embryonic stem (ES) cells. Conditioned media are known to contain numerous growth factors, cytokines, enzymes, hormones and other soluble mediators known to be essential to cell growth and differentiation, and to various other cellular events.

Here, we show for the first time that priming of undifferentiated hESC with HepG2 conditioned medium induces them towards DE and eventually can be differentiated to hepatic lineage. HLC differentiated by this protocol exhibited typical polygonal morphology of hepatocytes and were found to be highly enriched for liver specific proteins, albumin and CK8/18. The hepatic characteristic of the HLC was confirmed by the high expression of liver specific transcripts such as AFP, HNF4α and ALBUMIN. Importantly, these cells also expressed genes for drug metabolism enzymes, *CYP3A4* (Phase I) and glutathione transferase (*GSTA1*) (Phase II) that are

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responsible for metabolizing most pharmaceutical compounds. We aimed to demonstrate the functional characteristics of HLC by evaluating the albumin and urea secretion and glycogen storage. This represents a significant step towards the efficient generation of HLC for use in the discovery of new drugs and in regenerative medicine.

Materials and Methods

Cell culture and differentiation

hESC lines, BG01, Relicell®hES1 and Relicell®hES2 were maintained on inactivated mouse embryonic fibroblast (MEF) layer in hESC medium comprising of 79% DMEM/F12 (Lonza, Allendale, NJ, USA), 20% knockout serum (Invitrogen, Life Technologies Ltd., Paisley, UK), 1% non-essential amino acid solution (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1% β-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA), 15 ng/mL b fibroblast growth factor (bFGF) (R & D Systems, Minneapolis, MN, USA).[14] HepG2 cells were plated at 7×10^4 cells/cm^2 and cultured in DMEM (Lonza) supplemented with 10% FBS (Hyclone, Logan, UT, USA), 1 mM L-glutamine, 1% non-essential amino acids and 0.1% β-mercaptoethanol for three days. To obtain serum free conditioned medium (HepG2-CM), semi-confluent culture of HepG2 was washed twice with PBS and grown in DMEM/F12 media without serum.[15] After 48 h, the culture supernatant was collected and stored at -20°C until use. The supernatant was concentrated using 50 mL concentrators with a 3000 molecular weight cut off (Amicon® Ultra-15 Centrifugal filter unit with Ultracel-3 Membrane, UFC900324, Millipore Corp., Billerica, MA, USA) at 4000 rpm for 45 min. The conditioned medium was concentrated at 75:1. We have devised a 3-stage protocol to differentiate hESC into hepatocyte-like cells (Figure 1A). During stage 1, undifferentiated hESC clusters (20×10^4/cm^2) were cultured on matrigel coated dishes in hESC medium supplemented with bFGF for two days, followed by withdrawal of bFGF in the next two days. The hESC were then cultured for six days in the presence of HepG2-CM diluted with hESC media (1:30). In stage 2, the medium was replaced with the hepatocyte basal medium composed of DMEM/F12, supplemented with 5% knock out serum, 1 mM L- glutamine, 100 ng/mL aFGF, 30 ng/mL HGF, and 10 ng/mL oncostatin (all from R&D Systems), 5 µg/mL ITS (GIBCO, Life Technologies Ltd.), 20 ng/mL EGF (Sigma), 10^{-3}M dexamethasone (Sigma) to generate HLC. In stage 3, the differentiated HLC were allowed to mature in hepatocyte culture media (HCM) supplemented with SingleQuots (HCM BulletKit Lonza, CC3199 and CC4182) and additional aFGF, HGF, oncostatin, EGF, ITS and dexamethasone in the concentrations specified above. Media were changed every second day during the entire differentiation process.

Gene expression profiling

Total RNA was extracted from the cell pellets collected on Days 0, 10, 20 and 30 of differentiation using the RNAeasy Kit (Qiagen GmbH, Hilden, Germany). Human fetal liver RNA (Clonetech, Takara Holdings Inc., Kyoyo, Japan; cat. n. 636540) was used as the positive control. One microgram of RNA was converted to cDNA using superscript reverse transcriptase (Invitrogen). Pre-designed assays on Demand TaqMan® probes and primers were obtained from Applied Biosystems (Life Technologies Ltd.). Quantitative real-time polymerase chain reaction (RT-PCR) analysis was conducted using ABI 7500HT Fast Real Time System (Applied Biosystems). The conditions were: an initial denaturation cycle of 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 60°C for 1 min. Relative changes in the gene expression were normalized with 18S rRNA (ABI) expression levels. Fold change was calculated as $2^{-\Delta\Delta Ct}$. The results were analyzed using qbase software (Sequence Detection Software Ver 1.2.2, 7500 Systems, ABI).

Immunofluorescence

Differentiated HLC (Day 30) were fixed in 4% paraformaldehyde (Sigma) and permeabilized with 0.2% Triton X-100 (Sigma) for 15 min. The non-specific binding sites were blocked with 1% bovine serum albumin (Sigma) in PBS for 1 h at RT followed by incu-

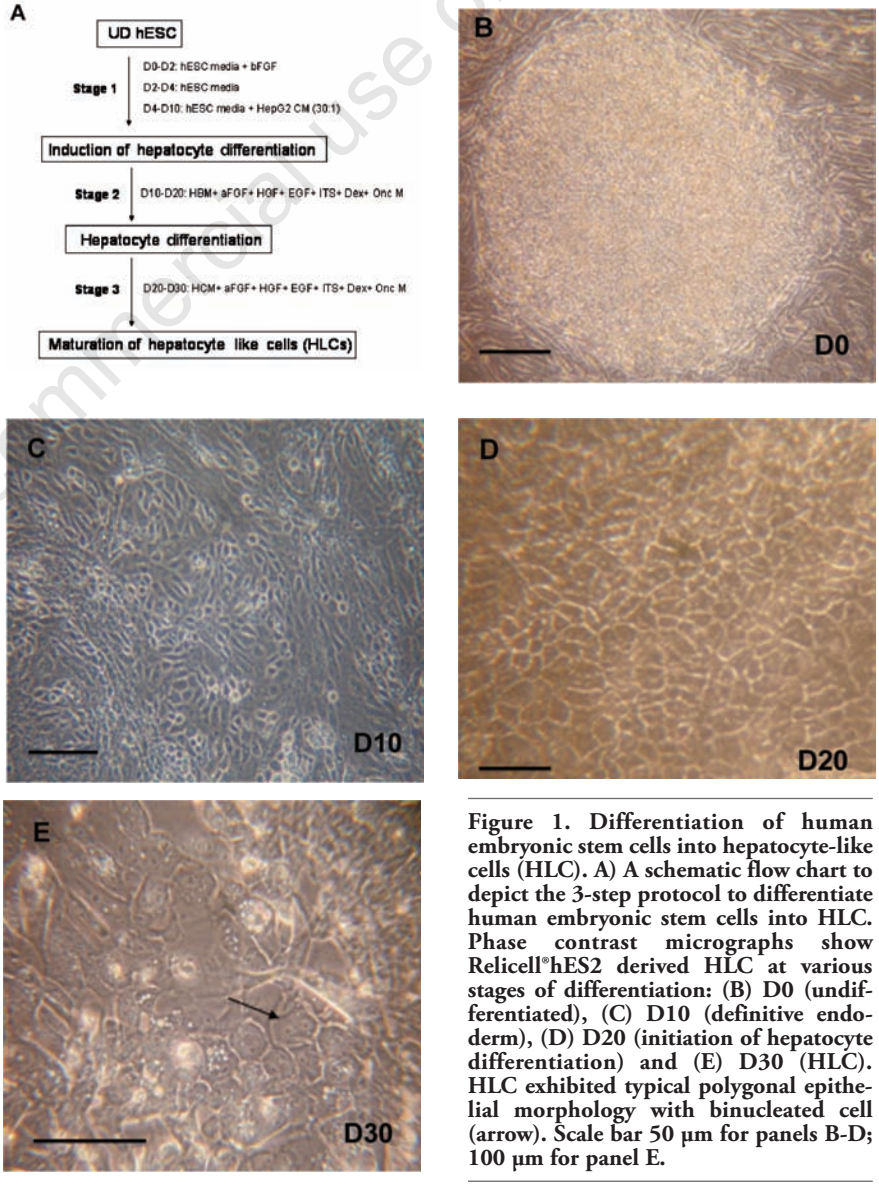

Figure 1. Differentiation of human embryonic stem cells into hepatocyte-like cells (HLC). A) A schematic flow chart to depict the 3-step protocol to differentiate human embryonic stem cells into HLC. Phase contrast micrographs show Relicell®hES2 derived HLC at various stages of differentiation: (B) D0 (undifferentiated), (C) D10 (definitive endoderm), (D) D20 (initiation of hepatocyte differentiation) and (E) D30 (HLC). HLC exhibited typical polygonal epithelial morphology with binucleated cell (arrow). Scale bar 50 µm for panels B-D; 100 µm for panel E.

bation of the cells with primary antibody [Sox 17, R & D Systems, 1:50; GATA4, Santa Cruz Biotechnology (Santa Cruz, CA, USA), 1:50; HNF4α, Santa Cruz Biotechnology, 1:50; albumin, Sigma, 1:100; CK8/18, Chemicon (Millipore Corp., Billerica, MA, USA), 1:100; EpCAM, BD Biosciences, 1:100] at 4ºC overnight. Next day the cells were incubated with FITC labeled secondary antibody for 1 h at RT and counterstained with DAPI. Images were captured using fluorescent microscope (Nikon Eclipse, E600; Nikon Corp., Tokyo, Japan). Appropriate negative controls were included.

Flow cytometric analysis

Differentiated cells (Days 10 and 30) were harvested using 0.05% trypsin EDTA (Invitrogen). For staining of intracellular markers, Oct3/4 (Santa Cruz Biotechnology), HNF3β (Santa Cruz Biotechnology), Sox17 (R & D Systems), albumin (Sigma), CK8/18 (Santa Cruz Biotechnology), AFP (Santa Cruz Biotechnology) and HNF4α (Santa Cruz Biotechnology) were used and the cells were fixed and permeabilized with Cytofix/Cytoperm™ reagent kit (BD Biosciences, San José, CA, USA) for 20 min. The antibodies were diluted 1:100 in all cases. Detection of surface markers such as CD73 (1:100, BD Biosciences) and NCAM (1:200, Chemicon) was carried out without permeabilization. After permeabilization, the cells were incubated with the primary antibody at 4ºC overnight. Next day the cells were labeled with appropriate FITC labeled secondary antibodies (Sigma) at RT for 40 min. The cells were analyzed on FACScaliber™ Flow Cytometer (BD Biosciences) using Cell Quest software.

Measurement of hepatocyte secretory proteins

The human albumin content was determined in culture supernatant, collected from both the differentiated HLC (Day 30) and HepG2 cells using Human Albumin ELISA Quantitation kit (Alpha Diagnostic, 1190; Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) according to the manufacturer's instructions. In brief, 100 µL of the culture supernatant, controls and standards were loaded onto each well in duplicates and incubated for 60 min, and were then washed 4 times. The plate was incubated with 100 µL/well of Anti-human Albumin-HRP Conjugate for 30 min, washed 5 times followed by incubation with 100 µL of TMB substrate for 15 min. The color development was terminated by addition of 100 µL Stop Solution and the plate was read at 450 nm using an ELISA plate reader. The albumin content in culture supernatants was calculated after normalizing with the media controls.

The human urea content in culture supernatant, collected from both the differentiated HLC (Day 30) and HepG2 cells. The urea content was determined by quantitative colorimetric urea determination kits (BioAssay Systems, Hayward, CA, USA) using a 100 µL sample according to the manufacturer's instructions.

Periodic acid Schiff assay

The differentiated HLC (Day 30) along with HepG2 (positive control) and MEF cells (negative control) were fixed with 4% paraformaldehyde for 15 min at RT and then washed 3 times with PBS. Fixed cells were allowed to oxidize in 1% periodic acid (Sigma) for 5 min and rinsed 3 times in deionized water. The cells were incubated with Schiff's reagent (Sigma) for 15 min, stained with hematoxylin (Sigma) for 1 min and then observed under the microscope.

Results

Our protocol was able to efficiently differentiate all the three hESC lines to HLC (Figure 1A). During the differentiation process, hESC underwent a series of profound morphological changes (Figure 1B).

Formation of definitive endoderm

In stage 1, the undifferentiated hESC (Figure 1B, panel A) were primed with HepG2-CM to initiate their transition to definitive endoderm stage. This resulted in the appearance of typical endodermal cells in the culture (Figure 1B, panel B).

In a parallel and independent experiment, we had used Activin A (100 ng/mL) in combination with EGF (10 ng/mL) without HepG2-CM to induce DE formation and differentiated further to generate HLC.

When cell numbers from both the methods were compared, hESC seeded at 20×10^4 cells/cm^2 induced by HepG2-CM yielded 14×10^4 cells/cm^2 differentiated cells. In contrast, induction of hESC seeded at the same cell density with Activin A yielded only 1.9×10^4 cells/cm^2 (Figure 2A).

Molecular analysis showed that HepG2-CM treated Day 10 cells during stage 1 expressed elevated levels of SOX17, GATA4 and HNF4α as compared to undifferentiated hESC (Figure 2B). At the same time, the level of OCT3/4, the pluripotency indicator in hESC was found to be down-regulated. The expression of the mesendoderm marker T (Brachury) was not detected (*data not shown*). This confirms the cells had completed the intermediate mesendoderm transition and progressed towards endoderm lineage. The expression of ectoderm markers were either absent (SOX1) (*data not shown*) or very low (NFH), demonstrating negligible ectoderm differentiation. Similarly, the expression of mesoderm lineage marker, NKX2.5 was also very low (Figure 2B). Immunostaining of the HepG2-CM treated (Day 10) cells showed positive expression of endoderm markers such as Sox 17, GATA4 and HNF4α (Figure 2C). Quantification of these protein markers by flow cytometric analysis (FACS) revealed that Sox 17 (83%), HNF3β (32%), HNF4α (49%) were highly expressed by these cells whereas the level of Oct3/4 (10%) was low. This provided further confirmation that the cells had differentiated towards endodermal lineage (Figure 2D).

The expression of various hepatocyte related genes observed during the differentiation of hepatocytes induced by Activin A treatment in stage 1 are as shown in Figure 2E.

Differentiation into hepatocyte-like cells

Further differentiation and maturation were achieved by allowing the cells to proliferate in the basal medium supplemented with pro-hepatic growth factors aFGF, HGF, OSM, ITS, Dexamethasone and EGF. By Day 30, the final cell population (25×10^4 cells/cm^2) exhibited the characteristic polygonal epithelial morphology of hepatocyte-like cells (HLC) some of which were binucleated under the microscope (Figure 1B, panel D).

Characterization of hepatocyte-like cells derived from human embryonic stem cells

The HLC differentiated from the three hESC lines, BG01, Relicell®hES1, Relicell®hES2 showed the expression of genes involved in hepatic differentiation and metabolic functions (Figure 3A). Even though HLC derived from different hESC lines showed the expression of all the genes, there were subtle differences in the expression amongst the samples. OCT3/4 expression was down-regulated while the expression level of HNF4α gene was up-regulated in HLC derived from all the three hESC lines. HNF4α and ALBUMIN levels were highest (19.9-fold and 52.fold, respectively) in the Relicell®hES2 derived HLC. Similarly, the level of AFP, a marker for embryonic liver was elevated in all the three hESC lines, the highest expression was seen in Relicell®hES2 derived HLC (699-fold). This was higher than in human fetal liver, indicating that the maturation level of hESC derived HLC is lower than that seen in the fetal liver. The genes associated with the functionality of the hepatocytes such as *CYP3A4* and *GSTA1* were seen in all the hESC derived HLC but were many times lower than human fetal liver. The maximum expression of phase I drug metabolizing enzyme CYP3A4 was seen in BG01 while the

phase II drug metabolizing enzyme, GSTA1, was highly expressed in BG01 and Relicell®hES2 derived HLC. These results suggest that Relicell®hES2 derived hepatocytes expressed higher levels of mature genes such as ALBUMIN, GSTA1 than BG01 and Relicell®hES1 (Figure 3A).

Immunofluorescence analysis revealed that hESC derived HLC were strongly positive for hepatocyte specific intracellular markers such as albumin and CK8/18 (a skeletal protein of hepatocytes) and surface marker, EpCAM (Figure 3B).

The expression of various markers in HLC was further quantitated by FACS (Figure 3C). Seventy percent of Relicell®hES2 differentiated cells showed the expression of albumin and other liver specific markers such as EpCAM (44%) and CK8/18 (55%). The expression of early stage markers such as Sox17 and HNF3β was evaluated to be relatively low, 25% and 15%, respectively (*data not shown*). BG01 derived HLC showed high expression of hepatic and mesenchymal lineage marker (CD73-46%) and lower expression of neuronal marker (NCAM- 12%). Interestingly, in the present study, the Relicell®hES1 cell line with known propensity for ectodermal lineage[19,20] had once again demonstrated this affinity, as 65% of the

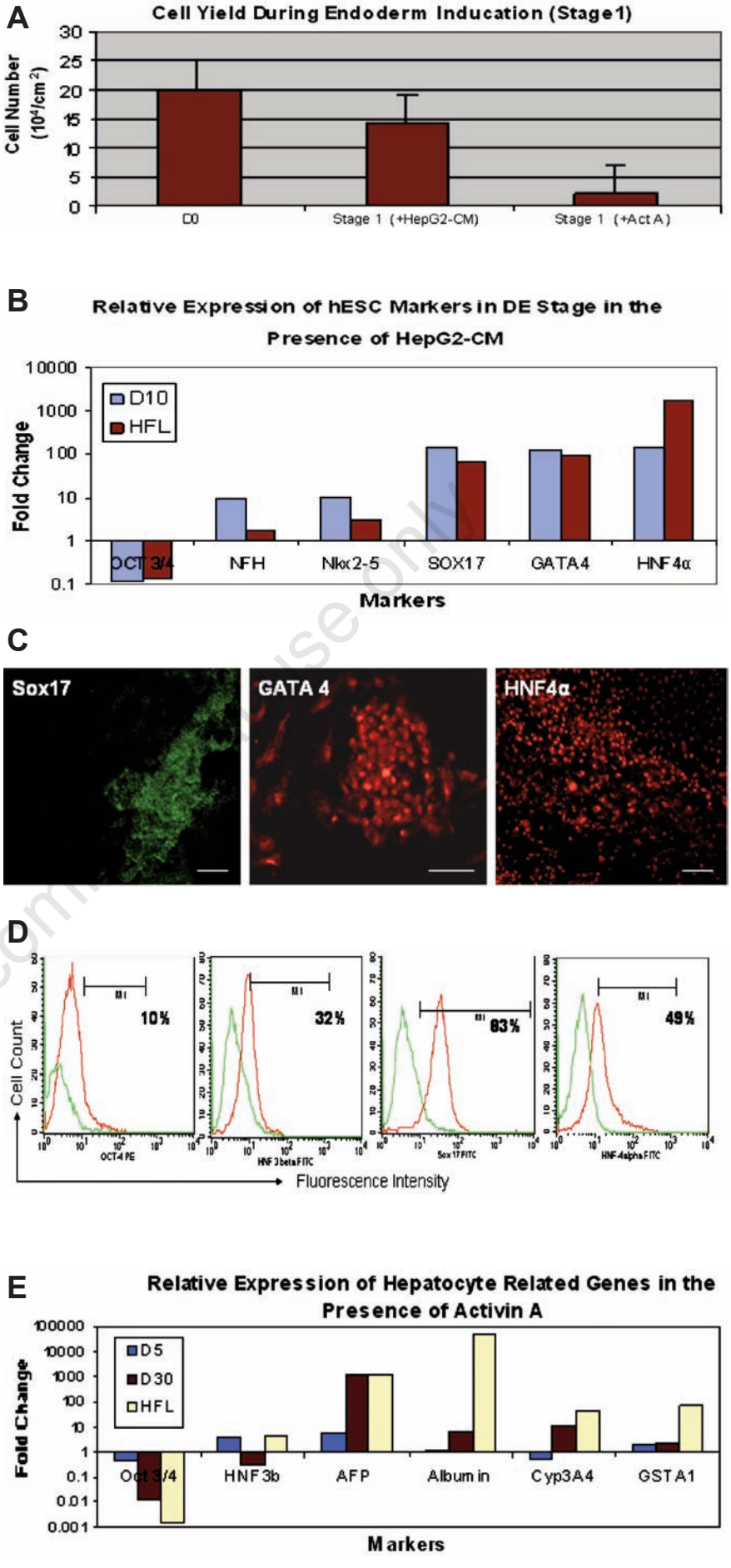

Figure 2. Induction of definitive endoderm formation from human embryonic stem cells using HepG2 conditioned media. A) Comparison of the effect of HepG2-CM and Activin A on cell number during induction of endoderm formation (stage I of differentiation). Reduction in cell number was higher in the Activin A treated cultures than those treated with HepG2-CM. B) Gene expression analysis of D10 cells by qRT-PCR shows that Relicell®hES2 cells expressed high levels of definitive endoderm genes such as *SOX17, GATA4* and *HNF4α* and negligible levels of other lineage markers (NFH, NKx2.5). The expression of each gene was normalized to corresponding levels of 18s rRNA gene and fold expression was compared to undifferentiated cells. HFL, hepatocyte fetal liver. C) Immunostaining of Relicell®hES2 cells induced by HepG2-CM on Day 10 of differentiation shows strong expression of stage specific markers, Sox 17, HNF4α and GATA4. Scale bar 50 µm for Sox 17 and HNF4α and 100 µm for GATA4. D) Flow cytometry analysis of Day 10 cells shows immunoreactivity (red line) of these cells to HNF3β, Sox 17 and HNF4α and low level of Oct3/4. Isotype-antibody staining (green line) shows background fluorescence. E) Differentiated HLC from Relicell®hES2 in the presence of Activin A evidenced regulation of hepatic specific markers as analyzed by qRT-PCR.

differentiated cells were positive for the neuronal marker, NCAM. Our results indicate that the HLC derived from both Relicell®hES2 and BG01 showed higher expression of hepatic lineage markers and lower expression of mesenchymal and neuronal markers.

Functional evaluation of the differentiated hepatocyte-like cells

We then evaluated the liver specific properties of hESC derived HLC such as glycogen storage, production of plasma proteins, albumin and urea. Periodic acid Schiff staining revealed cytoplasmic deposits of glycogen (pink staining) in hESC derived HLC as well as in HepG2 cells (positive control). However, no staining was observed in the MEF samples which were used as the negative control (Figure 4A). The level of albumin secreted by the Relicell®hES2 cells derived HLC in the culture supernatant was measured by ELISA. Differentiated HLC secreted 90 µg/mL of albumin as compared to 78 µg/mL secreted by HepG2 cells (Figure 4B). Detectable levels of urea (8.56 mg/mL) were produced and released by hESC derived HLC in the culture supernatant on Day 30 (Figure 4C) whereas undifferentiated cells did not show any urea production.

Discussion

The development of a successful differentiation protocol depends on the basic understanding of the *in vivo* functioning of several molecular signals and processes. During embryogenesis, the differentiation of progenitor cells into fully mature hepatocytes depends on the initiation of complex pathways triggered by various signals released from adjacent cells. With clarification of the intricate mechanisms of liver development through recent advances in mouse, zebrafish and chicken embryos,[21,22] new protocols of hepatocyte differentiation have been established to mimic the development of hepatocytes *in vivo*.[23]

During gastrulation, epiblast cells ingress into the primitive streak to form mesendoderm and DE. During embryogenesis, the DE layer gives rise to pancreas and liver.[24] High levels of Nodal signaling bring about the endoderm specification.[25] Activin A, a member of the TGFβ superfamily, is generally used to induce the ES cells to form definitive endoderm *in vitro* as it binds to the same receptor as Nodal.[26] Most of the published protocols have demonstrated the use of a very high concentration of Activin A with either Wnt3a[27] or sodium butyrate[28] to achieve this *in vitro.* Due to the high cost of Activin A and high cell mortality caused by both Activin A and sodium butyrate,[29] an alternative method to induce DE is desirable. We have attempted to address this issue by using HepG2-CM since we assume that it may contain biologically active liver specific soluble factors and other extracellular proteins to induce hepatic commitment. Proteomic analysis of HepG2-CM has identified the presence of liver specific proteins such as α-fetoprotein, transferin, α1-antitrypsin and albumin. Other proteins such as vimentin and fibronectin, which are associated with cell adhesion and migration, are also known to be present in HepG2-CM.[30] Transcriptional profiling of HepG2-CM treated hESC has shown upregulation of a number of genes involved in the TGFβ1/NODAL pathway which is related to primitive streak cells and nascent mesoderm formation.[31] Further study is needed to clarify the role of the proteins identified and their interactions in the signaling pathways involved in differentiation of hESC.

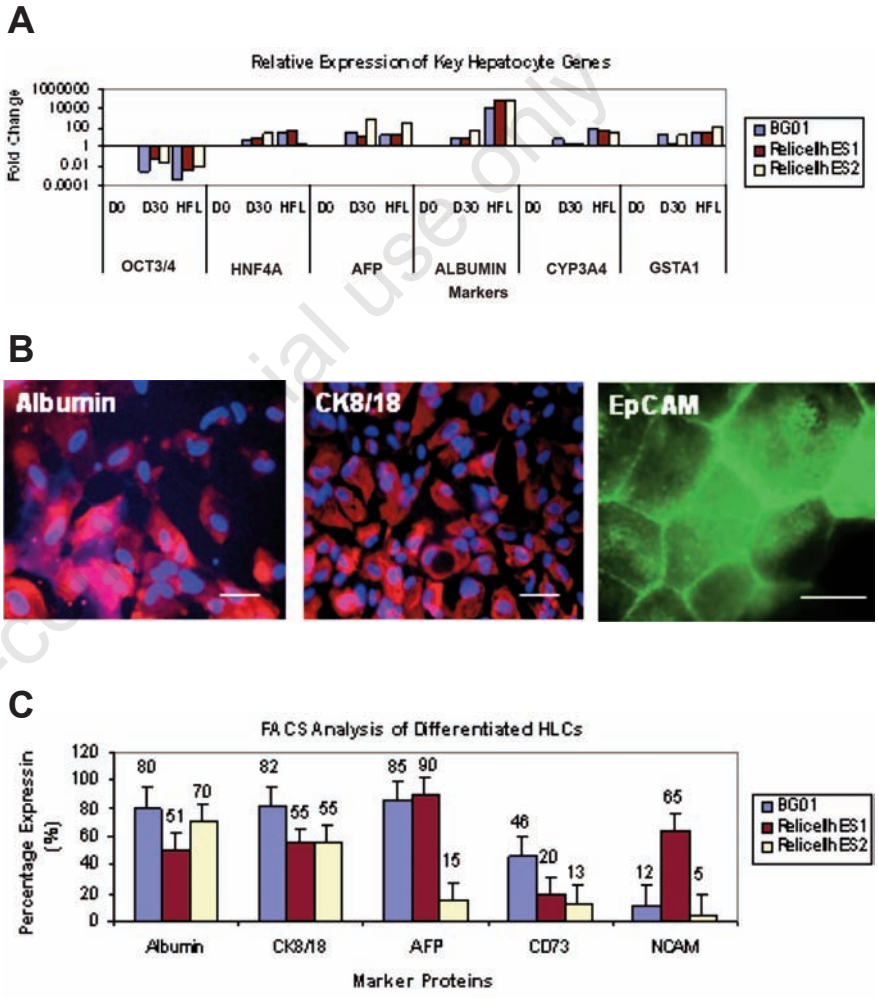

Figure 3. Characterization of the hepatocyte-like cells (HLC) from BG01, Relicell®hES1 and Relicell®hES2 cell lines. A) A comparative molecular profiling of D30 HLC derived from BG01, Relicell®hES1 and Relicell®hES2 cell lines by qRT-PCR shows downregulation of OCT3/4 and upregulation of hepatocyte related markers such as HNF4α, AFP, Cyp3A4 and GSTA1. The values are expressed as the fold change over undifferentiated hESC. B) The immunofluorescence assay shows that the Day 30 cells strongly express the key functional protein of hepatocyte, albumin as well as other hepatocyte associated markers such as CK8/18 and EpCAM. Representation of HLC derived from Relicell®hES2 cell line; similar results were also obtained from BG01 and Relicell®hES1 cell differentiation experiments. (Scale bar 50 µm for albumin and CK8/18, 100 µm for EpCAM). C) Protein expression of HLC derived from the three different cell lines were quantitated by FACS analysis.

Priming of undifferentiated hESC with HepG2-CM in stage 1 of our differentiation protocol was helpful in inducing the hESC towards DE, evident from the upregulation of SOX17, GATA4 and HNF4α and downregulation of OCT3/4. SOX17 is specifically expressed by endoderm cells and not by ectoderm, mesoderm or trophectoderm.[32] High levels of SOX17 expression in our culture demonstrate that the cells are differentiating towards the endodermal lineage. In an independent and parallel experiment using only Activin A, we observed that Activin A promotes the formation of DE as expected (Figure 2E), but the hepatocyte specific marker expression was much lower than that seen during induction with HepG2-CM (Figure 2B-D).

In addition, it is known that serum free media promotes DE differentiation[8,25,33] as is also evident in our differentiation process.

Furthermore, the initiation of liver ontogeny requires FGFs secreted from the pre-cardiac mesoderm and bone morphogenetic proteins from the septum transversum mesenchyme.[34] The rapid expansion and maturation of the fetal hepatic cells require HGF and Oncostatin-M (OSM) which are secreted from the surrounding mesenchymal stromal cells and hematopoietic stem cells.[35] The production of HGF is greater in the postnatal than in the prenatal period and is known to stimulate maturation of cultured fetal hepatocytes, suggesting that it may play a similar role *in vivo*.[36]

In our protocol, the sequential application of suitable combinations of pro-hepatic factors at appropriate stages resulted in visibly coordinated differentiation of the cells and exhibited a temporal regulation of hepatic stage specific gene expression (Figures 2B and 3A). Increasing hepatic characteristics were acquired over time. The majority of the cells in culture resembled HLC morphologically, expressing a repertoire of mature hepatic genes, *HNF4α*, ALBUMIN, *CYP3A4* and *GSTA1*. The prevalence of drug-metabolizing enzymes CYP3A4 and GSTA1 in hESC-derived hepatocytes are critical to their application both in cell replacement, the study of drug metabolism and *in vitro* hepatotoxicity screening. Cyp3A4 is the most abundant form of CYP protein in the liver and is involved in phase I metabolism of a large number of pharmaceutical drugs.[37] GSTs catalyze the conjugation of xenobiotics with glutathione and are a vital part of the phase II detoxifying system.[38]

The method developed here is suitable to obtain high yielding, *in vitro* functional HLC from three independently derived hESC lines. However, this study clearly identified finer differences between the marker expression profiles of the different cell lines indicating an inherent spontaneous differentiation potential. Specifically, the Relicell®hES1 cell line has a lower inclination to differentiate into hepatocytes than the BG01 and Relicell®hES2 cell lines. This may possibly be attributed to their inherent propensity of development. Earlier studies also suggest that independently derived hESC may differ in their developmental potential.[39]

Although the hESC-derived hepatocytes described here exhibit characteristics of mature hepatocytes, they also appear to retain some immature characteristics, such as a relatively low level of cytochrome P450 transcript and persistent AFP expression, a marker of fetal rather than adult hepatocytes. This is consistent with earlier reports.[26,40] Since by this robust protocol we could successfully generate hepatocyte-like cells from different hESC lines, we envisage that this method can probably be employed to generate patient specific hepatocytes leading to the development of personalized drug regimens which may circumvent the need for life-long immune suppression following hepatocyte transplantation.

In conclusion, the hepatic-like cells generated by this unique protocol have a tremendous potential in the discovery and development of new drugs, and also as therapy for the treatment of human liver failure by their incorporation into liver assist devices. However, from a clinical perspective, it is essential to clarify the biological as well as the potential carcinogenic molecules and pathogens in HepG2-CM prior to the application of this methodology to generate functional hepatocytes.

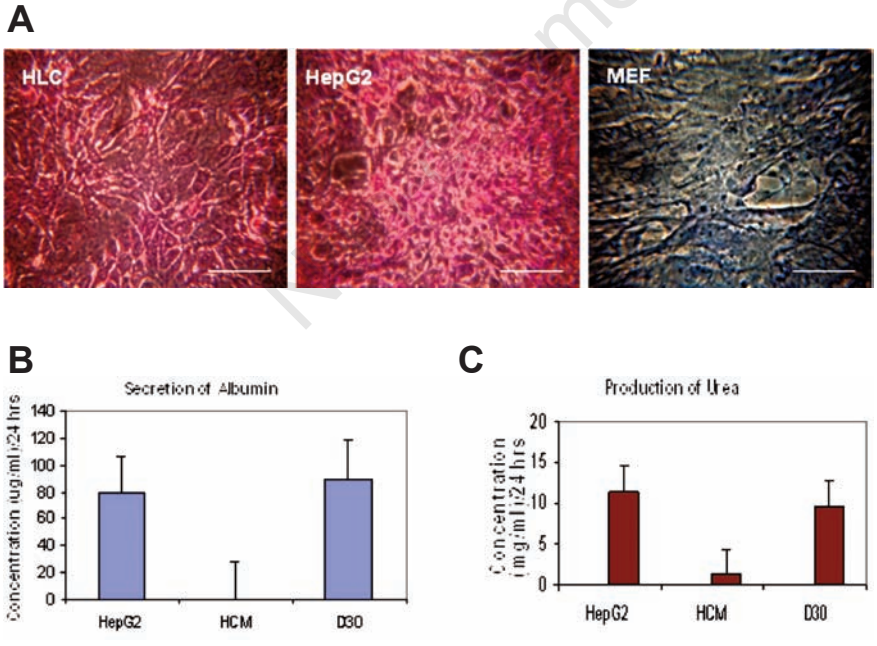

Figure 4. Functional assays of human embryonic stem cell derived hepatocyte-like cells (HLC). A) Periodic acid Schiff staining to demonstrate glycogen storage in HLC seen here as areas stained in pink. The controls are seen as dark pink in HepG2 (positive) and no stain in mouse embryonic fibroblast (negative). (Scale bar 100µm). B) Albumin secreted by the Day 30 HLC in the culture supernatant as compared with HepG2 (positive control) and hepatocyte culture media (negative control). C) Urea production by the Day 30 HLC as compared with the appropriate controls.

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