Enhanced Differentiation of Adult Bone Marrow-Derived Stem Cells to Liver Lineage in Aggregate Culture

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Hepatocyte-like cells derived from stem cells hold great potential for clinical and pharmaceutical applications, including high-throughput drug toxicity screening. We report a three-dimensional aggregate culture system for the directed differentiation of adult rat bone marrow-derived stem cells, rat multipotent adult progenitor cells, to hepatocyte-like cells. Compared to adherent monolayer cultures, differentiation in the aggregate culture system resulted in significantly higher expression level of liver-specific transcripts, including an increased albumin mRNA level, and higher levels of albumin and urea secretion. This coincides with the presence of significantly more cells that express intracellular albumin at levels found in primary hepatocytes. The differentiated cell aggregates exhibited cytochrome P450-mediated ethoxyresorufin-O-dealkylation and pentoxyresorufin-O-dealkylation activity. Consistent with these increased mature functions, cells within the aggregates were shown to have many ultrastructural features of mature hepatocytes by transmission electron microscopy. With the scalability of the aggregate culture system and the enhanced differentiation capability, this system may facilitate translation of generating hepatocytes from stem cells to technology.

Introduction

THE LIVER IS THE LARGEST visceral organ in the body with pivotal roles in metabolism and biosynthesis. The primary cells of the liver, the hepatocytes, are involved in a wide variety of functions, including metabolism of carbohydrates, proteins, and fats; the synthesis of plasma proteins such as albumin; maintenance of blood hemostasis by the production of coagulation factors; storage of glucose and vitamins; and detoxification of drugs and xenobiotics. Functional hepatocytes may be exploited in treatment of liver failure by cell transplantation and bioartificial liver devices. They are also needed in the pharmaceutical applications of toxicity testing and drug development. Therefore, for both clinical and pharmaceutical discovery applications, large numbers of functional hepatocytes are required. Cultured primary hepatocytes lack proliferative capacity and lose their liver-specific function a few days after isolation and culture. Thus, an alternative, preferably renewable source of functional hepatocytes would be of great significance.

The unique capacity of stem cells to undergo extensive self-renewal, and their ability to differentiate into multiple cell types, including hepatocytes, make them promising candidates as a source of functional hepatocytes for wide-

ranging applications.^{1–7} This promise of stem cell technology is, however, critically dependent on the ability to direct the lineage specification of stem cells to hepatocytes with high efficiency and in scalable systems. Efforts to differentiate pluripotent stem cells to hepatocytes initially relied mainly on spontaneous embryoid body (EB) differentiation. Given the low extent of differentiation to hepatocyte-like cells in EBs, subsequent efforts focused on developing directed differentiation protocols based on time-dependent treatment of soluble factors, designed to mimic embryonic liver development.^{8–14} Instead of employing EBs, these studies were carried out in monolayer cultures on extra-cellular matrix protein coatings. We have recently developed such a multi-step protocol capable of directing the differentiation of both bone marrow-derived rat stem cells (multipotent adult progenitor cells [MAPCs])^{15–18} and human embryonic stem cells (hESCs) toward a functional hepatocyte-like cell state.¹⁹ However, these directed differentiation protocols lead to a heterogeneous population with a relatively small fraction of hepatocyte-like cells. Further optimization of culture systems may increase the yield and functional maturity of these stem cell-derived hepatocyte-like cells.

It has been previously demonstrated by our group and others that primary hepatocytes exhibit higher levels of

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hepatic-specific function, such as albumin and urea synthesis and cytochrome P450 activity, for a longer period *in vitro* when cultured as three-dimensional (3D) spheroids as compared to monolayer culture.^{20–22} A number of factors such as the microenvironment, cell–cell interactions, and cellular polarization may play a positive role in sustaining liver-specific functions in those hepatocyte spheroids. Thus, it is worth exploring whether such positive effects of 3D cultivation translate to the differentiation of stem cells to hepatocyte-like cells. Differentiation to the hepatic lineage in 3D culture has been attempted using both ESC^{23,24} and mesenchymal stem cells^{25,26} with encouraging results. However, those early studies were performed with single-step protocols, not with the multistep directed differentiation methods that have been employed recently with more success. hESCs differentiated using a multistep directed hepatic differentiation protocol²⁷ in a 3D four-compartment hollow fiber capillary membrane perfusion platform exhibited enhanced hepatic differentiation.²⁸ However, further exploration is needed to optimize the differentiation protocol and culture conditions to yield mature hepatocyte-like cells capable of functioning at the level of primary hepatocytes.

In this study, we applied the multistep directed differentiation protocol, previously optimized for monolayer differentiation,¹⁹ to a spheroid-like aggregate culture system. We recently demonstrated that MAPCs as aggregates can be expanded 85-fold within 4 days of stirred spinner flask culture to reach a final cell concentration of approximately 10^6 cells/mL.²⁹ The propensity of differentiation of these spinner-expanded cell aggregates to hepatocyte-like cells was also shown. Given the ease of scalability of the aggregates and the established ability to generate cells with hepatic functions in 2D monolayer culture, we explored whether hepatic maturation would be further increased in differentiations carried out using a directed differentiation protocol on cells cultured as aggregates.

Materials and Methods

Cell culture of rat MAPC

The isolation of rat MAPC lines from rat bone marrow has been previously described.^{16,30} Briefly, cells were harvested from the bone marrow of the hind limbs of a 4–6-week-old rat and cultured under rat MAPC conditions until cells with the rat MAPC phenotype arose. MAPC cell lines were maintained in MAPC medium at 37°C in a 5% oxygen and 5%–6% CO₂ incubator at a starting cell density of 300 cells/cm² and passaged using 0.05% (w/v) Trypsin-EDTA (5 mg/L Cellgro) every 2 days.¹⁶ The expression level of transcripts (*Oct4*, *Cd31*, and *Rex1*) and protein (*Oct4*) characteristic of MAPCs, as well as multilineage differentiation potential, were routinely evaluated to assure the undifferentiated status of the rat MAPC lines.^{15,16}

MAPC medium

MAPC medium consisted of a basal medium that was a 60/40 (v/v) mixture of low-glucose Dulbecco's modified Eagle's medium (Gibco) and MCDB-201 (Sigma) supplemented with ascorbic acid 3-phosphate (Sigma) (0.026 µg/mL), linoleic acid–bovine serum albumin (LA-BSA; Sigma; final concentrations of 10^3 µg/mL BSA and 8.13 µg/mL LA),

insulin–transferrin–selenium (ITS; Sigma; final concentration 10 µg/mL insulin, 5.5 µg/mL transferrin, 0.005 µg/mL sodium selenite), dexamethasone (Sigma; 0.02 µg/mL), β-mercaptoethanol (4.3 µg/mL), and qualified fetal bovine serum (Hyclone) (2% (v/v)). MAPC medium also contained three growth factors: human platelet-derived growth factor (R&D; 10 ng/mL), mouse epidermal growth factor (EGF; Sigma; 10 ng/mL), and mouse leukemia inhibitory factor (10^3 units/mL; Chemicon, ESGRO). All media used were also supplemented with penicillin (100 IU/mL) and streptomycin (100 µg/mL) (Gibco).

Formation of 3D aggregates

Aggregates were formed from single-cell suspensions of MAPCs using the forced aggregation method.²⁰ Briefly, cells in suspension were seeded in each well of ultralow attachment round-bottomed 96-well plates (Corning) at 1000 cells/well in 100 µL of media, and centrifugal force (1500 rpm, 4 min) was applied to settle them to the bottom of the well, thus facilitating agglomeration. The plates were subsequently incubated in a 37°C incubator at 5% oxygen and aggregates formed with time.

Hepatocyte harvest and spheroid culture

Hepatocytes were harvested from 4- to 5-week-old female Sprague-Dawley rats weighing 200–250 g using the two-step collagenase perfusion procedure.³¹ The isolated hepatocytes were plated in Williams' E medium (Sigma) supplemented with 2.2 g/L sodium bicarbonate (Sigma), 20 ng/mL EGF (Sigma), 10^{-7} M dexamethasone, $1 \times$ ITS (Sigma), 10^{-4} M L-Ascorbic Acid, and 100 IU/mL Penicillin and 100 µg/mL Streptomycin (Gibco) on 35 mm Falcon Primaria dishes (Becton Dickinson) at approximately 10^6 cells/plate and kept in a 21% O₂, 5.8% CO₂ incubator to form the spheroids.

Hepatic differentiation

For the monolayer differentiation, rMAPCs were plated in 24- or 12-well plates, precoated with 2% Matrigel (BD Biosciences) at a starting cell density of 25,000–50,000 cells/cm² in rMAPC medium, and maintained in a 5% O₂, 5.8% CO₂ incubator until 80%–90% confluency was reached. For the aggregate differentiation, aggregates were allowed to form for 4 days after forced aggregation, as previously described,³² and differentiation was continued in the 96-well U-bottom plates with one aggregate/well. To initiate differentiation, maintenance medium was removed, the wells were rinsed with PBS, and the differentiation medium was added. During differentiation, the cells were maintained in a 21% O₂, 5% CO₂ environment. The basal medium for hepatic differentiation was similar to MAPC medium except for the following: ITS and LA-BSA were at 25% of the level in MAPC medium, dexamethasone was at 0.4 µg/mL, and the three growth factors and serum were absent. Differentiations were carried out in four stages for a total of 20 days. On day 0 (D0), D6, D10, and D14, complete medium was replaced with fresh medium with the following growth factor supplements: (i) D0: Activin A at 100 ng/mL and Wnt3a at 50 ng/mL (Note: on D2.5 and D4, in 50% medium exchange, fresh medium containing only Activin A was added), (ii) D6: bFGF at 10 ng/mL and BMP4 at 50 ng/mL, (iii) D10: FGF8b

at 25 ng/mL, aFGF at 50 ng/mL and FGF4 at 10 ng/mL, (iv) D14: HGF at 20 ng/mL and Follistatin at 100 ng/mL. During each stage, 50% of medium was exchanged every 2 days.

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was isolated from cell lysates using the RNeasy microkit (Qiagen) according to instructions provided in the kit. cDNA was synthesized from the extracted RNA using the Superscript III reverse transcriptase (Invitrogen) method. The PCR mix consisted of the cDNA samples (20 ng), 1×SYBR Green Mix PCR buffer (Applied Biosystems), and primers (100 nM) in a 12 µL reaction mixture. The quantitative real-time polymerase chain reaction (qRT-PCR) was run on a Realplex mastercycler (Eppendorf) using the following program: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min followed by a dissociation protocol to obtain a melting curve. Transcript abundance relative to *Gapdh* was expressed as $\log_2(\text{Expression Relative to } Gapdh)$ and calculated as ΔCt , which is $Ct(\text{gene of interest}) - Ct(Gapdh)$. [Note: Expression Relative to *Gapdh* = $2^{-\Delta Ct}$]

Albumin secretion by ELISA

For albumin secretion measurements, medium was completely replenished on D19, collected on D20 (24 h after replenishment), and stored at -80°C until albumin concentration measurement was performed. For rat hepatocyte spheroids, the medium was replenished on D5 after isolation and collected on D6 for albumin measurement. Hepatocyte differentiation medium was used as a negative control. Rat albumin was measured using an ELISA kit (Starters Kit Bethyl E101 and, respectively, Bethyl E110-125), following the manufacturer's instructions. An internal standard, provided with the kit, was used to obtain a four-parametric logistic fit to enable the estimation of albumin concentration. The albumin concentration in the fresh differentiation medium, which was added to the cells on D19, was subtracted from the amount present in the D20 samples to quantify the amount secreted by the cells from D19 to D20. Cells were counted at D20, and albumin secretion was reported on a per cell basis.

Urea secretion

To quantify urea secretion, cells were rinsed with PBS and then grown in hepatocyte differentiation basal medium containing 1 mM ammonia-bicarbonate and 0.5% fetal bovine serum (FBS) for 24 h. Supernatant was kept at -80°C until urea was measured using the QuantiChrom™ Urea Assay Kit (BioAssay Systems DIUR-500). Cells were counted at D20, and urea secretion was reported on a per cell basis.

Intracellular staining for albumin by flow cytometry

Cells were harvested by trypsinization, washed with PBS thrice, and suspended at 100,000 cells per tube. Cells were fixed with 4% paraformaldehyde for 15–20 min at room temperature and then permeabilized in SAP buffer (PBS with 0.1% [w/v] saponin and 0.05% [w/v] sodium azide) for 30 min at 4°C. An incubation at 4°C with 1 µg/mL rat-albumin-specific antibody (Dako; A0001) or 1 µg/mL Rabbit IgG isotype control (Jackson Immunoresearch) in SAP buffer

for 15 min was followed by three washes of SAP buffer and incubation with Cy5-labeled anti-rabbit IgG (Jackson Immunoresearch; 1:500 in SAP buffer) for 30 min in the dark. Finally, cells were washed thrice with SAP buffer, filtered, and resuspended in 500 µL PBS for flow cytometric analysis using FACS Calibur (Becton Dickinson).

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde for 30 min and washed with PBS. The samples were incubated in 5% sucrose in PBS overnight and supercooled with isopentane before freezing in OCT for sectioning. H₂O₂ was used to inhibit endogenous peroxidase, and FBS was used to reduce nonspecific binding. Cells were then incubated with antibodies against AFP (Neomarkers; 1:75 dilution), CK18 (Abcam; 1:75 dilution), albumin (MP Biomedicals; 1:5000 dilution), or E-cadherin (BD Biosciences; 1:150 dilution). The antibody-treated samples were then treated with EnVision-Peroxidase with DAB substrate (Dako) and examined under microscope. To rule out nonspecific binding, samples were stained with the secondary antibodies only without primary antibodies.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on D0 and D20 aggregates. Cell aggregates were washed thrice (3×5 min) with 0.1 M cacodylate buffer and fixed in 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 7.2) for 40 min at room temperature. After postfixation in 1% osmium tetroxide and 0.1 M of cacodylate buffer, samples were dehydrated in graded series of ethanol followed by propylene oxide treatment, and embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined using a JEOL 1200 EXII electron microscope.

Ethoxyresorufin-O-dealkylation activity assay

Ethoxyresorufin-O-dealkylation (EROD) activity was measured in 96-well tissue culture dishes by monitoring the release of fluorescent reaction product into the medium using the SpectraMax M2 (Molecular Devices) fluorescent spectrometer. For monolayer samples, cells were seeded directly into the assay dish on Matrigel under differentiation conditions. Two aggregates were placed in each well for the measurement, whereas primary hepatocytes were seeded at 35,000 cells/well. For induction the cells were treated with 50 µM β-naphthoflavone (BNF) for 24 h. Resorufin at known concentrations was used to construct a standard curve and ethoxyresorufin without cells were used as a background control. Cells were washed once with assay media (Williams' E medium without phenol red supplemented with 25 µM dicumarol at pH 7.4) and incubated for 10 min at 37°C before the assay medium was replaced with fresh assay medium containing 39 µM ethoxyresorufin with 0.1% v/v DMSO, and incubated in a 37°C incubator. Data were collected every 30 min for up to 3 h.

Statistical analysis and reproducibility

All differentiations were performed in biological triplicates with monolayer and aggregate cultures run in parallel.

FIG. 1. Gene expression during differentiation by quantitative real-time-polymerase chain reaction (average of $n=3$). Temporal expression of the pluripotency gene transcript *Oct4* in monolayer (**a**) and aggregate (**b**) cultures; temporal expression of definitive endoderm gene transcripts *Gsc* (□), *Eomes* (○), *Mixl1* (Δ), and *Foxa2* (◇) in 2D (**c**) and aggregate (**d**) cultures; temporal expression of hepatic endoderm gene transcripts *Afp* (□) and *Ttr* (○) and hepatoblast/hepatocyte marker *Alb* (Δ) in 2D (**e**) and aggregate (**f**) cultures; (**g**) day 20 (D20) expression of mature hepatic gene transcripts *G6pc*, *Pepck*, *mGst1*, *Hnf4a*, *Arg1*, *Ttr*, *Aat*, *Tat*, and *Mrp2* in monolayer (□) and aggregate (■) cultures; (**h**) D20 expression of nonliver lineage gene transcripts *Pdx1*, *Ngn3*, *Ve-cad*, and *Pax6* in monolayer (□) and aggregate (■) cultures.

FIG. 2. Expression of intracellular albumin in differentiation cultures. Flow cytometric comparison of cells expressing intracellular albumin in primary rat hepatocytes (a) and in cells differentiated in aggregate cultures (b) or monolayer cultures (c), with isotype controls. Percentages were generated from gating based on 0.5% false-positive in a negative control population (not shown). Color images available online at www.liebertonline.com/tea

qRT-PCR, albumin secretion, urea secretion, and EROD kinetic activity for D20 samples are each presented as the mean of three replicates with conventional standard deviation. The Student's *t*-test was used to calculate *p*-values for statistical significance. Remaining assays are presented as representative samples.

Results

Liver differentiation in 3D aggregates

Rat MAPC aggregates were allowed to form by the forced aggregation method for 4 days, yielding one cell aggregate in each well of a 96-well plate. Differentiation was initiated by the addition of 100 μ L differentiation medium per well for a starting cell concentration of 1.5×10^5 cell/mL. In addition, rMAPC differentiation to the hepatic cell lineage was also carried out in monolayer cell culture on Matrigel-coated plates at a similar cell concentration. Three biological replica cultures were performed for each condition. A four-step differentiation protocol designed to mimic embryonic liver development was used to direct the cells to hepatocyte-like cells over a period of 20 days.¹⁹ The cells were treated with

Activin-A and Wnt3a till D6 to induce differentiation to mesendoderm and definitive endoderm.^{33–37} The transition through this stage was monitored by the expression of Goosecoid (*Gsc*), eomesodermin (*Eomes*), *Mixl1*, and *Foxa2* transcripts. Next, to specify the cells to the ventral foregut endoderm/hepatoblast, FGF2 and BMP4 were supplemented to the medium from D6 to D10^{38–41}; expression of *Afp* and *Ttr* transcripts, typical hepatoblast markers, was evaluated. To promote proliferation of hepatoblasts and maturation to hepatocyte-like cells, FGF8b, FGF1, and FGF4 were added between D10 and D14,⁴² and HGF and Follistatin-288 from D14 to D20.

Generation of cells with mature hepatic functions was assessed by qRT-PCR for several hepatic-specific genes: albumin (*Alb*), a protein secreted by hepatoblasts and mature hepatocytes; Arginase 1 (*Arg1*) of the urea cycle; phosphoenolpyruvate carboxykinase (*Pepck*) and glucose 6-phosphatase (*G6pc*) of the gluconeogenesis pathway; microsomal GST1 (*mGst1*) of the glutathione-S-transferase (GST) pathway; Factor 7 (*F7*) of the coagulation factor cascade; *Mrp2*, a bile acid transporter; and Hepatocyte nuclear factor 4a (*HNF4a*), a liver-enriched transcription factor.

FIG. 3. Higher functional activity in aggregate differentiation cultures. (a) Albumin secretion by ELISA and (b) urea secretion quantification in monolayer (□) and aggregate (■) cultures on D20 of differentiation and in primary rat hepatocytes spheroids (■) (average of *n* = 3; *denotes *p* < 0.05).

FIG. 4. Ethoxyresorufin-O-dealkylation activity. Kinetic measurements of resorufin release into the media without drug treatment (□) and after β -naphthoflavone treatment (■) in monolayer and aggregate cultures on D0 and D20 of differentiation and in primary hepatocyte spheroids (D20 samples average of $n=3$).

We first assessed if MAPC aggregate cultures remained undifferentiated after 4 days of formation by assessing expression of *Oct4* transcript and several genes expressed early during differentiation. *Oct4* transcripts were at a similar high level in aggregate and monolayer cultures on D0 (Fig. 1a, b), whereas levels of *Gsc*, *Afp*, *Alb*, *G6pc*, and *Tat* were all undetectable (data not shown). This is consistent with published data in that *Oct4* remained high after MAPC aggregate formation, whereas differentiation markers were low or undetectable.³² Thus, at the start of differentiation, cells in both monolayer and aggregate cultures were undifferentiated. The expression level of *Oct4* transcripts decreased by 16–32-fold in both cultures as early as D6 and subsequently remained relatively unchanged. Transcripts for the definitive endoderm genes *Gsc*, *Eomes*, *Mixl1*, and *Foxa2* increased and peaked on D6 in both cultures (Fig. 1c, d). A similar increase in expression of transcripts for the hepatic endoderm markers *Afp* and *Ttr* was observed on D10 in both culture systems and remained unchanged till D20 (Fig. 1e, f), suggesting that cells progress through definitive endoderm and hepatoblast stages with similar efficiencies in both monolayer and aggregate cultures.

The transcript level for *albumin*, which was similar for both conditions during the first two stages of differentiation (to D10), became more than 17-fold higher in the aggregate culture as compared to the monolayer culture on D20 (Fig. 1e, f). Similarly, transcript levels of several other mature hepatic genes such as *G6pc* (11-fold), *Pepck* (69-fold), *mGst1* (8-fold), and *Hnf4a* (4-fold) were significantly higher in aggregate cultures on D20, based on p-values of less than 0.05 (Fig. 1g). A trend of higher levels of transcripts was observed for several other mature hepatocyte genes, such as *Arg1* (6-fold), *Ttr* (2-fold), *Aat* (2-fold), *Tat* (2-fold), and *Mrp2* (1.4-fold), in aggregate cultures compared to monolayer cultures on D20, although the higher p-values cast doubt on the significance of the differences (Fig. 1g). Therefore, although

the dynamics of expression of stage-specific genes were similar between the monolayer and aggregate differentiations through D10, the expression levels of some hepatocytes-specific transcripts were significantly higher in aggregate differentiation on D20. The expression levels of several nonhepatic lineage transcripts, including pancreatic and duodenal homeobox 1 (*Pdx1*), neurogenin-3 (*Ngn3*), VE-cadherin (*Ve-cad*), and paired box gene 6 (*Pax6*), on D20 were either similar in both conditions or significantly lower in aggregate cultures (Fig. 1h).

Albumin expression in hepatocyte-like cells

We next compared the fraction of albumin-positive cells in the entire population by fluorescence-activated cell sorting (FACS) in aggregate and monolayer cultures (Fig. 2). Primary hepatocytes isolated from adult rat liver were used as a positive control. rMAPC-progeny cultured for 6 days without cytokines, in which no *albumin* transcripts were detected, were used as a negative control. Gating was set such that false-positives in the negative control were limited to 0.5%. With this gating, 92.3% of the primary hepatocyte population was albumin positive (Fig. 2a). The data, including the samples and their isotype-matched controls, are presented as histogram plots wherein the relative abundance of cells expressing albumin protein at a particular intensity can be enumerated. In the D20 aggregate differentiation sample, 12% of the population expressed albumin protein at levels comparable to adult hepatocytes (Fig. 2b). In contrast, 0.67% of the population from the D20 monolayer culture expressed albumin protein at similar levels (Fig. 2c), and this population virtually overlapped with the negative control. To further investigate the fraction of cells expressing albumin in each population, data of albumin and side scattering intensities were shown as a contour plot (Supplementary Fig. S1, Supplementary Materials and Methods; Supplementary Data are available online at www.liebertonline.com/tea). Gating on the side scatter intensity axis was set such that >99% of hepatocytes fell in the positive region (upper two quadrants). These data demonstrate that 5.9% of the cells from the aggregate culture had similar side scatter characteristics as primary hepatocytes (Supplementary Fig. S1a), suggesting a similar level of cell complexity and granularity between the two samples, in addition to their comparable levels of albumin expression. Only 0.3% of cells from the monolayer differentiation had similar side scatter characteristics as primary hepatocytes (Supplementary Fig. S1b). Thus, a larger fraction of cells from the aggregate differentiation appear to possess primary hepatocyte-like features.

Albumin secretion by aggregate progeny

We next tested whether the increased albumin transcript and protein levels in the aggregate culture were reflected in increased albumin secretion. Albumin production was measured, normalized to the number of cells, and compared to primary rat hepatocytes cultured as spheroids. The albumin secretion rate of the aggregate culture on D20 was 1.8 ± 1.0 pg/cell/day, which was at least 10 times that of the secretion in the monolayer culture (0.1 ± 0.06 pg/cell/day) (Fig. 3a) and 20% of that of adult hepatocyte spheroids (8.3 ± 3.7 pg/cell/day). Considering that only 12% of aggregate-progeny contained significant amounts of albumin

FIG. 5. Ultrastructural evaluation of 3D differentiated aggregates. Transmission electron microscopic evaluation of undifferentiated aggregates on D0 (**a**, **b**) and fully differentiated aggregates on D20 (**c**–**f**) of differentiation. (**a**, **b**) Undifferentiated aggregates demonstrate a spindle-like morphology and contain a high nucleus (N)-to-cytoplasm (C) ratio with very few cytoplasmic structures (scale bars 5 μ m). (**c**) Whole-cell micrograph of a differentiated aggregate reveals a polygonal morphology, a significantly reduced nucleus (N)-to-cytoplasm (C) ratio, and an abundance of cytoplasmic structures including lipid vesicles (arrow) (scale bar 5 μ m). (**d**) Micrograph of a portion of a single cell reveals an abundance of mitochondria (arrows) and rough endoplasmic reticulum (arrowheads) (scale bar 2 μ m). (**e**) Micrograph of a cell–cell border reveals a canaliculus-like structure (CS) between the cells delimited by tight junctions (arrows) (scale bar 1 μ m). (**f**) Micrograph centered on a canaliculus-like structure (CS) reveals microvilli (arrows) protruding into the CS space (scale bar 2 μ m).

protein, it is possible that this small population of cells may produce albumin at levels comparable to primary hepatocytes cultured as spheroids.

Other mature hepatocyte functions

The urea secretion rate of aggregates from D20 was measured. On a per cell basis the rate, 32 ± 12 urea pg/cell/day, was 2.5 times of the corresponding D20 monolayer culture (13 ± 5 pg/cell/day) and at a 25% level of primary hepatocyte spheroids (132 ± 20 pg/cell/day), respectively (Fig. 3b). Cytochrome P450 activity in the aggregates was assessed by CYP1A (EROD) activities. The rate of resorufin generation from ethoxyresorufin and its subsequent release into the culture media was quantified on undifferentiated and differentiated cells with or without BNF treatment and compared to activity in rat hepatocyte spheroids (Fig. 4). Negligible activity was observed in undifferentiated rMAPCs in both monolayer and aggregate cultures. However, on D20 of the differentiation, cells maintained in the aggregate culture exhibited higher EROD activity (1008 pmol/h/ 10^6 cells) than cells from the

monolayer culture (172 pmol/h/ 10^6 cells). EROD activity increased in both cultures after a 24h treatment with BNF to 5150 and 1280 pmol/h/ 10^6 cells in aggregate and monolayer cultures, respectively. These EROD activity levels corresponded to 34.5% (aggregate) and 8.6% (monolayer) of the activity observed in BNF-treated rat hepatocyte spheroids (14900 pmol/h/ 10^6 cells).

We also assessed EROD and pentoxoresorufin-O-dealkylation (PROD) activities by imaging intracellular accumulation of the fluorescent reaction product. Although the method is qualitative, it provides a more sensitive assessment than the resorufin release assay. Higher EROD- and PROD-induced fluorescence can be seen in the D20 differentiated aggregates as compared to D0 undifferentiated samples (Supplementary Figs. S2 and S3). The expression of *Cyp1A1* and *Cyp2B1/2* transcripts was also investigated by RT-PCR. Expression levels of *Cyp1A1* and *Cyp2B1/2* transcripts were higher in the aggregate culture samples than in the monolayer cultures (Supplementary Fig. S4). No *Cyp1A1* or *Cyp2B1/2* transcript expression was observed in the undifferentiated samples.

FIG. 6. Immunohistochemistry for hepatic protein expression in aggregates. **(a)** AFP protein expression on D16 of differentiation is observed in the cytoplasm of cells on the exterior of the aggregate, whereas no AFP protein is observed in the undifferentiated D0 sample (inset); **(b)** CK18 protein expression on D20 of differentiation is observed in the cytoplasm of cells on the exterior of the aggregate, whereas no CK18 is observed in the undifferentiated D0 sample (inset); **(c)** E-Cadherin protein expression is observed on the membrane of cells in both the D0 undifferentiated sample (inset) and the D16 differentiated sample; **(d)** albumin protein expression on D20 of differentiation is observed in the cytoplasm of cells on the exterior of the aggregate, whereas no albumin protein is observed in the undifferentiated D0 sample (inset). Positive stains are indicated with solid arrows, whereas nucleus counterstains are indicated with dashed arrows. Color images available online at www.liebertonline.com/tea

Morphological and structural features in differentiated aggregates

The morphological and structural characteristics of the differentiated aggregates were examined by TEM (Fig. 5). Cells in the aggregates on D0 (Fig. 5a, b) had a high nucleus-to-cytoplasm ratio. In differentiated aggregates, cells had developed a polygonal epithelial shape with low nucleus-to-cytoplasm ratio and much more complex cytoplasmic features (Fig. 5c–e). Abundance of mitochondria, rough ER, canaliculi-like structures between cells delimited by tight junctions on both ends, and microvilli (Fig. 5f), features indicative of a differentiated hepatocyte-like cell structure,⁴³ were identifiable within the cells.

Immunostaining was performed on the aggregates on D0 and D16 or D20 for AFP, CK18, E-Cadherin, and Albumin (Fig. 6). No AFP, CK18, or Albumin immunoreactivity was found in the aggregates on D0. On D16 of differentiation, AFP was localized to cells on the exterior of the aggregate (Fig. 6a), and on D20, CK18 (Fig. 6b), and albumin (Fig. 6d) were also observed to localize to the cytoplasm of cells on the exterior of the aggregate. Cell-cell adhesion protein

E-cadherin is seen at both D0 and D16 in the cell membranes of some cells (Fig. 6d).

Discussion

In this study, in which we combined directed multistep differentiation of MAPCs toward the hepatocyte lineage with a 3D aggregate culture system, we observed an enhanced level of differentiation and a higher degree of maturity of MAPC-derived hepatocytes as compared to differentiation in monolayer culture. Many mature hepatocyte-specific genes were expressed at higher transcript levels in the aggregate culture than in the monolayer, including members of the gluconeogenesis pathway (*Pepck*, *G6pc*), glutathione conjugation (*mGst1*), and liver-enriched transcription factors (*Hnf4a*).

The enhanced hepatocyte differentiation in aggregate cultures, as compared to the monolayer culture, could be attributed either to a larger fraction of hepatocyte-like cells in the population with similar levels of maturation, to a higher level of maturation in a similar fraction of hepatocyte-like cells within the population, or to a combination of both. It is also conceivable the aggregate culture sustains differentiated hepatocyte-like properties better and contributes to the observed higher activities. The expression levels of mesoderm, definitive endoderm, and hepatoblast transcripts were similar between the aggregate and monolayer cultures up to the midstage of differentiation, suggesting that a similar fraction of cells were induced toward liver-endoderm in both differentiation systems. It is therefore likely that the increased hepatocyte-specific transcript levels observed in the aggregate differentiation are at least partly due to an enhancement in the terminal stage of differentiation to more mature hepatocytes. The larger fraction of cells with increased maturity is indeed shown by intracellular albumin protein expression using flow cytometry. The albumin-positive cells seen in the aggregate culture, comprising 12% of the bulk population, had levels of intracellular albumin similar to primary hepatocytes. They also exhibited similar granularity and cellular complexity to primary hepatocytes as suggested by the side scatter profile. The albumin staining protocol was optimized to eliminate background staining in undifferentiated MAPCs; the substantially reduced false-positive staining may have been at the expense of a lower sensitivity in identifying the positive cells, as suggested by the relatively low level of positive cells in the monolayer culture.

The relative differentiation efficiency can be estimated from the input of stem cells and the resulting hepatocyte-like cells. Using the initial seeded rMAPC density and the number of albumin-positive cells on D20, the efficiency of hepatocyte-like cell generation is estimated to be 60% and 3.85% for aggregate and monolayer culture, respectively.

Overall, the results suggest that the increased transcript levels of hepatocyte-specific genes in the aggregate culture is likely the result of enhanced differentiation of the hepatoblasts to cells with more mature functions in the final stages of differentiation. Indeed, the maturation of fetal liver cells into functional hepatocytes has been demonstrated in 3D-based systems using various cytokines, including acidic FGF, FGF4, and HGF, which are present in the late stages of our protocol.^{44,45} However, it is not clear if and why these growth factors are more effective in aggregate culture than in monolayer. Hepatocytes cultured as spheroids sustain higher

levels of liver-specific functions over a longer period than when maintained in the conventional monolayer culture. The sustained functions are synchronous to structural and morphological characteristics of spheroids, including the existence of tight cell-cell contact, retention of cell-cell junctions, and polarization.^{17,20–22,46,47} Under TEM, cells in the differentiated aggregates appeared to be abundant in tight junctions at cell-cell boundaries. Bile-canalicular-like openings possessing microvilli and delimited by tight junctions were also highly visible.

A key feature to the 3D aggregate system is the extensive cell-cell contact, which may have contributed to the enhanced differentiation. E-cadherin, which plays a key role in cell polarity and junction formation, is seen at cell-cell contacts. It has been shown to regulate signaling in some systems (in Caco-2 enterocytes) by controlling the nuclear abundance of liver transcription factor Hnf4a.⁴⁸ Using a surface deposited with different amount of E-cadherin, it was shown that hepatocytes have higher levels of albumin and urea secretion when exposed to higher concentrations of cadherin.⁴⁹ Similarly, mouse ESCs grown as EBs overexpressing E-cadherin were shown to have higher transcript levels of *G6pc* in response to growth factor cocktails than EBs in which E-cadherin expression was downregulated.⁵⁰ Other than cell-cell interactions, polarization of cells in 3D configurations has been shown to help in enhancing and stabilizing the differentiated functions of hepatocytes.^{47,51} Whether similar positive effects of 3D culture on hepatocytes are in play in enhancing differentiation of stem cell certainly requires further investigation.

In spite of the enhanced differentiation in the aggregate culture, the degree of maturation we observed still falls short of genuine hepatocytes. For many applications, further improvement in terms of the percentage of cells and their extent of differentiation will need to be demonstrated. Coculture of hepatocytes with other nonparenchymal cells such as endothelial cells, bone marrow cells, and fibroblasts has been shown to enhance maturation and stabilize differentiated function over a long period.^{52–54} Coculture with nonparenchymal cells in a 3D culture system and further optimizing the protocol are worth exploring for generating organized liver-tissue equivalents.

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Disclosure Statement

No competing financial interests exist.

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