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The Differentiation of Hepatocyte-Like Cells from Monkey Embryonic Stem Cells

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Abstract

Embryonic stem cells (ESC) hold great potential for the treatment of liver diseases. Here, we report the differentiation of rhesus macaque ESC along a hepatocyte lineage. The undifferentiated monkey ESC line, ORMES-6, was cultured in an optimal culture condition in an effort to differentiate them into hepatocyte-like cells *in vitro*. The functional efficacy of the differentiated hepatic cells was evaluated using RT-PCR for the expression of hepatocyte specific genes, and Western blot analysis and immunocytochemistry for hepatic proteins such as α-fetoprotein (AFP), albumin and α1-antitrypsin (α1-AT). Functional assays were performed using the periodic acid schiff (PAS) reaction and ELISA. The final yield of ESC-derived hepatocyte-like cells was measured by flow cytometry for cells that were transduced with a liver-specific lentivirus vector containing the α1-AT promoter driving the expression of green fluorescence protein (GFP). The treatment of monkey ESC with an optimal culture condition yielded hepatocyte-like cells that expressed albumin, α1-AT, AFP, hepatocyte nuclear factor 3β, glucose-6-phophatase, and cytochrome P450 genes and proteins as determined by RT-PCR and Western blot analysis. Immunofluorescent staining showed the cells positive for albumin, AFP, and α1-AT. PAS staining demonstrated that the differentiated cells showed hepatocyte functional activity. Albumin could be detected in the medium after 20 days of differentiation. Flow cytometry data showed that 6.5 $\pm$ 1.0% of the total differentiated cells were positive for GFP. These results suggest that by using a specific, empirically determined, culture condition, we were able to direct monkey ESC toward a hepatocyte lineage.

Introduction

Chronic liver disease is one of the most prevalent health problems worldwide. Although some people with end-stage liver disease can be effectively treated with orthotopic liver transplantation (OLT), considerable morbidity and mortality are associated with this form of treatment. In addition, a shortage of available donors and the high cost of the surgical procedure have rendered this treatment unavailable to many patients suffering from liver diseases in the United States and throughout the world. As a result, thousands of patients die each year while on a waiting list for transplantation, and many more are never put on the list. In view of these problems, cell-based therapy would offer a safer and readily available alternative source of treatment for patients with chronic liver diseases. The applicative potential of cell replacement therapy is evident in studies that have successfully demonstrated the use of primary adult hepatocytes in animal models of hepatic failure and liver-based metabolic diseases (Laconi et al., 2006; Wege et al., 2001). Despite the encouraging results and continued advances in the field, technical difficulties that are associated with isolating adequate number of transplantable adult hepatocytes have hindered the progress of this form of therapy toward clinical application. Moreover, adult hepatocytes dedifferentiate and do not proliferate in culture.

In recent years, embryonic stem cells (ESCs) have emerged as an attractive source of cells that may be used for cell replacement therapy and tissue regeneration because of their pluripotent status and unlimited capacity for self-renewal. For these reasons, an efficient and reproducibly robust method for differentiating an unlimited source of human ESC to functional hepatocytes in an *in vitro* system would provide a means to overcome the challenges of using adult hepatocytes. However, the use of human ESC as a model for cell therapy has been plagued with multiple scientific, ethi-

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cal and legal questions that are highly controversial and have yet to be resolved. As such, nonhuman primate ESC are similar to human ESC (Kuo et al., 2003; Wolf et al., 2004; Thomson et al., 1995, 1996), without the associated controversies and regulatory rules. Indeed, comparative genomics studies have shown that the genomes of human and several nonhuman primate species are highly conserved throughout evolution, and thus share many biological similarities (Patterson et al., 2006; Gibbs et al., 2007). Among the nonhuman primate species, rhesus macaque in particular has been shown to be very similar to humans during embryonic and fetal development (Golos et al., 2004). Rhesus ESC therefore represents a useful model to perform basic and applied research.

The primary objective of this paper is to efficiently differentiate rhesus monkey ESC towards hepatocyte-like cells by employing an optimal culture condition (Shirahashi et al., 2004) for directing the differentiation of human and mouse ESC toward a hepatocyte lineage. The nonhuman primate ESC-derived hepatocyte-like cells are characterized and evaluated for functional efficacy. In addition, they are isolated from a heterogeneous population of total differentiated cells by using an α1-AT-GFP lentiviral transduction approach, and are fluorescence-activated cell sorting (FACS) analyzed for GFP-positive cells. The isolated cells will be an important source material for future studies that will include the transplantation of these cells into nonobese-diabetic/severe combined immunodeficiency disease (NOD-SCID) mice and nonhuman primates to examine the efficacy of ESC derived hepatocyte-like cells in an *in vivo* system.

Materials and Methods

Materials

Tissue culture reagents were from Invitrogen (Grand Island, NY), unless otherwise stated. Mitomycin C, β-mercaptoethanol, monothroglycerol, dexamethasone, basic fibroblast growth factor (FGF) and collagen type I were from Sigma–Aldrich (St. Louis, MO). Western blot reagents were from Invitrogen. All the primary antibodies were from Sigma (St. Louis, MO), unless otherwise stated. Second antibodies, Cy3-conjugated rabbit antigoat IgG and Cy3-conjugated goat antimouse IgG were from Jackson ImunoResearch (West Grove, PA), goat antimouse IgG-HRP and donkey antigoat IgG-HRP were from Santa Cruze Biotechnology (Santa Cruz, CA).

Culture of monkey ES cells

Rhesus monkey ESC (ORMES) were obtained from Oregon National Primate Research Center (Beaverton, OR). ESC were grown on passage 2 mouse embryonic fibroblast (MEF) feeder layers prepared at CNPRC according to previously published methods (Li et al., 2006). To prepare feeder layers, MEF were cultured until confluent and treated with 5 μg/mL mitomycin C for 2 h. The ESC were propagated in Dulbecco modified Eagle Medium: Nutrient Mix F-12 (DMEM/F12) supplemented with 10% (vol/vol) fetal bovine serum (Hyclone, Logan, UT), 100 μg/mL penicillin G, 100 μg/mL streptomycin sulfate in 0.85% saline, 100μM MEM nonessential amino acid solution, 1 mM glutamine and 0.1 mM β-mercaptoethanol at 37°C with 5% CO_2. The medium was changed daily, and ESC colonies were split every 3 or 4 days by a manual method to select for undifferentiated ESC (Pau and Wolf, 2004). ESC were stained weekly for Oct 4, SSEA 1 and 4, TRA1-60, TRA1-81, and alkaline phosphatase to confirm that they were maintained in an undifferentiated state.

The ESC colonies were manually cut into small clumps of approximately 100–150 cells. These cell clumps were maintained in suspension culture in Iscove's modified Dulbecco's medium containing 20% fetal bovine serum, 0.3 mM monothroglycerol, 0.126 U/ml human insulin (Eli Lilly and Company, Indianapolis, IN), 100 nM dexamethasone, 100 U/mL penicillin G, 1% streptomycin sulfate, and 1 mM glutamine. After 12–14 days of suspension culture, embryoid bodies (EBs) were harvested and plated in six-well culture dish coated with collagen type I. This point in time was designated as the first day of differentiation.

RNA isolation and RT-PCR analysis

Total RNA was extracted from undifferentiated or differentiated ESC. Complementary DNA was synthesized from 1 μg total RNA using a superscript III firststrand synthesis system (Invitrogen) according to the manufacturer's protocol. PCR amplification of different genes was performed using Gene Amp PCR 9600 (Perkin-Elmer Corporation, Norwalk, CT), with a program of 94°C for 4 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, and extension at 72°C for 10 min. Primer for GAPDH was from Biosource (Carlsbad, CA), primers for albumin and AFP were from Roche (Indianapolis, IN). Other sets of primers include the following: for α1-AT, 5-tcgc tacagcctttgcaatg, 5-tgagggtacggaggagttcc; for HNF3b, 5-gacaagtgagagagcaagtg, 5-acagtagtggaaaccggag; for G-6-P, 5-cttctggacactgcatgatcacag, 5-ccagtgcagtcaacccatagaagc; for CYP3A4, 5-tgtgcctgagaacaccagag, 5-gcagaggagccaaatctacc; for CYP2E1, 5-ccgcaagcattttgactaca, 5-gctccttcacccttttcagac, for Cyp7A1, 5-gtgccaatcctcttgagttcc, 5-actcggtagcagaaagaatacatc, and for CYP1A1, 5-aggcttttacatccccaagg, 5-gcaatggtctcaccgataca. The amplified PCR products were analyzed by electrophoresis on 1.2% agarose gel and were stained with ethidium bromide for visualization.

Immunohistochemistry

Cells were rinsed twice with phosphate-buffered saline and fixed with 4% paraformaldehyde for 20 min at room temperature. Then cells were blocked with 2% BSA in PBS for 1 h. After washing three times with PBS the cells were incubated with primary antibody overnight at 4°C. The primary antibodies were used as followed: mouse anti human AFP (1:100; Santa Cruz Biotechnology), goat antihuman α1-AT (1:100; Sigma), mouse antimonkey albumin (1:100; Sigma). After staining, the cells were washed three times and then incubated with Cy3-conjugatd second antibody in 1% BSA for 90 min at room temperature. The cells were washed three times again, and the cell nuclei stained with 1 μg/mL DAPI (Roche, Germany), and then mounted with Fluoromount-G solution (SouthernBiotech, Birmingham, AL).

Western blot analysis

Western blot analysis was performed as described previously (Wege et al., 2003). All primary antibodies were purchased from Santa Cruz Biotechnology.

Periodic acid schiff (PAS) staining

PAS staining was performed on differentiated cells with a P.A.S. STAIN kit from PolySciences, Inc. (Warrington, PA). The differentiated cells were fixed in 4% paraformaldehyde for 20 min and then intracellular glycogen was stained using PAS staining according to the manufacturer's instructions.

ELISA

Every 48 h the medium was changed with 4 mL of fresh medium for collecting the supernatant after EBs were seeded on coated six-well plates. The human ALB values secreted in the supernatant was determined by Human Albumin ELISA Quantitation kit (Bethyl Laboratory, Inc., Montgomery, TX) following the manufacturer's instructions.

FACS analysis

After EBs were plated and cultured for 12–14 days, differentiated monkey ESC were transduced with the lentivirus vector containing the α1-AT promoter driving the GFP marker gene. The transduced differentiated ESC were cultured for another week, then FACS analysis was performed on a DakoCytomation MoFlo Cell Sorter, for detection of GFP-positive cells. Forward and side scatter plots were used to exclude dead cells and debris from the histogram analysis plots. The mean fluorescent intensity was determined using cells that had signal intensities higher than the control (nontransduced) cells, which avoids the intrinsic background fluorescence of the cells.

Results

We performed the evaluation of hepatic differentiation by the analysis of cell morphology, expression of hepatic proteins including α-fetoprotein (AFP), albumin and α1-antitrypsin (α1-AT) by Western blots and immunocytochemistry, and hepatocellular gene expression by RT-PCR. After monkey ESC had been differentiated for 7 days, the morphology of the cells was observed under light microscopy. The hepatocyte-like cells were round or polyhedral shaped and some were arranged in trabeculae from day 22 (Fig. 1).

To determine the time course and degree to which ESC were induced to differentiate into hepatocytes, we separated PCR products by electrophoresis on a 1.2% agarose gel containing ethidium bromide at various times during the culture process to analyze markers for liver-specific gene expression; AFP, α1-AT, albumin, hepatocyte nuclear factor (HNF-3β), glucose-6-phosphate (G-6-P), and P450 cytochrome enzymes. Most of the gene expression such as α1-AT, G-6-P, CYP1A1, HNF-3β, and CYP2E1, occurred by day10 and continued to increase from 20 to 30 days of dif-

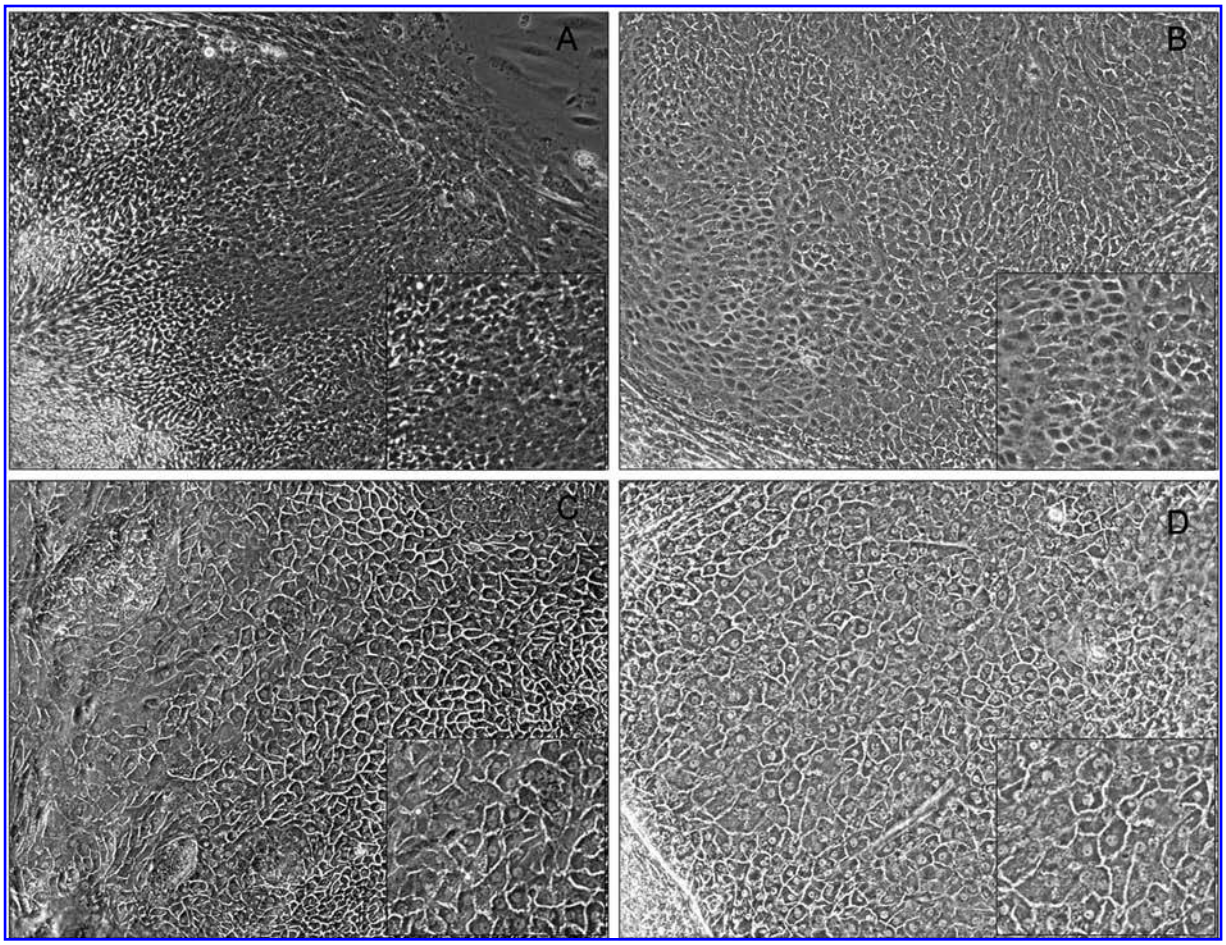

FIG. 1. The morphology of the hepatocyte-like cells derived from monkey ESC. The cells were differentiated for 7 days (**A**), 12 days (**B**), 22 days (**C**), and 30 days. Original magnification: ×100.

ferentiation. Albumin, a mature hepatocyte differentiation marker, was expressed later than the other proteins investigated. It occurred from day 20 of differentiation and the expression increased until day 30. However, the expression of AFP, the endodermal marker of early fetal hepatocyte differentiation, gradually decreased over time in differentiation culture (Fig. 2). CYP7A1 and CYP3A4, two key members of the cytochrome P450 superfamily of metabolism enzymes, were not expressed until day 20. These results suggested the cells were maturing along a hepatocyte lineage and were not primitive endoderm.

AFP, albumin, and α1-AT were also tested at the protein level by immunostaining or Western blot analysis. The results of both assays confirmed the RT-PCR findings. For immunocytochemistry, the monkey ES-derived cells were cultured in differentiated medium for 30 days and stained with albumin, AFP, and α1-AT antibody. All of them showed positive staining (Fig. 3). Western blot analysis assayed levels of AFP, α1-AT, and albumin protein in the differentiated cells during 30 days in culture. As with the PCR data, AFP expression began early and peaked at day 20, then decreased dramatically by day 30 (Fig. 4B). In normal monkey liver, there is no AFP expression. α1-AT levels increased during the differentiation process and albumin levels had the same trend as with RT-PCR analysis (Fig. 2), with later initiation of expression, but enhanced protein levels with time in differentiation culture (Fig. 4A).

We examined albumin secreted in the supernatant by ELISA and glycogen storage, which are liver-specific functions used for the identification of differentiated hepatocytes *in vitro*. The PAS staining assay showed that the differentiated cells were stained dark pink, and that the stained cells had typical hepatocyte morphology (Fig. 5). ELISA results also showed that the differentiated monkey ESC may have produced some albumin as early as day 10 after EBs were seeded; however, the level of expression was not clearly over background. These cells secreted higher levels at later time points (Fig. 6).

To detect the hepatic differentiation ratio, we used the α1-AT promoter as an internal promoter to drive the GFP marker gene and transfected this lentivirus into the cells that had been differentiated for 2 weeks. The GFP-positive cells showed green fluorescence under fluorescence microscopy (Fig. 7A). Approximately 6.5 $\pm$ 1.0% of the cells was positive for GFP by FACS analysis (Fig. 7E).

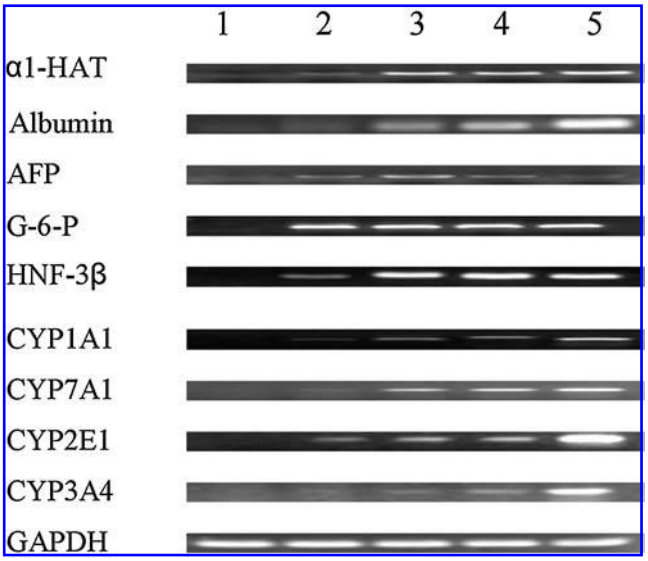

FIG. 2. Hepatocyte-specific gene expression by PCR. Undifferentiated ESC (lane 1), 10-day differentiated cells (lane 2), 20-day differentiated cells (lane 3), 30-day differentiated cells (lane 4), and adult monkey liver (lane 5). RNA was extracted from cells at different time points after differentiation, and cDNA was generated and used for PCR to detect mRNA. This is representative of three experiments. Abbreviations: PCR, Polymerase chain reaction; AFP, alpha-fetoprotein; α1-AT, alpha-1-antitrypsin; G-6-p, glucose-6-phophatase; HNF3β(Foxa2), hepatocyte nuclear factor 3β; CYP, cytochrome P450; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Discussion

The therapeutic potential of ESC has led to the derivation of various cell lines from mouse, rabbit, nonhuman primate, and human embryos (Evans and Kaufman, 1981; Thomson et al., 1995, 1998; Wang, et al., 2007). Although the majority of published literature has focused on the differentiation processes that are involved in mouse and human embryonic stem cells becoming hepatocyte-like (Agarwal et al., 2008; Chinzei et al., 2002; Duan et al., 2007; Hay et al., 2008; Teratani et al., 2005), monkey ESC serve as a clinically relevant model for studies that cannot be conducted in humans because of practical or ethical limitations, and because nonhuman primates represent a much better model for human physiology than do rodents. Considering these benefits that are associated with using nonhuman primate ESC as a model system, here we report a new method for differentiating rhesus macaque ESC along a hepatocyte lineage to enhance the potential of ESC-based therapy for liver-related diseases.

The differentiation of hepatocytes from monkey ESC was characterized by analyzing liver-specific serum protein genes such as albumin, AFP, α1-AT, G-6-P, and P450 cytochrome enzymes, as well as the endoderm-specific transcription factor gene, HNF3β, with RT-PCR. Our data showed that AFP expression was present 10 days after the initiation of the differentiation process, increased until 20 days after the beginning of differentiation, and decreased at 30 days. This is consistent with the fact that AFP is expressed in endoderm and in fetal hepatocytes, and that after birth the AFP gene is selectively silenced (Jones et al., 2001); thus, we postulated that at the earliest stage of differentiation, a precursor hepatic phenotype was present, and a more mature hepatic phenotype occurred with longer periods of differentiation culture. In further support of this view, we observed that albumin expression was initiated at 20 days after differentiation, as assessed by RT-PCR, Western blot analysis, and ELISA. Albumin is the most abundant protein synthesized by mature hepatocytes (Kamiya et al., 2001); hence, between 20 and 30 days of differentiation appears to be the transition between precursor hepatocytes and more mature hepatocytes. In addition, G-6-P, predominantly expressed in hepatocytes during late gestational or perinatal stages (Imamura et al., 2004), as well as CYP1A1, CYP2A3, CYP2E1, CYP7A1, and CYP3A4, examples of cytochrome genes that play key roles in the detoxification of drugs or the metabolic

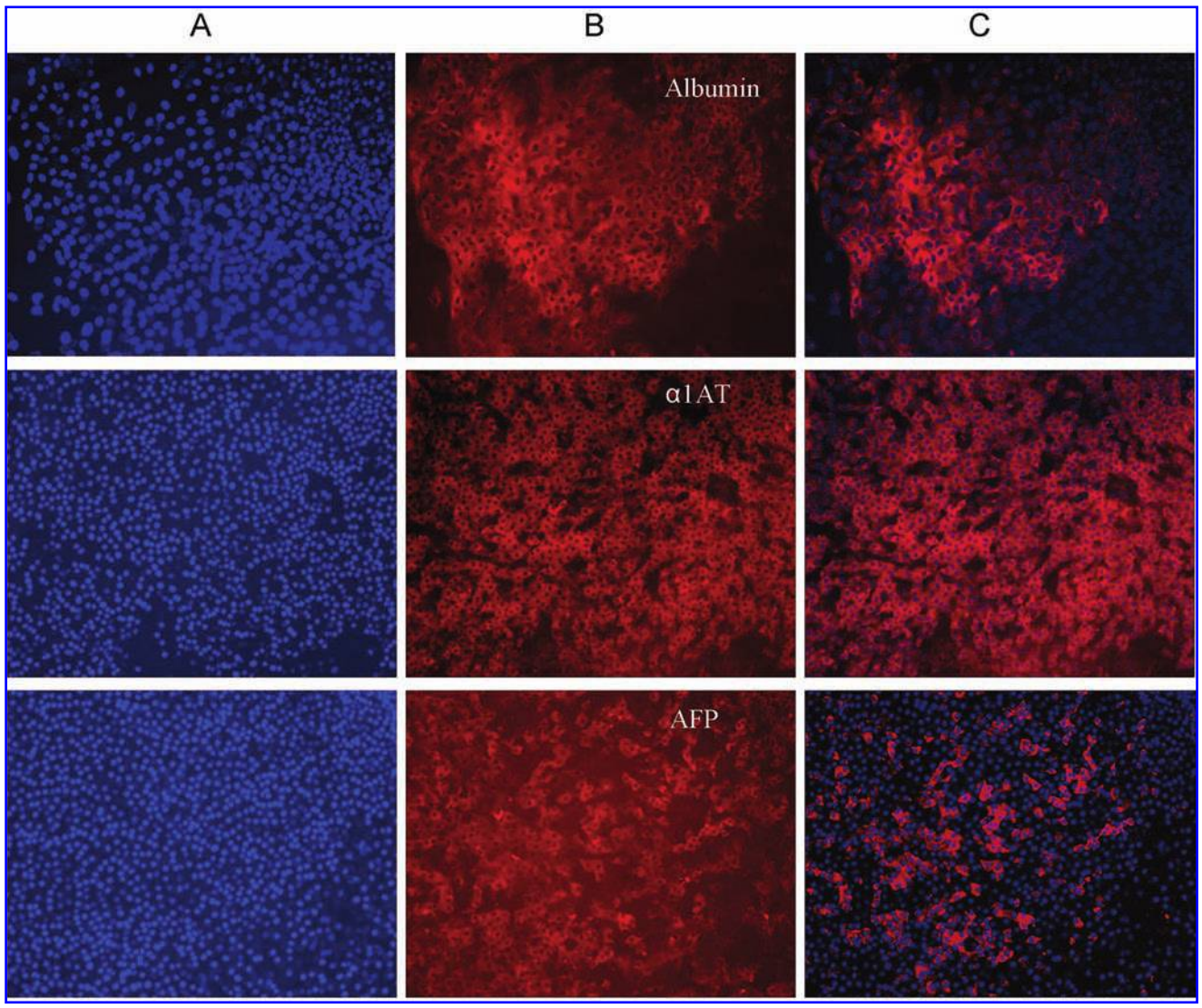

FIG. 3. Immunocytochemical analysis of monkey ESC differentiated for 30 days. (**A**) Nuclei were stained by DAPI. (**B**) Monkey ESC were immunostained with antialbumin, anti-alpha-1-antitrypsin, and anti-AFP antibodies. (**C**) Merged figure. After 30 days differentiation, monkey ESC were fixed and sequentially stained with monoclonal antibodies against either albumin, alpha-1-antitrypsin, or AFP, with a Cy3 conjugated IgG secondary antibody, and finally with DAPI. Original magnification: ×100. Abbreviations: AFP, alpha-fetoprotein; α1-AT, alpha-1-antitrypsin

activation of xenobiotics (Gonzalez and Gelboin, 1994; Wrighton and Stevens, 1992), were expressed during the differentiation culture.

Liver-enriched transcriptional factors play critical roles in hepatocyte development (Cereghini, 1996; Jochheim et al., 2004). Hepatocyte nuclear factor 3 (HNF3) is required for hepatocyte-specific expression of genes that are expressed during the differentiation of definitive endoderm (Duncan et al., 1998; Kaestner, 2000). Moreover, Jochheim et al. (2004) indicated that ESC expressing the HNF-3β gene as a transgene predominantly differentiate into cells with a hepatic phenotype (Jochheim et al., 2004). In our study, HNF3β expression occurred within 10 days of differentiation and continued to be expressed during the whole process of differentiation culture, further indicating that our culture condition appears to aid in the progressive differentiation toward hepatocytes.

We showed that the monkey-derived differentiated stem cells had morphological characteristics of hepatocytes, and molecular analysis revealed that liver-specific genes such as albumin, AFP, and α1-AT, were expressed in differentiated cells. However, the expression of these genes can also be found in the yolk sac, a derivative of the extraembryonic endoderm, as well as in the liver (Makover et al., 1989; Meehan et al., 1984; Sellem et al., 1984).The visceral yolk sac and liver have different embryonic origins yet express some common genes, and AFP is the major secreted protein in the yolk sac (Meehan el al., 1984). Both AFP and albumin are expressed in the yolk sac and in fetal hepatic cells (Sellem et al., 1984); however, during fetal liver development, AFP levels decrease as the levels of albumin gradually increases (Selten et al., 1982). Thus, the fact that AFP expression decreased with time in our differentiation culture, while at the same

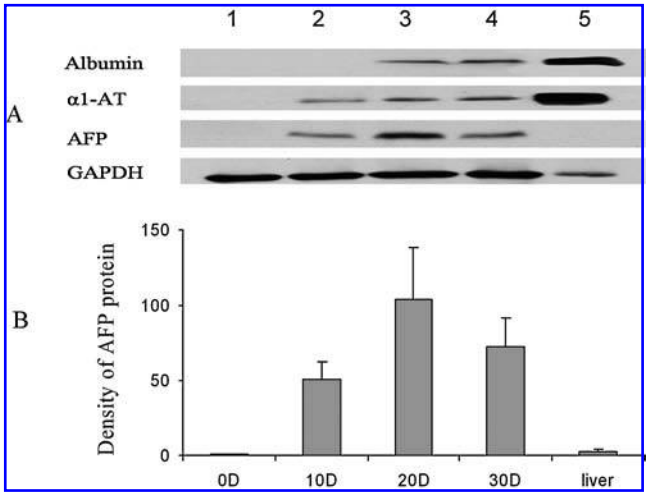

FIG. 4. Expression of hepatocyte-specific proteins. (**A**) Western blot analysis. Undifferentiated ES cells (lane 1), 10-day differentiated cells (lane 2), 20-day differentiated cells (lane 3), 30-day differentiated cells (lane 4), and adult monkey liver (lane 5). Twenty micrograms of protein from monkey stem cell extracts was loaded and 1 μg of protein from adult monkey liver tissue extract was loaded as a positive control. GAPDH as the housekeeping control was used to ensure equal loading of lanes. This figure is representative of three experiments. (**B**) The expression of AFP was quantitated by densitometry. Abbreviations: AFP, alpha-fetoprotein; α1-AT, alpha-1-antitrypsin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

time albumin expression increased strongly, suggests that the differentiated cells are of a hepatocyte lineage, not primitive endoderm. This is further confirmed by the presence of a series of liver-specific genes as well as the functional studies we employed. ELISA of albumin secretion and PAS staining for glycogen have been used as means to evaluate liver function. Both glycogen production and storage are representative functions of hepatocytes. The ELISA further revealed that the differentiated monkey ESC secreted increasing amounts of albumin over time in the differentiation culture. Thus, the ability of our differentiated cells to express these functions is highly suggestive of their having a hepatocyte phenotype.

Immunochemistry staining for AFP, α1-AT, and albumin showed positive results only in localized areas of the well. To evaluate the differentiation level more accurately, we introduced a reporter gene expressed under the control of a liver-specific promoter to analyze the percentage of hepatocyte-like cells in the whole cell population. We used a lentivirus vector to transfect the differentiated ESC (Follenzi and Naldini, 2002). The self-inactivated transfer construct contains the posttranscriptional regulatory element of the woodchuck hepatitis virus and the central polypurine tract, both of which have been shown to enhance lentiviral gene expression in several cell lines, including stem cells (Deglon et al., 2000; Follenzi et al., 2000; Zennou et al., 2000). To achieve liver-specific transgene expression, the α1-AT promoter was used as an internal promoter to drive the GFP marker gene (Duan et al., 2007). The generation and concentration of the lentiviral vectors were done as described previously (Follenzi and Naldini, 2002). During the differentiation process, α1-AT-expressing, GFP-positive cells were isolated by a cell sorter MoFlo, and GFP expression was confirmed by fluorescence microscopy. The result showed that $6.5 \pm 1.0\%$ of the cells were found to be GFP positive with FACS analysis.

In this study, we demonstrate that the combination of human insulin with dexamethasone contributes to the differen-

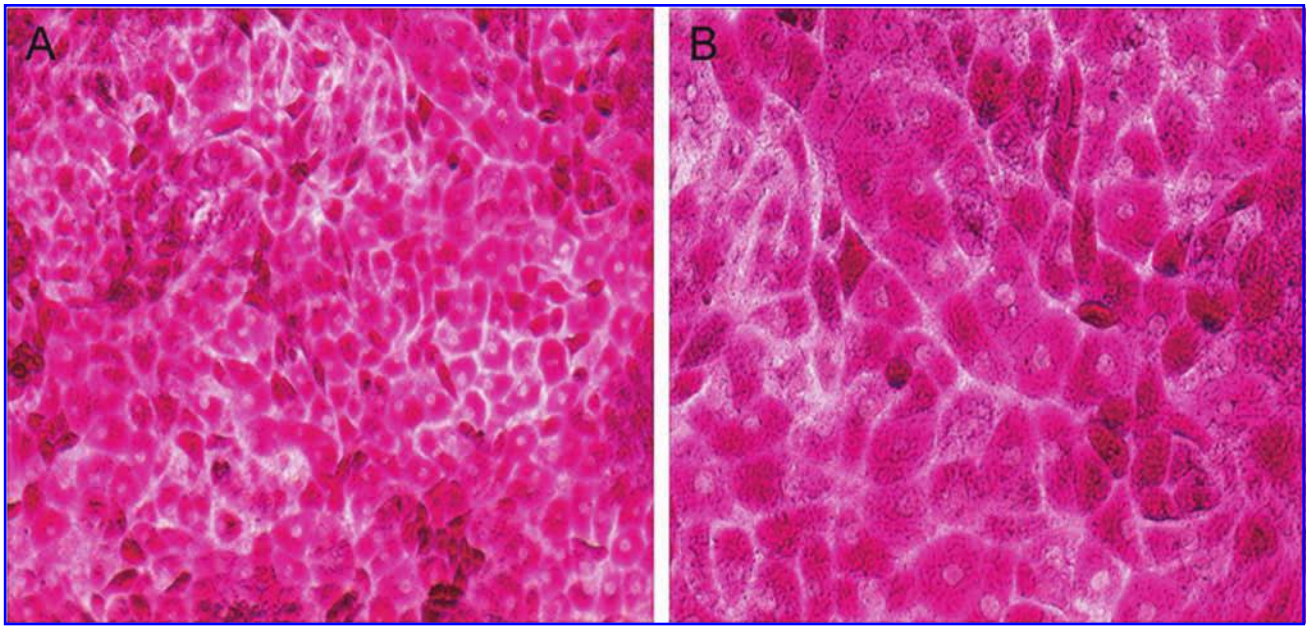

FIG. 5. Periodic acid-Schiff (PAS) staining of monkey ESC differentiated for 30 days. Chamber slides containing cells were fixed in 4% paraformaldehyde for 20 min at room temperature, and then were incubated with Periodic acid then in Schiff's reagent as described in Materials and Methods: (**A**) Differentiated cells (original magnification: $\times$100), (**B**) Differentiated cells (original magnification: $\times$200).

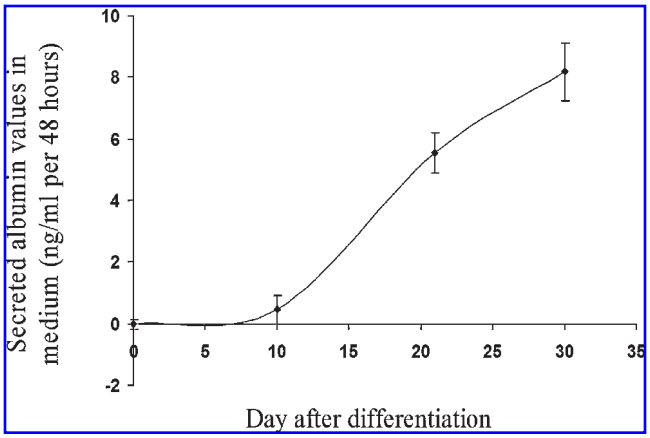

FIG. 6. Albumin secretion by the differentiated monkey ESC was determined by enzyme-linked immunosorbent assay test during differentiation. ($n \geq 3$ in each time point)

tiation of monkey ESC into hepatic-like cells. This is not surprising, since dexamethasone was reported to be involved in the differentiation of fetal liver cells into mature cells (Kamiya et al., 2001). Dexamethasone has been shown by others to be active in induction of enzymes involved in gluconeogenesis such as phosphoenolpyruvate carboxykinase and tyrosine aminotransferase (McGrane et al., 1990). It also induces albumin expression (Jefferson et al., 1985; Woodworth and Isom, 1987), and cytochrome P450 activity (Kim et al., 1995) in hepatocyte cultures, and it can alter the activities of hepatic interconvertible enzymes (Tsukada et al., 2006). It is an important substance in hepatocyte culture because the activation of glycogen synthesis by insulin is strongly enhanced by dexamethasone. When dexamethasone was removed from hepatocyte culture, insulin was incapable of activating glycogen synthesis and inactivating glycogen phosphorylase. Dexamethasone does not alter insulin binding, insulin receptor number, or kinase activity, but insulin receptor substrate was

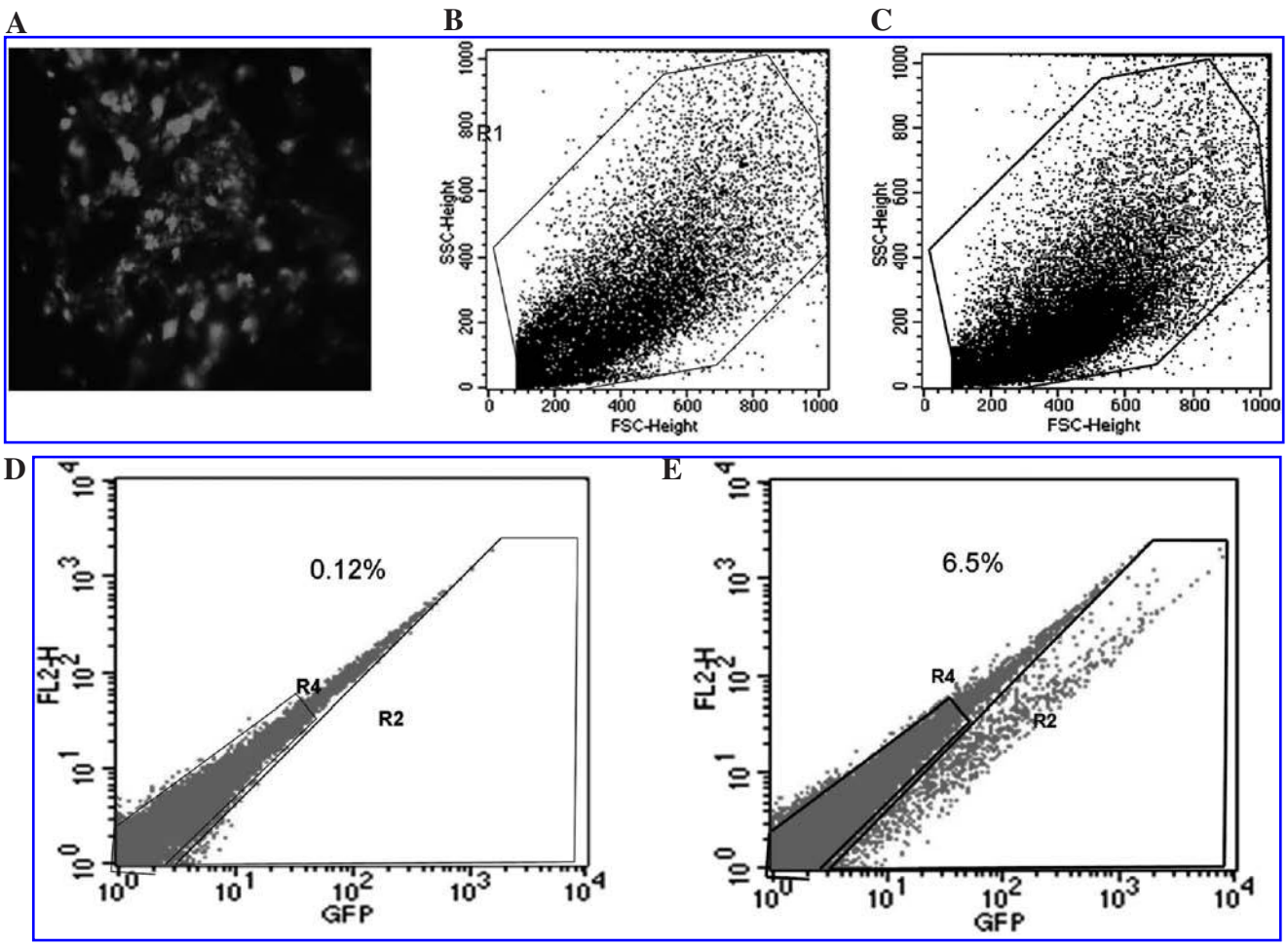

FIG. 7. GFP expression evaluated by fluorescent microscopy and fluorescence-activated cell sorting (FACS). Differentiated monkey ESC were transduced with the liver-specific lentivirus vector containing the α1-AT promoter driving the GFP gene at 2 weeks after EBs were plated. The GFP-positive cells were shown 1 week after the transduction under fluorescence microscopy (**A**) (Original magnification: ×100). FACS analysis was performed using a MoFlo cell sorter. For detection of GFP+ cells, dead cells and debris were excluded by forward scatter and SSC plots in the histogram analysis plots from untransduced cells (**B**) and transduced cells (**C**). Differentiated monkey ESC not transduced with the lentivirus vector were used as control cells (**D**). The mean fluorescent intensity was determined using cells that had signal intensities higher than that of the control cells (**E**). Abbreviations: EBs, embryoid bodies; GFP, green fluorescence protein

increased in the presence of dexamethasone in hepatocytes culture (Klein et al., 2002). This implies that both insulin and dexamethasone are crucial factors in hepatocyte differentiation cultures, and they appear to have synergistic effects on many aspects of hepatocyte metabolism, differentiation and growth. Although the precise mechanism by which human insulin and dexamethasone affect hepatocyte differentiation is not defined in this study, the monkey ESC-derived hepatocyte-like cells exhibited characteristic hepatocyte morphology, expressed hepatocyte markers, including AFP, albumin, HNF-3β, G-6-P, α1-AT, and exhibited glycogen storage, albumin secretion, and cytochrome P450 activity.

Most researchers have used mouse or human ESC to investigate the differentiation of embryonic stem cell into hepatocyte-like cells (Agarwal et al., 2008; Chinzei et al., 2002; Duan, et al., 2007; Hay et al., 2008; Teratani et al., 2005). Only two teams of investigators have reported the differentiation of hepatocyte-like cells from monkey ESC (Saito et al., 2006; Tsukada et al., 2006). Tsukada et al. evaluated the hepatic differentiation of cynomolgus monkey ESC with the induction of acidic fibroblast growth factor (aFGF). However, their cells had no TAT expression and increasing α-fetoprotein expression at the latest period of culture, indicating early hepatic cells that were not differentiating further, or endoderm. Saito et al. demonstrated the differentiation of cynomolgus monkey ESCs into hepatocyte-like cells by use of a coculture system with mouse fetal liver-derived cells (MFLCs). However, their cells also had high level α-fetoprotein expression during later stages of culture. In neither previous study was the differentiation efficiency quantitatively assessed, nor were Western blot analyses undertaken, a technique requiring high levels of protein expression. In our study, Western blot analyses confirmed high levels of liver-specific gene expression, α-fetoprotein expression decreased, and marker gene expression quantitated differentiation expression. To do this latter analysis, we used a lentivirus vector with a liver-specific α1-AT promoter driving GFP expression. The hepatocyte-like cells that were GFP+ could then be FAC sorted from a heterogeneous population of total differentiated cells. The results suggest that the use of the liver-specific lentivirus vector appear to be effective in enhancing the purity of our hepatocyte-like, differentiated monkey ESC.

In conclusion, we tested the expression of hepatocyte-specific markers, and performed functional assays, as well as immunochemistry and FACS analysis to evaluate the differentiation of monkey ESC toward a hepatocyte phenotype. The data in our present study underline the view that our in vitro ESC differentiation system has the potential to generate mature hepatocyte-like cells from a monkey embryonic stem cell line. Future directions will include enhancing the purity of the differentiated cell population by further optimizing the culture conditions, and by selecting a population of GFP-positive cells that have been transduced with the liver-specific promoter, by reculturing following FACS or laser microdissection and pressure catapulting. With further purification they can then be employed in transplantation studies using monkey models of liver injury.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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