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Differentiation of Mouse Embryonic Stem Cells Into Hepatocytes Induced by a Combination of Cytokines and Sodium Butyrate

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ABSTRACT

There is increasing evidence to suggest that embryonic stem cells (ESCs) are capable of differentiating into hepatocytes in vitro. In this study, we used a combination of cytokines and sodium butyrate in a novel three-step procedure to efficiently direct the differentiation of mouse ESCs into hepatocytes. Mouse ESCs were first differentiated into definitive endoderm cells by 3 days of treatment with Activin A. The definitive endoderm cells were then differentiated into hepatocytes by the addition of acidic fibroblast growth factor (aFGF) and sodium butyrate to the culture medium for 5 days. After 10 days of further in vitro maturation, the morphological and phenotypic markers of hepatocytes were characterized using immunohistochemistry, immunoblotting, and reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, the cells were tested for functions associated with mature hepatocytes, including glycogen storage and indocyanine green uptake and release, and the ratio of hepatic differentiation was determined by counting the percentage of albumin-positive cells. In the presence of medium containing cytokines and sodium butyrate, numerous epithelial cells resembling hepatocytes were observed, and ~74% of the cells expressed the hepatic marker, albumin, after 18 days in culture. RT-PCR analysis and immunohistochemistry showed that these cells expressed adult liver cell markers, and had the abilities of glycogen storage and indocyanine green uptake and release. We have developed an efficient method for directing the differentiation of mouse ESCs into cells that exhibit the characteristics of mature hepatocytes. This technique will be useful for research into the molecular mechanisms underlying liver development, and could provide a source of hepatocytes for transplantation therapy and drug screening. J. Cell. Biochem. 109: 606–614, 2010.

KEY WORDS: EMBRYONIC STEM CELLS; DIFFERENTIATION; HEPATOCYTES; SODIUM BUTYRATE; CYTOKINES

Embryonic stem cells (ESCs) are continuously growing cell lines initially isolated from the inner cell mass of blastocysts (Martin, 1981; Thomson et al., 1998). They are endowed with self-renewal and multi-lineage differentiating capabilities, and are more "plastic" than their adult counterparts. Earlier studies have shown that mouse ESCs possess differentiating potential in vitro (Keller, 1995), including the abilities to differentiate into cells of the hematopoietic lineage, cardiomyocytes of mesodermal origin and neuronal cells of ectodermal origin, as well as into endoderm (Abe et al., 1996). Hepatic differentiation from murine and human ESCs both in vitro and in vivo has been reported in recent studies (Hamazaki et al., 2001; Chinzei et al., 2002; Choi et al., 2002; Ishizaka et al., 2002; Jones et al., 2002; Miyashita et al., 2002; Yamada et al., 2002; Yin et al., 2002; Kanda et al., 2003). These hepatic cells derived from ESCs expressed some markers typical of mature hepatocytes and demonstrated functions able to rescue experimental liver injury when transplanted in vivo, thus raising hopes of generating a transplantable cell source for the treatment of end-stage liver diseases. At the same time, the large-scale differentiation of ESCs into hepatocytes could also provide a valuable model system for novel pharmaceutical drug discovery assays, as well as for new drug metabolism and cytotoxicity screening, particularly in light of the liver's major role in detoxification (Kulkarni and Khanna, 2006).

The liver, like the pancreas, develops from the primitive gut tube, which is formed by a flat sheet of cells called the definitive endoderm (Cascio and Zaret, 1991; Martinez Barbera et al., 2000). During early development, the inner cell mass of the pre-implantation blastocyst differentiates into the epiblast (which gives rise to the three germ layers: ectoderm, mesoderm, and definitive endoderm) and the primitive endoderm (which forms the extra-embryonic tissues, visceral endoderm and parietal endoderm that do

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not contribute directly to the developing embryo proper, Rossant, 2004). The definitive endoderm is formed during gastrulation when pluripotent epiblast cells migrate through the primitive streak of the embryo to form either mesoderm or definitive endoderm, which then displaces the pre-existing primitive (visceral) endoderm (Zaret, 2002). Thus, the specification and subsequent maturation of hepatocytes from the definitive endoderm is governed by a series of interactions with other juxtaposed cell types in the developing embryo. Potentially, elucidation of these developmental mechanisms could help to develop culture conditions that would allow the derivation of hepatocytes from mouse ESCs in vitro in a manner that mimics differentiation in vivo. Although these cutting-edge findings have unveiled the reality of differentiation of ESCs into hepatocytes, the optimal inducing conditions and the mechanisms responsible for the differentiation of hepatic cells remain unclear.

Several recent studies have reported the differentiation of hepatocytes from ESCs (Matsumoto et al., 2008; Mizumoto et al., 2008; Moore et al., 2008; Shiraki et al., 2008). Many reports have highlighted the differentiation of hepatocytes from ESCs both in vitro and in vivo, though most protocols have involved the formation of embryoid bodies, and the acquired differentiated cells represented heterogeneous populations. The residual ESCs had the propensity to develop teratomas and would thus not be suitable for cell transplantation. Low efficiency has also been a problem associated with hepatic differentiation of stem cells. Hepatic progenitor cells are bipotential and can differentiate into both functional hepatocytes and bile duct cells with high efficiency under suitable conditions (Snykers et al., 2008). In addition to providing a potential cell source for transplantation, the availability of hepatic progenitor cells at different stages of differentiation is also an important factor.

In the present study, we established a novel procedure to direct the differentiation of ESCs into hepatic cells using a combination of cytokines and sodium butyrate. Using this novel procedure, ESCs were efficiently induced to differentiate into morphologically and biologically functional hepatocytes. These results suggest that this method can provide a useful model system for studying key events during early liver development and a potential source of transplantable cells for cell replacement therapies.

MATERIALS AND METHODS

GROWTH FACTORS, CHEMICALS, AND ANTIBODIES

Activin A, acidic fibroblast growth factor (aFGF), and hepatocyte growth factor (HGF) were purchased from Peprotech (Rocky Hill, NJ). Oncostatin M (OSM) was purchased from R&D Systems (Minneapolis, MN). Dexamethasone (Dex) and sodium butyrate were obtained from Sigma–Aldrich (St. Louis, MO). Antibody against mouse cytokeratin-18 (CK-18) was purchased from Invitrogen (Grand Island, NY). Antibodies against mouse α-fetoprotein (AFP) and albumin (ALB) were purchased from DAKO (Glostrup, Denmark). Antibodies against mouse nuclei were obtained from Chemicon (Temecula, CA). Fluorescein isothiocyanate (FITC)- and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

MOUSE ESC CULTURE

Undifferentiated murine E14 ESCs and ES-D3 cells were maintained on 0.1% gelatin-coated dishes in Glascow minimum essential medium (GMEM; Sigma), supplemented with 10% fetal bovine serum (FBS, Hyclone, Rockville, MD), 2 mM L-glutamine (Gibco-BRL, Grand Island, NY), 0.1 mM 2-mercaptoethanol (Sigma), 1% non-essential amino acids (Invitrogen), 1% sodium pyruvate (Invitrogen), and 10 ng/ml mouse leukemia inhibitory factor (Chemicon) to prevent spontaneous differentiation. Murine ES-D3 cells were cultured on mitomycin C-treated mouse embryonic fibroblast feeder cells.

HEPATIC DIFFERENTIATION

For hepatic differentiation, mouse ESCs were cultured on gelatin-coated dishes in medium comprising GMEM supplemented with 2% FBS and 100 ng/ml Activin A for 3 days. To establish an effective directional differentiation method, a series of combinations of cytokines and sodium butyrate were devised, as shown in Table I. After 3 days of Activin A treatment, the differentiated cells were cultured in medium comprising GMEM supplemented with 10% FBS, 30 ng/ml aFGF and 2.5 mM sodium butyrate for 5 days. The differentiated cells then underwent further maturation in GMEM containing 20 ng/ml HGF for 5 days, followed by 5 days in 10 ng/ml OSM plus 0.1 μM Dex. The medium was changed every day. The same medium without cytokines and sodium butyrate was used as a negative control.

IMMUNOHISTOCHEMISTRY

On days 0, 1, 2, 3, 6, 12, and 18 of cell differentiation, cells were fixed with methanol (Merck) for 10 min and subsequently incubated with primary antibodies against mouse ALB for 45 min and with second antibody for 45 min. The antigens were visualized using the APAAP protocol (DAKO). For 3,3′-diaminobenzidene (DAB)

TABLE I. Compositions of Hepatocyte-Inducing Media

Inducing medium	Groups	Cytokines and sodium butyrate
Early inducing medium for endoderm induction	Control I	None
	Control II	None
	Experiment I, Experiment II	100 ng/ml Activin A (3 days)
Mid-term inducing medium for hepatic induction	Control I	None
	Control II	2.5 mmol/L sodium butyrate (5 days)
	Experiment I	30 ng/ml aFGF (5 days)
	Experiment II	30 ng/ml aFGF + 2.5 mmol/L sodium butyrate (5 days)
Terminal inducing medium for maturation	Control I	None
	Control II, Experiment I, Experiment II	20 ng/ml HGF (5 days) 10 ng/ml OSM + 0.1 μM Dex (5 days)

staining, a biotinylated second goat anti-mouse antibody (Sigma) was used, followed by a streptavidin complex (DAKO). After washing, cells were incubated with DAB reagent (DAKO). The reaction was stopped with tap water. Stained material was mounted with Kaiser's glycerin gelatin (Merck).

For immunofluorescence, cells were fixed at days 0, 6, 12, and 18 of differentiation in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 20 min, and blocked with PBS containing 0.1% Triton X-100 and 5% bovine serum albumin at room temperature for 1 h. Cells were incubated with primary antibodies at 4°C overnight. Isotype mouse IgG or normal rabbit serum were used as negative controls. No fluorescence was observed in the negative controls. The primary antibodies against mouse AFP, ALB, CK18, and Oct-4 were diluted at 1:200, antibody against mouse nuclei was diluted at 1:1,000. After five washes with PBS, FITC-conjugated or TRITC-conjugated secondary antibody diluted at 1:200 was added and incubated at 37°C for 1 h. Then 1 g/ml DAPI (Roche, Germany) was used to stain the cell nuclei. Cells and sections were washed between each step with PBS containing 0.1% bovine serum albumin.

HEPATIC-SPECIFIC GENE EXPRESSION ANALYSIS BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Cells were collected on days 0, 3, 6, 9, 12, 15, and 18 of differentiation for analysis of mRNA expression of the following liver-specific genes: AFP, ALB, transthyretin (TTR), α1-antitrypsin (AAT), glucose-6-phosphate (G6P), and tyrosine aminotransferase (TAT). Adult liver and fetal liver were used as positive controls. β-Actin transcripts were used as an internal reference for amplification of cDNA. Total RNA was isolated from 1×10^6 differentiated cells using TRIzol reagent (Invitrogen) and reverse-transcribed using a reverse transcription system (Promega, Madison, WI). PCR amplification of different genes was performed using EXTaq polymerase (Takara, Japan), with a program of 94°C for 5 min, 35 cycles of 94°C for 30 s, 50–57°C for 30 s, 72°C for 30 s, and extension at 72°C for 10 min. The primers used are shown in Table II. The PCR reaction was performed with the following thermal profiles: denaturation at 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C. The program was then completed with a 10 min extension at 72°C. The amplified PCR products were analyzed by electrophoresis on 1.2% agarose gel and were stained with ethidium bromide for visualization. All the procedures were performed according to the manufacturers' instructions.

IMMUNOBLOTTING

On days 0, 6, 12, and 18 of cell differentiation, trypsin-EDTA-dislodged cells were lysed in suspension with Protease Inhibitor Cocktail (Sigma–Aldrich)-supplemented RIPA buffer (Boston Bioproducts, Worcester, MA). Protein concentrations were determined (BCA kit; Pierce, Rockford, IL), and equal amounts of total protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA), transferred to nitrocellulose, probed with primary antibodies (anti-ALB and anti-AFP from DAKO, anti-Oct-4 from Santa Cruz Biotechnology, Inc., anti-CYP46A1 from ProteinTech Group, Inc.), and detected by chemiluminescence (Pierce).

REDUCED INDOCYANINE GREEN (ICG) UPTAKE ASSAY

After washing the cultured cells with PBS, ICG solution was added to the plates at a final concentration of 1 mg/ml and incubated at 37°C for 30 min, then rinsed three times with PBS. The dishes were then refilled with DMEM containing 10% FBS. ICG was completely eliminated from the cells 6 h later. The uptake and secretion of ICG is a specific function of hepatocytes.

PERIODIC ACID SOLUTION (PAS) REACTION

Cells were fixed with 10% Carnoy's fixative solution for 15 min at 37°C, and then washed three times with PBS. Fixed cells were exposed to 0.8% periodic acid solution per well for 10–15 min. After rinsing with PBS, 1 ml of Schiff's reagent was added per well and cells were exposed for 20–30 min. Schiff's reagent is a mixture of pararosaniline and sodium metabisulfite that reacts to release a pararosaniline product, which stains glycol-containing cellular elements. Cells were washed for a third time to remove the reagent and then examined by image acquisition with an Olympus IX70 microscope and Olympus digital camera.

DETERMINATION OF THE HEPATIC DIFFERENTIATION RATIO

In order to evaluate the proportion of ALB-positive cells in day-18 cultures, we randomly picked 30 images from three independent cultures. ALB-positive cells and total cells counterstained with DAPI were counted using Image-Pro Plus software (Media Cybernetics). The values from each well were averaged to obtain a mean ± SD. Cells cultured with sodium butyrate and spontaneously differentiated cells were counted using the same method and used as controls.

TABLE II. Primers Used for Reverse-Transcription Polymerase Chain Reaction

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	Product (bp)
AFP	TCGTATTCCAACAGGAGG	AGGCTTTTGCTTCACCAG	173
AAT	AATGGAAGAAGCCATTCGAT	AAGACTGTAGCTGCTGCAGC	484
ALB	AGAAGACACCCTGATTACTCT	TCGAGAAGCAGGTGTCCTTGT	705
G6P	CAGGACTGGTTCATCCTT	GTTGCTGTAGTAGTCGGT	209
TTR	GGCTGAGTCTCTCAATTC	CTCACCACAGATGAGAAG	223
TAT	ACCTTCAATCCCATCCGA	TCCCGACTGGATAGGTAG	205
β-Actin	TTCCTTCTTGGGTATGGAAT	GAGCAATGATCTTGAACTTC	202

STATISTICAL ANALYSIS

The results are given as means ± SD. One-way analysis of variance (ANOVA) or Student's *t*-tests were used where applicable. Differences with *P*-values <0.05 were considered statistically significant.

RESULTS

ESC DIFFERENTIATION INTO HEPATOCYTE-LIKE CELLS

We designed a novel three-step strategy to induce hepatocytes from mouse ESCs using a combination of cytokines and sodium butyrate. In the first stage, mouse ESCs were induced to differentiate into definitive endoderm cells by 3 days of Activin A treatment. In the second stage, the definitive endoderm cells were differentiated into early hepatic cells using a combination of aFGF and sodium butyrate treatment for 5 days. In the third stage, the early hepatic cells were further matured by treatment with HGF for 5 days, followed by OSM plus Dex treatment for a further 5 days. Most experiments were carried out using the E14 cell line, but similar results were obtained for differentiation of the ES-D3 cell line.

After treatment with 100 ng/ml Activin A for 3 days, we tested the effects of aFGF and sodium butyrate on the induction of hepatic differentiation from definitive endoderm cells. The morphological changes in the ESCs during the process of cell differentiation were observed using light microscopy. In the absence of sodium butyrate, control ESCs spontaneously differentiated into multiple lineages. After incubation in culture medium containing cytokines and sodium butyrate, however, cell differentiation occurred. The cells assumed homogenous morphological characteristics. Most cells became epithelioid or fibroblast-like. In the presence of conditional selective medium, however, extensive cell proliferation occurred and numerous epithelial cells resembling hepatocytes were observed. The cells were polygonal in shape with large, round, central nuclei; bi-nuclei were also present (Fig. 1).

IDENTIFICATION AND CHARACTERIZATION OF ES-DERIVED HEPATIC CELLS

We examined the expression of hepatic markers in these hepatocyte-like cells at the RNA and protein levels to determine whether the ES-derived hepatic cells expressed markers typical of hepatic cells and had distinct phenotypes from ESCs. The results of RT-PCR analysis showed that undifferentiated ESCs failed to express any hepatic markers; however, hepatic differentiation markers were detected in the presence of cytokines and sodium butyrate. AFP, a marker of endodermal differentiation and an early fetal hepatic marker and ALB, a hepatocyte-specific gene, were expressed within 9 and 12 days of differentiation, respectively. Other endodermal and hepatic markers, AAT and TTR, were expressed within 6 and 9 days, respectively. The late fetal hepatic markers, TAT and G6P were not expressed until days 12 and 15, respectively (Fig. 2). The results of immunoblotting showed that the expression of hepatocyte markers such as ALB, AFP, and cholesterol 24-hydroxylase (CYP46A1) were significantly upregulated in hepatic lymphomyeloid cells compared with ESCs. Meanwhile, the expression of the undifferentiated ES cell

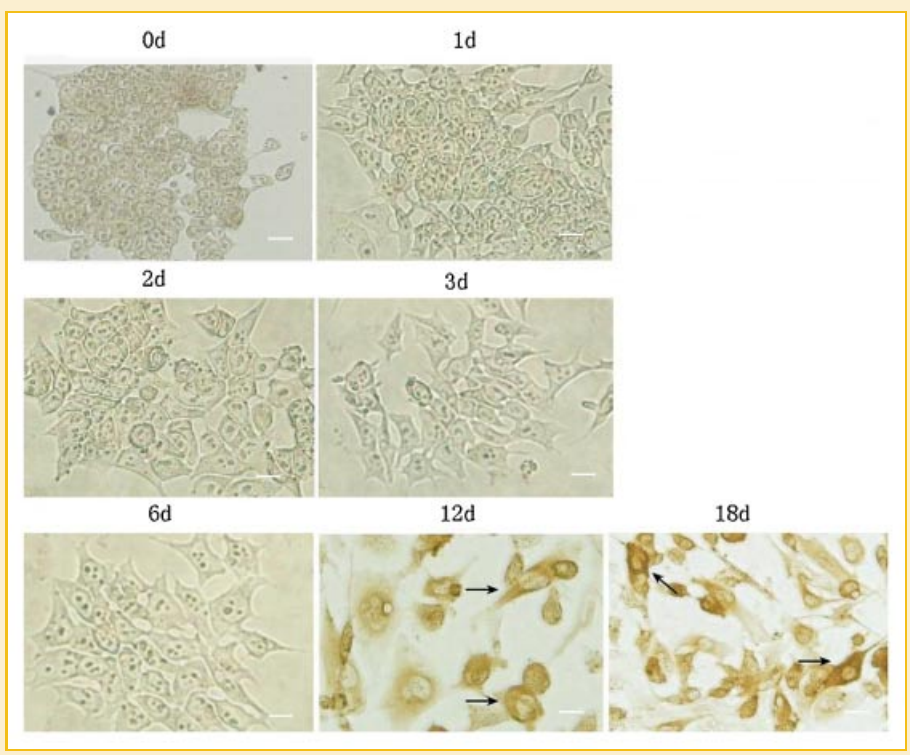

Fig. 1. Immunohistochemical images of liver cell-specific markers (ALB) at different stages of differentiation (days 0, 1, 2, 3, 6, 12, and 18). Arrows indicate ALB-positive cells. Bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

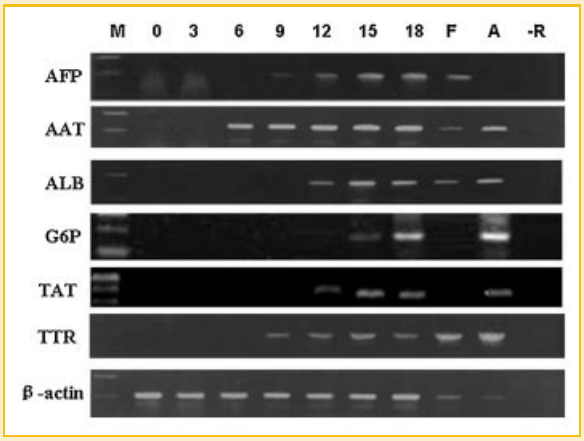

Fig. 2. Time course of hepatic gene transcription in the differentiating cells. Reverse transcription–polymerase chain reaction expression analysis of total RNA prepared from cells at various time points throughout differentiation, using primers specific for various hepatic genes and for the ubiquitous housekeeping gene β-actin. RNA from fetal and adult liver was included as a positive control and RNA from the induced differentiated cells without reverse transcription was used as a negative control.

marker, Oct-4 (Hay et al., 2004; Niwa et al., 2000) was dramatically reduced (Fig. 3).

Moreover, Immunohistochemical analysis showed that the differentiating cells exhibited a time-dependent, ordered pattern of stem cell- and liver cell-specific marker expression (Oct-4 and ALB) (Fig. 1). The expression profile of hepatic lineage genes throughout the time course of differentiation (days 0, 6, 12, and 18) was also analyzed by immunofluorescence (Fig. 4). AFP, ALB, and CK18 protein expression in the cells treated with sodium butyrate and conditional selective medium was first detected in a fraction of the cell population at day 12. The intensity and extent of their expression increased to include the majority of the cells by day 18, but they were not detected in the ESCs. The experiments were repeated three times.

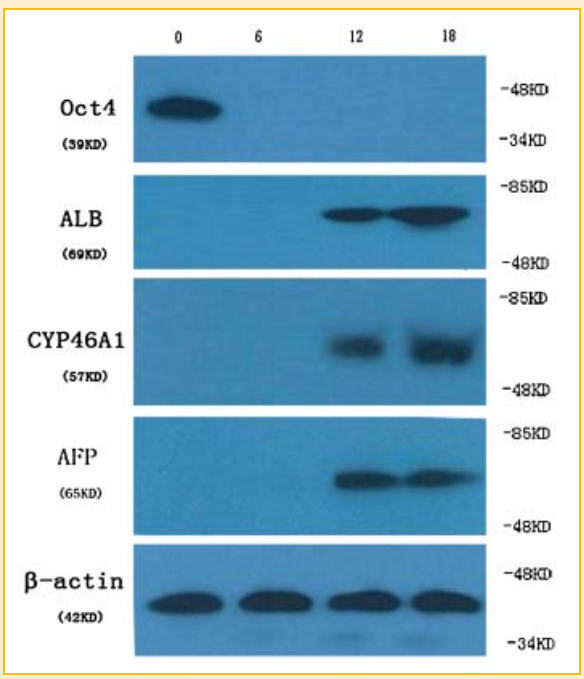

Fig. 3. Time course of immunoblotting analysis of protein extracts from the differentiating cells (days 0, 6, 12, and 18) for several marker proteins, as indicated on the left. The positions of molecular weight standards (in kDa) are indicated on the right. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CELL FUNCTION TESTS

Glycogen storage is an important metabolic function of hepatocytes. In this study, the glycogen storage capability of the differentiated cells was analyzed by the PAS reaction. As shown in Figure 5, glycogen storage was manifested as the accumulation of magenta staining in the cytoplasm of hepatocyte-like differentiated cells. Very few PAS-positive cells were detected amongst the spontaneously differentiated cells (Fig. 5). We also examined ICG take-up, which is a liver-specific function used to identify differentiated hepatocytes in vitro. The induced hepatocyte-like cells took up ICG from the medium and released it 6 h later (Fig. 6). ICG-positive cells were rarely observed among spontaneously differentiated mouse ESCs.

DETERMINATION OF HEPATIC DIFFERENTIATION RATIO

The ratio of hepatic differentiation has rarely been reported in previous studies involving ESCs. In the current study, the hepatic differentiation ratio was determined by evaluating the percentages of ALB-positive cells in the differentiated cells. Approximately 74% of the cells expressed the hepatic marker ALB by day 18 of culture in the medium containing cytokines and sodium butyrate. In contrast, in the same culture system without sodium butyrate, ALB was expressed in 66% of cells, and in 44% of cells cultured only with sodium butyrate. On average, 11% of spontaneously differentiated cells expressed ALB (Fig. 7).

DISCUSSION

Although ESCs can differentiate into hepatocyte-like cells either spontaneously or after induction by exogenous factors in vitro (Jones et al., 2002; Kuai et al., 2003; Yamamoto et al., 2003; Ishii et al., 2005), the resulting cells still contain multiple heterogeneous lineages which are not suitable for therapeutic transplantation (Kumashiro et al., 2005; Teramoto et al., 2005). To avoid the risk of teratoma formation after cell transplantation, recent reports have highlighted the importance of acquiring functional ES-derived hepatocytes with a uniform phenotype (Hu et al., 2004; Soto-Gutierrez et al., 2006). However, previous studies on the hepatic differentiation of human or mouse ESCs did not exclude the possibility of extraembryonic differentiation of ESCs, which could also lead to the expression of many liver cell markers.

In this study, we developed a novel and highly efficient method for generating functional hepatocytes from ESCs. A practical three-step strategy was designed based on the use of Activin A, aFGF, sodium butyrate, HGF, OSM, and Dex for the induction of mouse

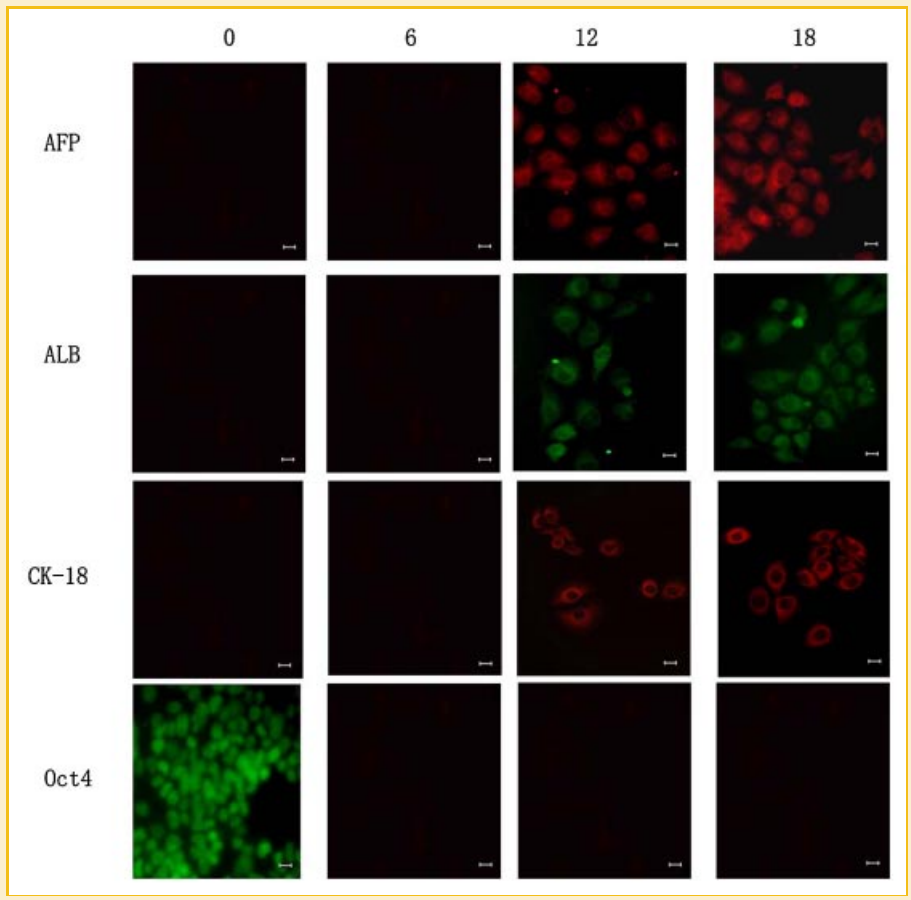

Fig. 4. Progression of the differentiating cells through a cascade of developmentally regulated hepatic gene induction in vitro. Immunofluorescence analysis of the expression of a panel of indicated stage-specific hepatic genes (AFP, ALB, and Oct-4) and ESC marker (Oct-4) in differentiating cells (days 0, 6, 12, and 18). Bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ESCs into hepatocytes. The results demonstrated that mouse ESCs could differentiate into progenitor cells and mature into hepatocytes with typical morphological, phenotypic, and functional characteristics. AFP and TTR mRNAs were expressed in the cultured cells throughout the time course of differentiation. TTR represents endodermal differentiation and is expressed throughout liver maturation (Thomas et al., 1990). AFP is a marker of endodermal differentiation, as well as an early fetal hepatic marker, and its

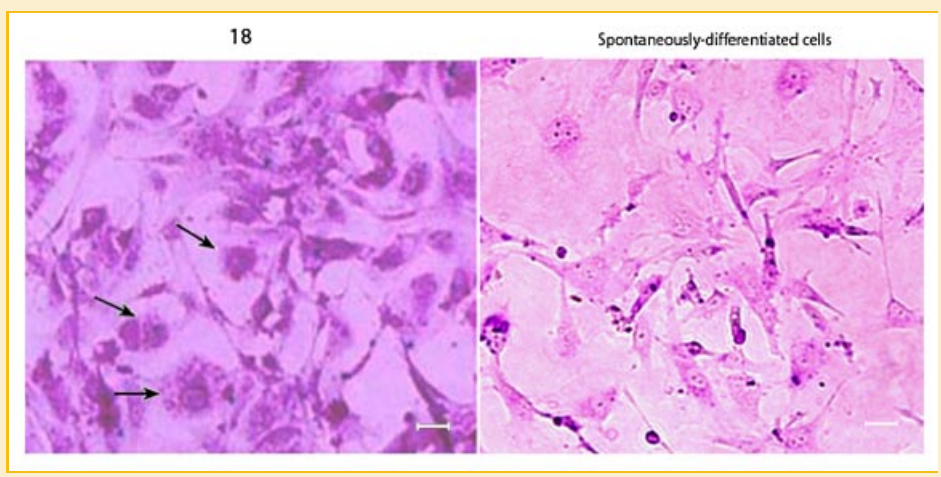

Fig. 5. Glycogen storage. Periodic acid-Schiff assay was performed on differentiating cells at day 18. Arrows show glycogen storage as indicated by pink or dark red-purple cytoplasm. Bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

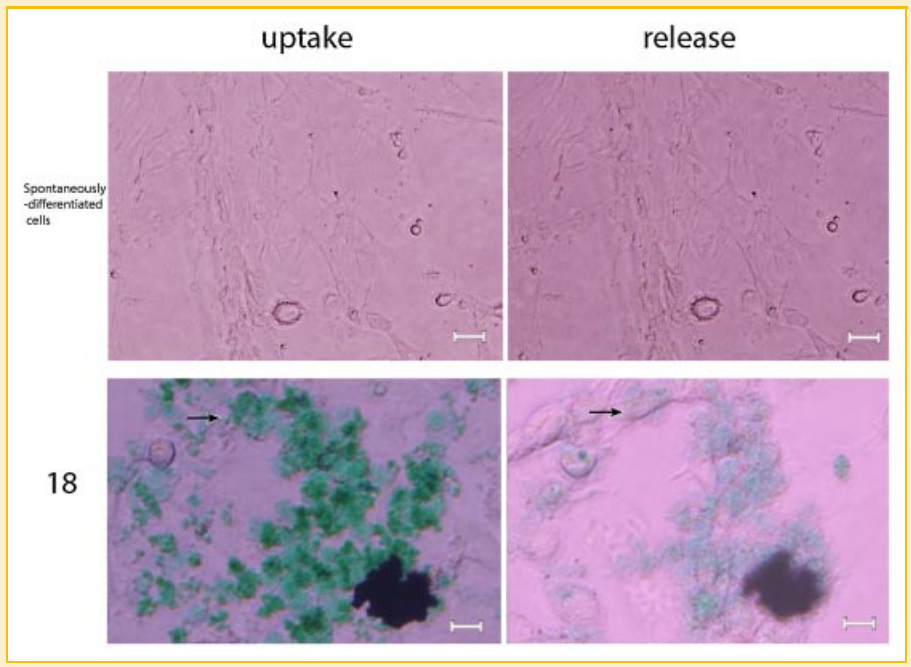

Fig. 6. Indocyanine green (ICG) uptake and release. Spontaneously differentiated cells and cells at the end of the differentiation process (day 18) were examined for their ability to take up ICG (left column) and release it 6 h later (right column). Arrows indicate internalized ICG that was allowed to internalize over 15 min and was then excluded 6 h later. Bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

expression decreases as the liver develops into the adult phenotype. Expression of ALB, the most abundant protein synthesized by mature hepatocytes, starts in early fetal hepatocytes and reaches a maximum in adult hepatocytes. ALB mRNA was detected after 12 days in the selective differentiation medium. TAT and G6P are produced in the developing liver during the late fetal and neonatal stages (Kamiya et al., 1999) and their mRNAs were detected by day 15 in the selective culture medium.

Most importantly, we demonstrated that sequential treatment of mouse ESCs with Activin A and aFGF/sodium butyrate was crucial for highly efficient hepatic differentiation. In the first stage, mouse ESCs were induced to form definitive endoderm cells by 3 days of Activin A treatment. In the second stage, these definitive endoderm cells differentiated into early hepatic cells after induction with a combination of aFGF and sodium butyrate for 5 days. In the third stage, the early hepatic cells were further matured by treatment with HGF for 5 days, followed by OSM plus Dex treatment for a further 5 days.

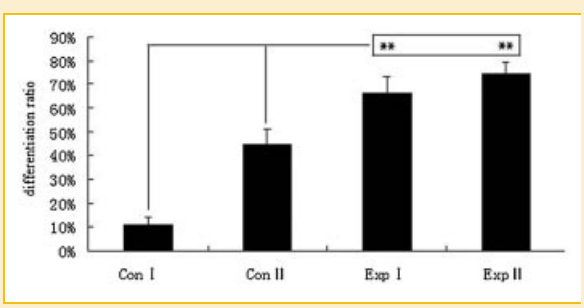

Fig. 7. Hepatic differentiation ratio of differentiated ESCs was determined by evaluating the percentages of ALB-positive cells. Data are expressed as mean ± SD (n = 24). $^{**}P < 0.01$. Control I: spontaneously differentiated cells; Control II: cells cultured with sodium butyrate; Experiment I: cells cultured with cytokines; Experiment II: cells cultured with sodium butyrate and cytokines.

Activin A is a peptide that enhances follicle-stimulating hormone (FSH) synthesis and secretion and participates in the regulation of the menstrual cycle, which is a potent growth and differentiation factor whose synthesis and bioactivity are tightly regulated (Gold et al., 2009). Activin A has recently been reported to be able to induce definitive endodermal differentiation of ESCs and is non-permissive for visceral endoderm. The highly efficient differentiation of hESCs into functional hepatic endoderm has specifically been shown to require Activin A (Hay et al., 2008). In the current study, we combined Activin A treatment with hepatic linage differentiation conditions and induced mouse ESCs to produce definitive endoderm cells. After Activin A treatment, the combination of aFGF and sodium butyrate efficiently initiated hepatic differentiation from the definitive endoderm.

Previous studies have confirmed that aFGF is involved in mammalian liver development and recovery from liver injury, and participates in the hepatic differentiation of ESCs (Tsukada et al., 2006). During the embryonic development of mice, aFGF derived from adjacent cardiac mesoderm commits the foregut endoderm to forming the liver primordium (Jung et al., 1999). The liver bud then proliferates and migrates into the surrounding septum transversum, which consists of loose connective tissue containing collagen. The concentration of aFGF used in the selective medium in the current experiments was based on the results of these preliminary studies.

Histone deacetylase (HDAC) inhibitors, such as suberoylanilide hydroxamic acid, valproic acid, trichostatin A, and sodium butyrate, have recently emerged as a new group of therapeutic agents that are effective in some human cancers, including some hematologic malignancies (Richon and O'Brien, 2002). HDACs catalyze the removal of acetyl groups from histones, thereby mediating condensation of the nucleosomes. HDAC inhibitors promote histone acetylation, relax the DNA wrapped around the core histones, and allow the transcription of genes involved in important cellular processes. HDAC inhibitors have been shown to induce growth arrest, terminal differentiation, and cell death in a wide variety of transformed cells in vitro and in vivo (Blanchard et al., 2002; Hsi et al., 2004; Cho et al., 2005; de Ruijter et al., 2005). The results of the present study were consistent with these previous results, and treatment with sodium butyrate allowed ESCs to differentiate into hepatic progenitor cells. Our data confirm that hepatocyte-like cells from spontaneously differentiated ESCs may arrest in an immature stage, while sodium butyrate not only promotes hepatic differentiation of ESCs, but also induces the maturation of ES-derived hepatocyte-like cells. These results provide evidence that sodium butyrate improves hepatocyte differentiation. Our differentiation protocol also showed that the combination of aFGF and sodium butyrate treatment primed mouse ESCs for further differentiation into hepatocytes more effectively than aFGF or sodium butyrate alone. This study demonstrated the interactions between aFGF and sodium butyrate, and showed that sodium butyrate had a synergistic effect with aFGF signaling to promote hepatic differentiation. However, the potential signaling pathway involved in the process remains uncertain and deserves further investigation.

It is currently widely accepted that HGF, OSM, and Dex are essential for the maturation of hepatocytes (Ong et al., 2006). HGF has been shown to support fetal hepatocytes during mid-stage hepatogenesis (Uehara et al., 1995). During and after the mid-stage of hepatogenesis, hematopoietic cells produce OSM that induces maturation of murine fetal hepatocytes (amiya et al., 1999). Furthermore, Shirahashi et al. (2004) showed that the addition of insulin and DEX to EBs cultured on collagen type I induced the expression of various endodermal genes. The protocol in the present study therefore included HGF, OSM, and DEX in the medium used to differentiate hepatocytes from mouse ESCs. Under the selective culture conditions, ~74% of the mouse ESCs could be manipulated to differentiate into ALB-positive cells and the hepatic differentiation ratio in differentiated cells was much higher than that in spontaneously differentiated ESCs.

In summary, we have developed a novel and efficient method for inducing mouse ESCs to differentiate into hepatocytes in vitro, using selective culture medium. However, the differentiated cells have not yet been tested by implanting into animals. This study only used two sources of ESCs and other sources and stages need to be tested to determine the general applicability of the method. This method provides not only a novel cell source for transplantation, but also an appropriate model for the study of hepatic linage differentiation, especially for the investigation of early hepatic determination from foregut endoderm.

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REFERENCES

Abe K, Niwa H, Iwase K, et al. 1996. Endoderm-specific gene expression in embryonic stem cells differentiated to embryoid bodies. Exp Cell Res 229(1):27–34.

Blanchard F, Kinzie E, et al. 2002. FR901228, an inhibitor of histone deacetylases, increases the cellular responsiveness to IL-6 type cytokines by enhancing the expression of receptor proteins. Oncogene 21(41):6264–6277.

Cascio S, Zaret KS. 1991. Hepatocyte differentiation initiates during endodermal-mesenchymal interactions prior to liver formation. Development 113(1):217–225.

Chinzei R, Tanaka Y, et al. 2002. Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. Hepatology 36(1):22–29.

Cho HH, Park HT, et al. 2005. Induction of osteogenic differentiation of human mesenchymal stem cells by histone deacetylase inhibitors. J Cell Biochem 96(3):533–542.

Choi D, Oh HJ, et al. 2002. In vivo differentiation of mouse embryonic stem cells into hepatocytes. Cell Transplant 11(4):359–368.

de Ruijter AJ, Meinsma RJ, et al. 2005. Gene expression profiling in response to the histone deacetylase inhibitor BL1521 in neuroblastoma. Exp Cell Res 309(2):451–467.

Gold E, Jetly N, et al. 2009. Activin C antagonizes activin A in vitro and overexpression leads to pathologies in vivo. Am J Pathol 174(1):184–195.

Hamazaki T, Iiboshi Y, et al. 2001. Hepatic maturation in differentiating embryonic stem cells in vitro. FEBS Lett 497(1):15–19.

Hay DC, Fletcher J, et al. 2008. Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling. Proc Natl Acad Sci USA 105(34):12301–12306.

Hay DC, Sutherland L, Clark J, et al. 2004. Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. Stem Cells 22(2):225–235.

Hsi LC, Xi X, et al. 2004. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces apoptosis via induction of 15-lipoxygenase-1 in colorectal cancer cells. Cancer Res 64(23):8778–8781.

Hu AB, Cai JY, et al. 2004. High-ratio differentiation of embryonic stem cells into hepatocytes in vitro. Liver Int 24(3):237–245.

Ishii T, Yasuchika K, et al. 2005. In vitro differentiation and maturation of mouse embryonic stem cells into hepatocytes. Exp Cell Res 309(1):68–77.

Ishizaka S, Shiroi A, et al. 2002. Development of hepatocytes from ES cells after transfection with the HNF-3beta gene. FASEB J 16(11):1444–1446.

Jones EA, Tosh D, et al. 2002. Hepatic differentiation of murine embryonic stem cells. Exp Cell Res 272(1):15–22.

Jung J, Zheng M, et al. 1999. Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science 284(5422):1998–2003.

Kamiya A, Kinoshita T, et al. 1999. Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. EMBO J 18(8):2127–2136.

Kanda S, Shiroi A, et al. 2003. In vitro differentiation of hepatocyte-like cells from embryonic stem cells promoted by gene transfer of hepatocyte nuclear factor 3 beta. Hepatol Res 26(3):225–231.

Keller GM. 1995. In vitro differentiation of embryonic stem cells. Curr Opin Cell Biol 7(6):862–869.

Kuai XL, Cong XQ, et al. 2003. Generation of hepatocytes from cultured mouse embryonic stem cells. Liver Transpl 9(10):1094–1099.

Kulkarni JS, Khanna A. 2006. Functional hepatocyte-like cells derived from mouse embryonic stem cells: A novel in vitro hepatotoxicity model for drug screening. Toxicol In Vitro 20(6):1014–1022.

Kumashiro Y, Teramoto K, et al. 2005. Isolation of hepatocyte-like cells from mouse embryoid body cells. Transplant Proc 37(1):299–300.

Martin GR. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 78(12):7634–7638.

Martinez Barbera JP, Clements M, et al. 2000. The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. Development 127(11):2433–2445.

Matsumoto K, Mizumoto H, et al. 2008. Hepatic differentiation of mouse embryonic stem cells in a three-dimensional culture system using polyurethane foam. J Biosci Bioeng 105(4):350–354.

Miyashita H, Suzuki A, et al. 2002. Evidence for hepatocyte differentiation from embryonic stem cells in vitro. Cell Transplant 11(5):429–434.

Mizumoto H, Aoki K, et al. 2008. Hepatic differentiation of embryonic stem cells in HF/organoid culture. Transplant Proc 40(2):611–613.

Moore RN, Dasgupta A, et al. 2008. Enhanced differentiation of embryonic stem cells using co-cultivation with hepatocytes. Biotechnol Bioeng 101(6):1332–1343.

Niwa H, Miyazaki, J, Smith AG. 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet 24(4):372-376.

Ong SY, Dai H, et al. 2006. Hepatic differentiation potential of commercially available human mesenchymal stem cells. Tissue Eng 12(12):3477–3485.

Richon VM, O'Brien JP. 2002. Histone deacetylase inhibitors: A new class of potential therapeutic agents for cancer treatment. Clin Cancer Res 8(3):662–664.

Rossant J. 2004. Lineage development and polar asymmetries in the peri-implantation mouse blastocyst. Semin Cell Dev Biol 15(5):573–581.

Shirahashi H, Wu J, et al. 2004. Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage. Cell Transplant 13(3): 197–211.

Shiraki N, Umeda K, et al. 2008. Differentiation of mouse and human embryonic stem cells into hepatic lineages. Genes Cells 13(7):731–746.

Snykers S, De Kock J, Rogiers V, et al. 2009. In vitro differentiation of embryonic and adult stem cells into hepatocytes: State of the art. Stem Cells 27(3):577-605.

Soto-Gutierrez A, Kobayashi N, et al. 2006. Reversal of mouse hepatic failure using an implanted liver-assist device containing ES cell-derived hepatocytes. Nat Biotechnol 24(11):1412–1419.

Teramoto K, Hara Y, et al. 2005. Teratoma formation and hepatocyte differentiation in mouse liver transplanted with mouse embryonic stem cell-derived embryoid bodies. Transplant Proc 37(1):285–286.

Thomas T, Southwell BR, et al. 1990. Plasma protein synthesis and secretion in the visceral yolk sac of the fetal rat: Gene expression, protein synthesis and secretion. Placenta 11(5):413–430.

Thomson JA, Itskovitz-Eldor J, et al. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282(5391):1145–1147.

Tsukada H, Takada T, et al. 2006. Acidic fibroblast growth factor promotes hepatic differentiation of monkey embryonic stem cells. In Vitro Cell Dev Biol Anim 42(3–4):83–88.

Uehara Y, Minowa O, et al. 1995. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature 373(6516):702–705.

Yamada T, Yoshikawa M, et al. 2002. In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green. Stem Cells 20(2):146–154.

Yamamoto H, Quinn G, et al. 2003. Differentiation of embryonic stem cells into hepatocytes: Biological functions and therapeutic application. Hepatology 37(5):983–993.

Yin Y, Lim YK, et al. 2002. AFP(+), ESC-derived cells engraft and differentiate into hepatocytes in vivo. Stem Cells 20(4):338–346.

Zaret KS. 2002. Regulatory phases of early liver development: Paradigms of organogenesis. Nat Rev Genet 3(7):499–512.