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In Vitro Differentiation of Hepatic Progenitor Cells From Mouse Embryonic Stem Cells Induced by Sodium Butyrate

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Abstract Recently it was shown that embryonic stem (ES) cells could differentiate into hepatocytes both in vitro and in vivo, however, prospective hepatic progenitor cells have not yet been isolated and characterized from ES cells. Here we presented a novel 4-step procedure for the differentiation of mouse ES cells into hepatic progenitor cells and then hepatocytes. The differentiated hepatocytes were identified by morphological, biochemical, and functional analyses. The hepatic progenitor cells were isolated from the cultures after the withdrawal of sodium butyrate, which was characterized by scant cytoplasm, ovoid nuclei, the ability of rapid proliferation, expression of a series of hepatic progenitor cell markers, and the potential of differentiation into hepatocytes and bile duct-like cells under the proper conditions that favor hepatocyte and bile epithelial differentiation. The differentiation of hepatocytes from hepatic progenitor cells was characterized by a number of hepatic cell markers including albumin secretion, upregulated transcription of glucose-6-phophatase and tyrosine aminotransferase, and functional phenotypes such as glycogen storage. The results from our experiments demonstrated that ES cells could differentiate into a novel bipotential hepatic progenitor cell and mature into hepatocytes with typical morphological, phenotypic and functional characteristics, which provides an useful model for the studies of key events during early liver development and a potential source of transplantable cells for cell-replacement therapies. J. Cell. Biochem. 100: 29–42, 2007.

Key words: embryonic stem cells; hepatic progenitor cells; in vitro differentiation; sodium butyrate; hepatocyte; bile duct structure

Embryonic stem (ES) cells are continuously growing stem cell lines isolated from the inner cell mass of developing blastocysts [Martin, 1981; Thomson et al., 1998], which have unlimited self-renewal capacity and pluripotential to differentiate into cells of all three primary germ layers under appropriate conditions. Because of these characteristics, ES cells have been an excellent in vitro model system for the studies of key events during early embryo development and may also provide a prospective source of transplantable cells for cell-replacement therapies. Presently remarkable progress has been made in understanding the differentiation of ES cells into neural [Hancock et al., 2000], hematopoietic [Baron, 2001; Perlingeiro et al., 2001; Feng et al., 2005], endothelial [Yurugi-Kobayashi et al., 2003], vascular [Sone et al., 2003; Yurugi-Kobayashi et al., 2003; Suzuki et al., 2005], and pancreatic stem/progenitor cells [Blyszczuk et al., 2003] and then various mature cells. However, limited knowledge has been acquired for the differentiation of hepatic progenitor/stem cells, although hepatic differentiation from murine and human ES cells 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; Yamamoto et al., 2003; Kania et al.,

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2004; Ogawa et al., 2005; Teramoto et al., 2005; Teratani et al., 2005]. These hepatic cells from ES cells expressed some typical markers of mature hepatocytes and showed sufficient functions to rescue experimental liver injury when transplanted in vivo, which raised the hope to generate a transplantable cell source for the treatment of end-stage liver diseases.

Hepatic progenitor cells, which express a series of early liver lineage specific markers and are capable of differentiating into hepatocytes or bile duct epithelial cells under proper conditions, have been identified in embryonic, adult liver or bone marrow of rodents and human [Brill et al., 1999; Saito et al., 1999; Azuma et al., 2003; Lazaro et al., 2003; Strick-Marchand and Weiss, 2003; Zhang et al., 2003; Petkov et al., 2004]. But none of previous articles have reported that ES cells can be directly induced to differentiate into hepatic progenitor cells in vitro. Recent experiments have demonstrated that hepatocyte-like cells could be generated from human ES cells by 6 days' treatment with sodium butyrate. The treated cells exhibited morphological features of primary hepatocytes and expressed liver-associated genes and proteins. In addition, the majority of the treated cells remained in cell cycle for 3 days after the addition of sodium butyrate, but the cycling fraction decreased to 17% 7 days later [Rambhatla et al., 2003]. It was now established that treatment of cells both in vitro and in vivo with sodium butyrate, a potential histone deacetylase (HDAC) inhibitor, could result in specific functional outcomes such as proliferation, cell cycle arrest, apoptosis, or differentiation [Janson et al., 1997; Sambucetti et al., 1999; Wang et al., 1999; Travers et al., 2002; Camphausen et al., 2004; Hsieh et al., 2004; Bug et al., 2005; Cho et al., 2005; Jiang et al., 2005; Rossig et al., 2005]. Therefore it was assumed that the treatment with sodium butyrate would lead to the cell cycle arrest of the ES cells, and the removal of sodium butyrate might be important for the treated cells to reenter into cell cycle and consequently the cells with the capacity to proliferate and express hepatic linage markers could be acquired.

In present study, we reported the in vitro differentiation of hepatic progenitor cells from mouse embryonic stem cells induced by sodium butyrate. The morphological and functional characteristics of the differentiated cells were carefully studied. The hepatic progenitor cells were identified as they expressed α-fetoprotein (AFP), α-anti-trypsin (*AAT*), Cytokeratin (CK)18 and 19, γ-glutamyltranspeptidase (γ-*GGT*), hepatocyte nuclear factor 3β (*HNF3β*) and Dipeptidylpeptidase IV (*DPPIV*), but not albumin (ALB), and the potential to differentiate into bile duct-like structures and hepatocytes, which acquired functional hepatocyte phenotypes as demonstrated by ALB secretion, glycogen storage and upregulated transcription of glucose-6-phophatase (*G6p*) and tyrosine aminotransferase (*TAT*). The result provides a useful model system for the studies of key events during early liver development and a potential source of transplantable cells for cell-replacement therapies.

MATERIALS AND METHODS

Culture of Mouse Embryonic Stem Cells

Murine ES D3 cells were cultured on mitomycin C–treated mouse embryonic fibroblast (MEF) feeder cells on gelatin (0.1%)-coated petri dishes in the Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 15% 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), penicillin-streptomycin (Invitrogen), and 10 ng/ml mouse leukemia inhibitory factor (mLIF, Chemicon, Temecula, CA) to prevent spontaneous differentiation. To avoid contamination by the feeder cells, ES cells were cultured without feeder cells according to the method previously described [Anisimov et al., 2002].

In Vitro Differentiation of ES Cells Into Hepatic Progenitor Cells

For hepatic progenitor cell differentiation, ES cells were cultured on gelatin-coated dishes in the medium comprised DMEM supplemented with 15% FBS, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol (basal medium I), and 0.8% dimethyl sulfoxide (DMSO; Sigma) for 4 days, then medium was changed to basal medium I supplemented with 2.5 mM sodium butyrate (Sigma) for 6 days, finally the treated cells were cultured in the medium withdrew sodium butyrate and supplemented 10 ng/ml recombinant mouse hepatocyte growth factor (rmHGF) for another 6–12 days until the hepatic progenitor cells became confluence.

Identification and Characterization of the ES-Derived Hepatic Progenitor Cells

Detection of hepatic-associated proteins by immunostaining assay. For immunostaining assay, cells were washed with PBS for three times and fixed in 4% paraformaldehyde for 10 min at room temperature followed by 20 min in methanol at −20°C, and then blocked with 10% normal goat serum in PBS for 10 min to inhibit unspecific binding. The cells were washed with PBS and then incubated with various primary antibodies overnight at 4°C The primary antibodies were used as followed: mouse anti AFP (1:50; Dako, Glostrup, Denmark), nestin (1:100; Dako), rabbit anti CK18 (1:100, Dako), CK19 (1:100, Dako), and sheep anti ALB (1:100; Biodesign, Saco). The secondary antibodies were used according to the manufacturer's instructions: FITC-conjugated bovine anti-sheep IgG (1:100; Santa Cruz Biotechnology, Santa Cruz), FITC-conjugated goat anti rabbit IgG (1:200; Sigma), TRITC-conjugated goat anti-mouse IgG (1:200; Sigma). The samples were incubated for 30 min at 37°C and examined under a confocal laser-scanning microscope (Carl Zeiss, Inc., Thornwood, New York).

Hepatic specific gene expression analysis by RT-PCR. Total RNA was extracted from ES cells, cells treated with sodium butyrate for 5 days, ES-derived hepatic progenitor cells, differentiated hepatocytes and adult mouse liver cells using NucleoSpin® RNA II Kits. RNA samples were prepared with RNase-free DNase I for 15min at room temperature. Complementary DNA was synthesized from 1 μg total RNA using a superscript III first-strand synthesis system according to the manufacturer's instruction. The resulting cDNA was amplified using Gene Amp PCR 9600 (Perkin-Elmer Corporation, Norwalk, CT) with the sets of primers as shown in the Table I. The primers were designed using different exons for each gene. PCR cycles were as follows: initial denaturation at 95°C for 4 min followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s and final extension at 72°C for 10 min. The amplified PCR products were analyzed by electrophoresis on 1.2% agarose gel and were stained with ethidium bromide for visualization.

Cell Cycle Analysis for ES-Derived Hepatic Progenitor Cells

Flow cytometry was performed to measure the DNA content and cell cycle distribution of the ES-derived hepatic progenitor cells. The measurements were made with a Becton Dickinson FACS Calibur machine, adapted for excitation with a 488-nm argon laser, and a 582/42-nm band-pass filter for detecting propidium iodide emission.

In Vitro Differentiation Potential Assay of ES-Derived Hepatic Progenitor Cells

Differentiation potential of ES-derived hepatic progenitor cell into hepatocytes. Hepatocyte differentiation was obtained by inoculating the hepatic progenitor cells in 50 ng/ml type I collagen-coated 96-well plate. The medium was changed into William'E medium supplemented with 10% FBS, 2 mM L-glutamine, and 0.1 mM 2-mercaptoethanol (basal medium II) containing 0.5 mM ascorbic

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

Gene	Sense primer (5′-3′)	Antisense primer (5′-3′)	Product (bp)
AFP	CACTGCTGCAACTCTTCGTA	CTTTGGACCCTCTTCTGTGA	300
AAT	GAACCATTATCAGGCAGAA	AATAAGGAACGGCTAGTAAGA	675
ALB	TCTTCGTCTCCGGCTCTG	CTGGCAACTTCATGCAAAT	475
G6p	TCAATCTCCTCTGGGTGGC	GGCAAAGGGTGTAGTGTCAAG	602
TAT	CTTCAGTGCTGGATGTTCGC	CAGGGATTGGACGGGTTGTT	619
DPPIV	GATTCCATACCCAAAGGC	GGTCACAACTAAGGCACT	587
CK19	GTCCTACAGATTGACAATGC	CACGCTCTGGATCTGTGACAG	570
BG	CGTGAAGGATACGGGAGT	CAGAGTTATTGACGAGGC	581
GGT	TGTCCCTGGTGAAATCCG	GGCATAGGCAAACCGAAA	577
CK8	CGTCTGTGGTGCTGTCTATG	CGCTGGTGGTCTTCGTAT	525
CK18	GTTGTCACCACCAAGTCTGC	CCACTCTGCCATCCACGA	415
HNF3β	GACCTCTTCCCTTTCTACCG	TTGAAGGCGTAATGGTGC	551
HNF4	CTTCCAAGAGCTGCAGATTG	CTTGTAGGATTCAGATCCCG	517
β-actin	TTCCTTCTTGGGTATGGAAT	GAGCAATGATCTTGATCTTC	200

Abbreviation: AFP, alpha-fetoprotein; AAT, alpha-1-antitrypsin; ALB, albumin; G6p, glucose-6-phophatase; TAT, tyrosine aminotransferase; DPPIV, dipeptidylpeptidase IV; CK, cytokeratin; BG, biliary glycoprotein; GGT, γ-glutamyl transpeptidase; HNF3β(Foxa2), hepatocyte nuclear factor 3β; HNF4, hepatocyte nuclear factor 4.

acid diphosphate, 10^{-6} M insulin, 10 mM nicontinamide, 10^{-7} M dexamethasone (Dex), 20 ng/ml mouse epidermal growth factor (mEGF) and 10 ng/ml rmHGF for 6 days, and then replaced with the maturation medium containing 10 ng/ml oncostatin M (OSM) and 10^{-6} M Dex for another 6–12 days. Finally, cells were collected for gene expression, immunostaining and functional analyses. For functional analysis, glycogen production and storage was determined by Periodic acid-Schiff agent according to the method previously described [Kania et al., 2004]. Briefly, cells were fixed in Carnoy for 15 min at room temperature, oxidized in 1% periodic acid for 10 min and rinsed three times in dH_2O. Afterwards, cultures were treated with Schiff 's reagent for 20 min, washed in dH_2O for 5∼10 min, and examined in Nikon microscope.

Differentiation potential of ES-derived hepatic progenitor cells into bile duct cells. Bile duct-like structure was induced through substrate-induced differentiation by seeding the progenitor cells on a layer of Matrigel (BD) basement membrane matrix. Pure Matrigel (500 μl) was spread on 60-mm dishes and allowed to settle for 1–2 h at 37 °C. Cells were then plated at a density of 2×10^5 cells/cm^2 and cultured in the medium supplemented with 100 ng/ml hepatocyte growth factor (HGF) (R&D) and 50 ng/ml epidermal growth factor (Peprotech).

RESULTS

Induction of Hepatic Progenitor Cells From ES Cells by Sodium Butyrate

Here we designed a novel 4-step strategy based on sodium butyrate, HGF, OSM, and Dex for the induction of mouse ES cells into hepatic progenitor cells and then hepatocytes. When cultured in the medium supplemented with recombinant mLIF and in the presence of MEF feeder layer, mouse ES cells grow as a homogeneous population with 95% greater of the colonies displaying distinctive domed colony morphology (Fig. 1A). When treated with 0.8% DMSO, the cultures contained a diversity of cell types. But after treated with 2.5 mM sodium butyrate for 6–8 days, the majority of cells detached and the residual cells contained large nuclei and dark granular deposits within the cytoplasm (named as granular-rich large cells, with a large cell diameter 20∼40 μm, Fig. 1B). The presence of sodium butyrate was important for the maintenance of these granular-rich large cell phenotypes. When cultured for another 6–12 days in the medium withdrew sodium butyrate, the granular-rich large cells changed into small round shape and finally gave rise to many proliferating colonies of small cells (Fig. 1C). In contrast to the granular-rich large cells, the small round cell population had a small diameter with 8–10 μm, scant cytoplasm and a high nuclear to cytoplasmic ratio, which resemble blast-like oval cells proliferating during severe liver injury or hepatoblasts found in fetal liver [Yasui et al., 1997; Tanimizu et al., 2003]. Different from the primitive round morphology on gelatin-coated plastic dishes, the small round cell population displayed typical epithelial-like characteristics when transferred them in the dishes coated with collagen I (Fig. 1D). Considering these small round cells were morphologically distinct and resembled hepatic progenitor cells, we named them as ES-derived hepatic progenitor cells.

Identification and Characterization of ES-Derived Hepatic Progenitor Cells

To determine whether the ES-derived hepatic progenitor cells have typical markers of hepatic progenitor cells and distinguished phenotypes from ES cells, we evaluated SSEA-1, AFP, CK18, CK19, and ALB by immunostaining assay. The result showed that the undifferentiated ES cells expressed SSEA-1, a pluripotent cell specific antigen, but not the liver-specific markers including AFP, CK18, CK19, and ALB. However, when ES cells treated with DMSO for 4 days and then sodium butyrate for 5 days, the granular-rich large cell population was generated, and they stopped expressing SSEA-1, and began to express CK19 and ALB (Fig. 2D–F), but not AFP (Fig. 2A–C), which was consistent with the process previously described [Rambhatla et al., 2003]. After removal of sodium butyrate, the granular-rich large cells changed into ES-derived hepatic progenitor cells accompanied with the increased expression of AFP and decreased expression of ALB until undetectable with immunofluorescence (Fig. 3A–C), while the expression of CK18 and CK19 was not significantly changed (Fig. 3B,G). Interestingly, the ES-derived hepatic progenitor cells also coexpressed nestin in the company of AFP

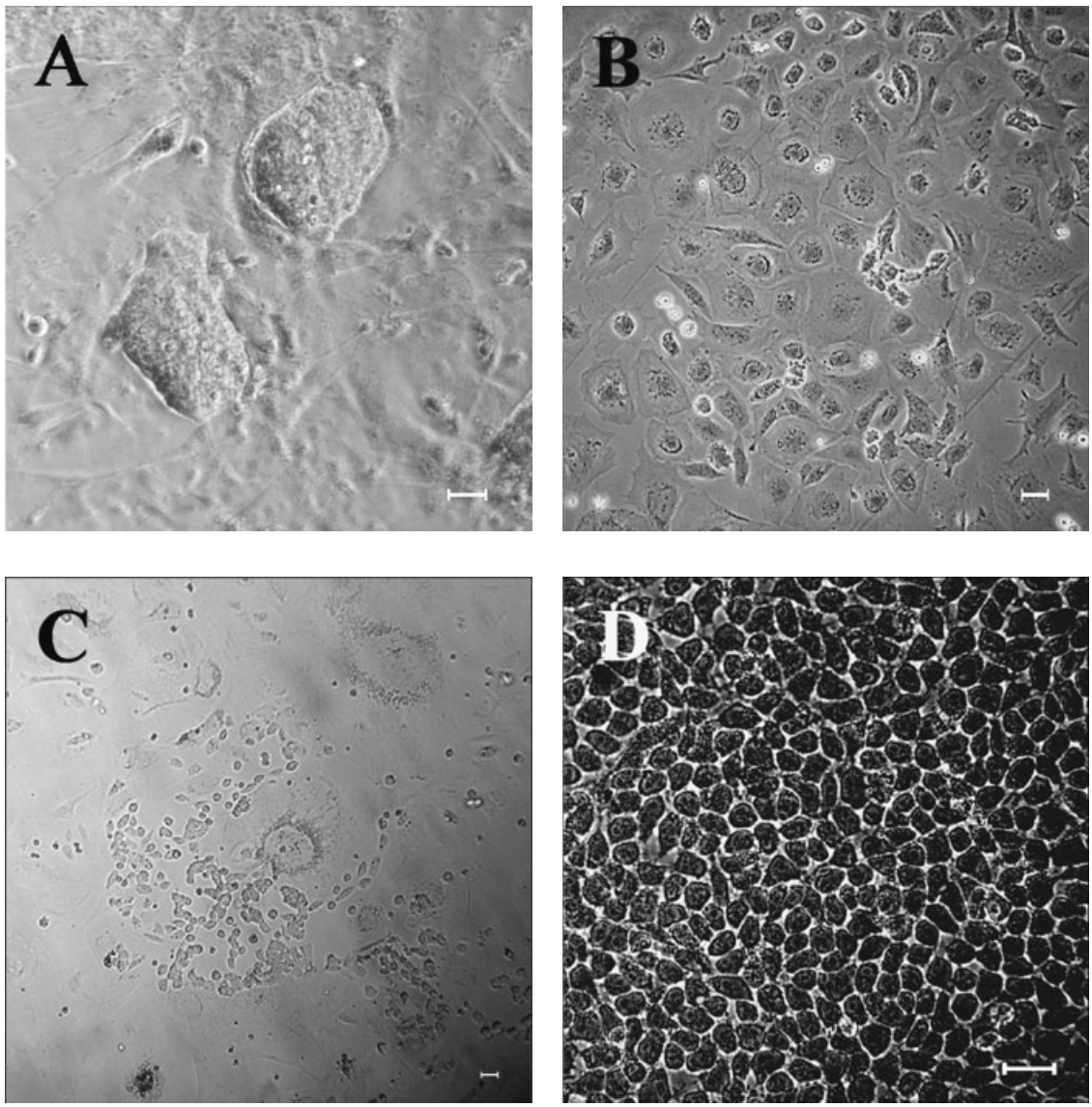


Fig. 1. Morphological changes of undifferentiated ES cells to differentiated hepatic progenitor cells with the treatment of sodium butyrate. (A) undifferentiated mouse ES cells cultured on the MEF feeder cells, (B) the cells treated with sodium butyrate for 6 days, (C) ES-HPCs appeared after cultured in the control medium for 3 days, (D) ES-HPCs cultured in the collagen-coated dish. Bar, 20 μm.

(Fig. 3D–F). By immunostaining assay, the ES-derived hepatic progenitor cells showed a homogeneous population of AFP, nestin, CK18 and CK19 positive, but ALB negative cells.

To further analyze the properties of the ES-derived hepatic progenitor cells, hepatic lineage-associated gene expression were examined by RT-PCR. As the results shown in Figure 4, undifferentiated control ES cells did not show transcripts of genes characteristic for hepatic lineage cells. However, after 5 days' induction of hepatic differentiation with sodium butyrate, the expression of a panel of liver associated genes, including *AAT*, *HNF3β*, and *ALB* were upregulated, which consistent with the results of immunostaining assay. In ES-derived hepatic progenitor cells, a number of early hepatocyte markers, such as *AFP* and *AAT*, the cholangiocyte markers, such as *CK19* and *γ-GGT*, the miscellaneous markers, such as *CK8*, *HNF3 β*, and *CK18* were detected. However, as a marker of mature hepatocytes, the ALB was not detected in ES-derived hepatic progenitor cells. The result showed that the ES-derived hepatic

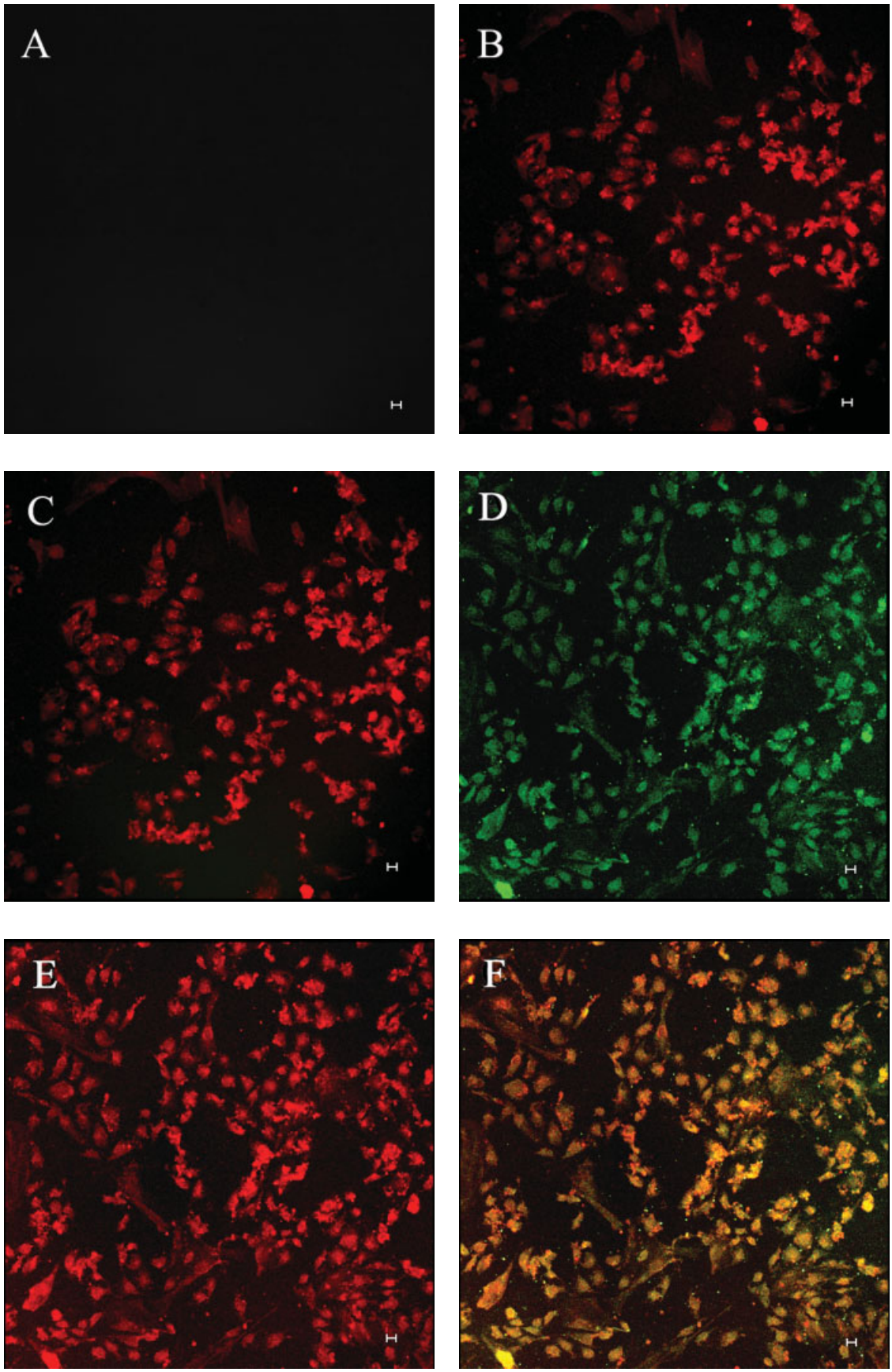


Fig. 2.

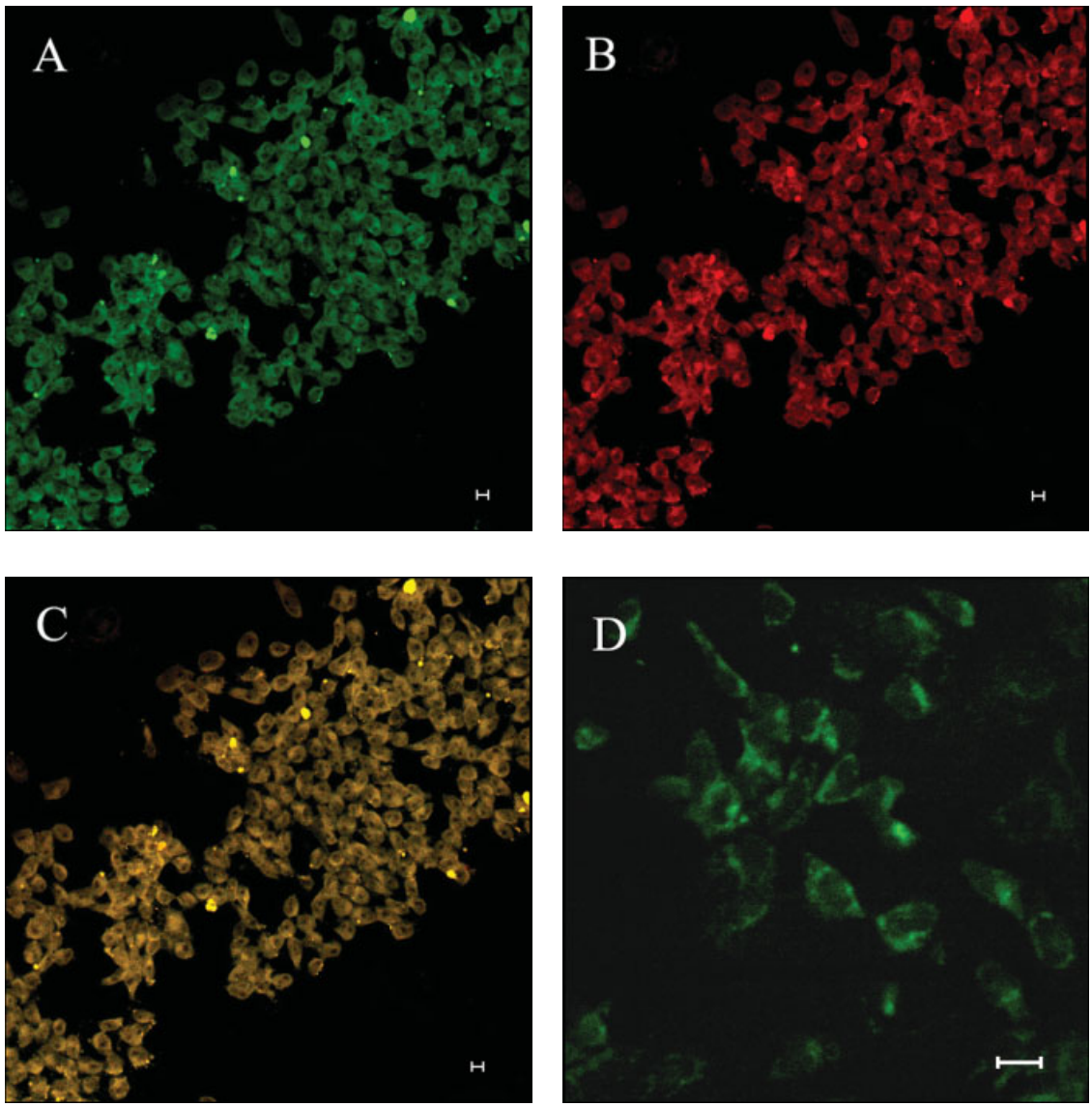


Fig. 3. Immunofluorescence analysis of ES-derived hepatic progenitor cells. (A; D) α-fetoprotein expression, (B) Cytokeratin 19, (E) Nestin, (G) Cytokeratin 18, (C) merged image of A and B, (F) merged image of D and E. Bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

progenitor cells have some similar molecular characterizations as the previously reported hepatic progenitor/stem cells [Brill et al., 1999; Saito et al., 1999; Azuma et al., 2003; Lazaro et al., 2003; Strick-Marchand and Weiss, 2003; Zhang et al., 2003; Petkov et al., 2004].

Cell Cycle Analysis for ES-Derived Hepatic Progenitor Cells

During the 6 days' treatment with sodium butyrate, the cells gradually differentiated into granular-rich large cells with liver lineage

Fig. 2. Double immunofluorescence analysis of the ES cells treated with sodium butyrate for 5 days. (A) Expression of α-fetoprotein were not detected, (B; E) red fluorescence indicates Cytokeratin 19 expression, (D) green fluorescence represents albumin expression. (C) merged image of panels A and B, (F) merged image of Panels D and E. Bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

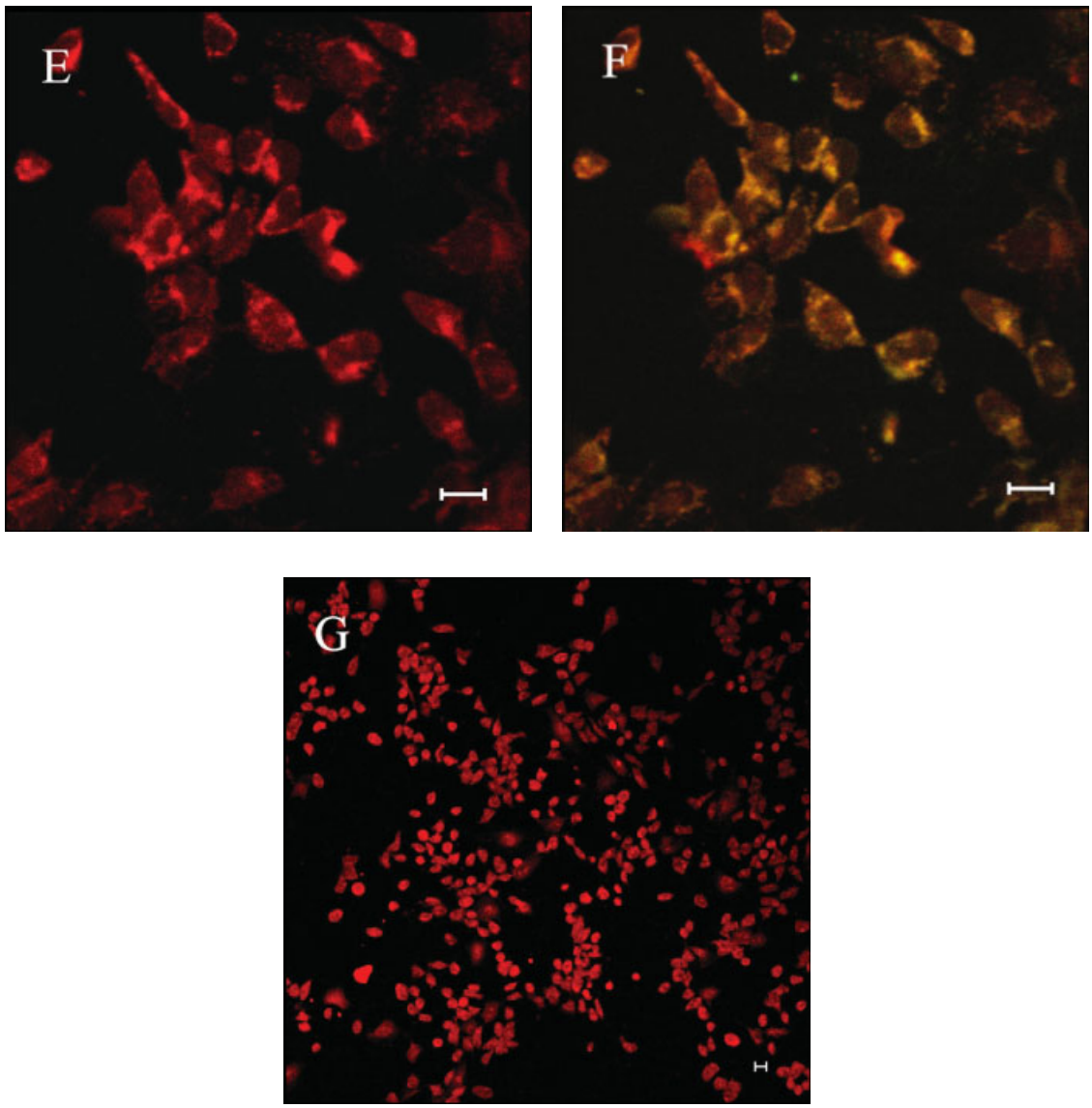


Fig. 3. (*Continued*)

molecular markers, and the cell proliferation was significantly decreased, which suggested that sodium butyrate could induce hepatic differentiation and cause mouse ES cell cycle arrested in the same way as human ES cells [Rambhatla et al., 2003]. However, when withdrawal of sodium butyrate, the granular-rich large cells further differentiated into ES-derived hepatic progenitor cells possessed rapid proliferation rate, which indicated that the withdrawal of sodium butyrate promoted the treated cells to reenter into cell cycle. As shown in Figure 5, the FACS results demonstrated that approximately 47% of the ES-derived hepatic progenitor cells were in S phase of the cells cycle, and the DNA content of cells in G_2/M phases is about twofold of that in G_0/G_1 phases, which is similar to normal diploid cells.

In Vitro Differentiation of ES-Derived Hepatic Progenitor Cells Into Hepatocytes

To assess whether the ES-derived hepatic progenitor cells could differentiate into hepatocytes, the cells were cultured in William' E medium supplemented with HGF, OSM, and Dex in collagen I-coated dishes, which was previously shown to favor hepatocyte maturation from hepatic stem/progenitor cells [Suzuki et al., 2003]. Early hepatocyte development was identified by the presence of AFP, *HNF3β*,

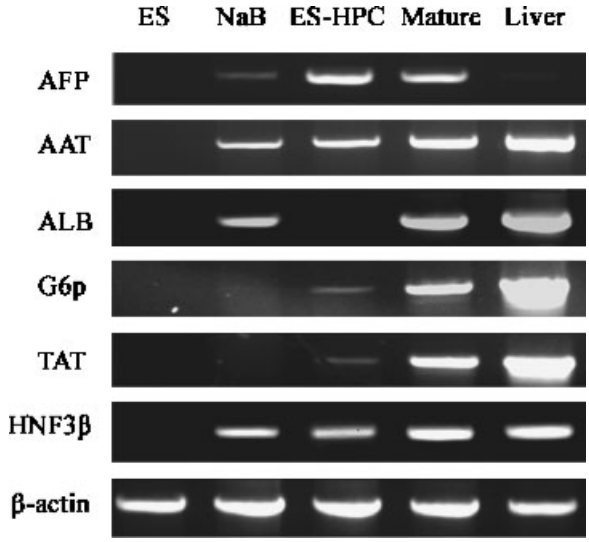
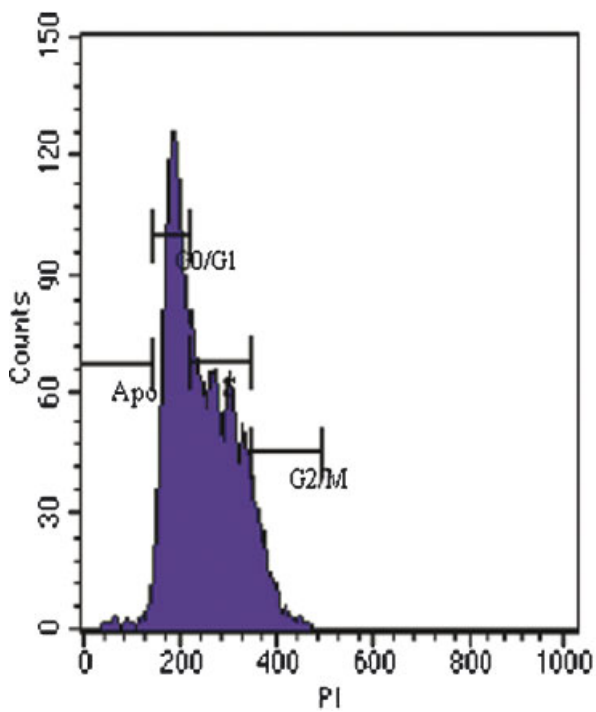


Fig. 4. Gene expression of the ES cells differentiated into hepatic lineage. ES cells didn't show transcripts of genes characteristic for hepatic lineage cells (ES). After 5 days' treatment with sodium butyrate, the expression of AAT and ALB were upregulated (NaB). The ALB expression was not detected in the sample of the ES-derived hepatic progenitor cells, the AFP expression was upregulated (ES-HPC). The mature hepatic markers were detected in the hepatocyte-like cells (Mature). Adult liver was the positive control (Liver).

HNF4, and *AAT* expression, while late development of the hepatic lineage was defined by the expression of *TAT*, *G6p* (Fig. 4), ALB and the synthesis of glycogen. As shown in Figure 6A and B, after differentiation for 2∼3 weeks, most cells strongly expressed ALB (Fig. 6A) and synthesized glycogen (Fig. 6B), whereas both AFP and CK19 were barely detectable. But the further maturation, which indicated by the expression of glutathione *S*-transferase (GST) and tryptophan 2,3-dioxygenase (TDO) [Nagao et al., 1986], was not achieved under the above-mentioned conditions. The results showed that the ES-derived hepatic progenitor cells could differentiate into partially mature hepatocytes in the above-mentioned conditions, the fully maturation conditions need to further investigate.

In Vitro Differentiation of ES-Derived Hepatic Progenitor Cells Into Bile Duct Cells

To determine whether the ES-derived hepatic progenitor cells can differentiate into bile duct cells, they were cultured in Matrigel, previously shown to favor bile duct cell differentiation [Spagnoli et al., 1998; Suzuki et al., 2002]. The



Marker	% Gated	Mean
All	100.00	243.82
G0/G1	46.78	190.22
S	46.98	281.85
G2/M	6.38	375.13
Apo	0.80	123.45

Fig. 5. Cell cycle analysis of ES-HPCs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

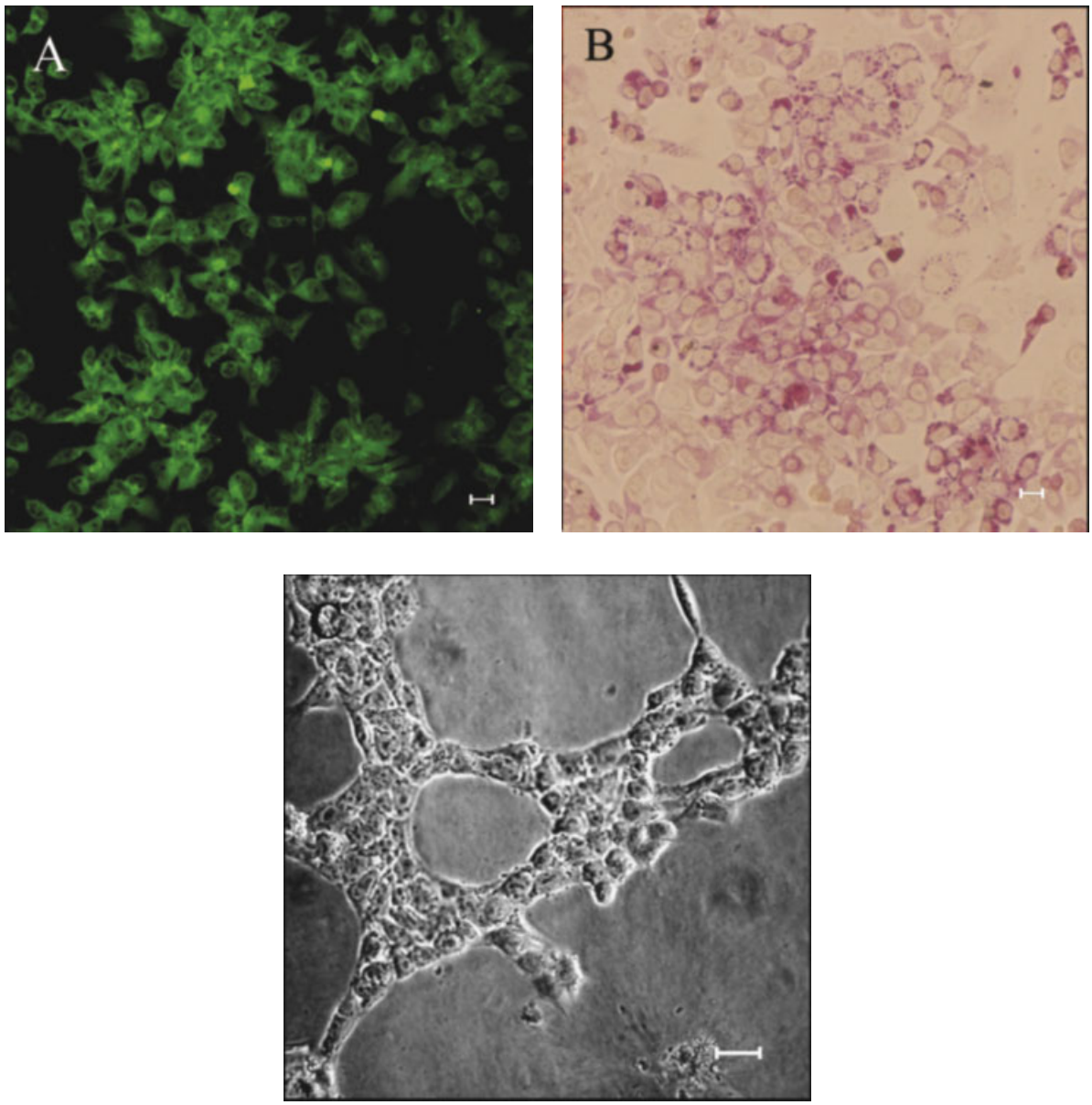


Fig. 6. Bipotential differentiation of the ES-HPCs. ES-HPCs gave rise to albumin- (A) and glycogen-positive (B) hepatocyte-like cells in the conditions that favor hepatic differentiation. (C) Composite of three overlapping phase micrographs highlighting duct-like structures with a well-defined lumen and circumscribed by neatly aligned cells. Bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cells underwent morphological changes and their proliferation was reduced. Many well-defined duct-like structures constituted of neatly aligned cells were found throughout the dish 1–2 days after inoculation, and maintained for about 5 days, and finally formed the spherical 3-dimensional structures of tightly packed columnar epithelium with a central lumen (Fig. 6C).

DISCUSSION

Recent reports had highlighted the differentiation of hepatocytes from ES cells both in vitro and in vivo, but most protocols involved the formation of embryoid bodies, and acquired differentiated cells represented heterogeneous populations. The residual ES cells had a propensity to develop teratomas and would not

be suitable for cell transplantation. In addition, the low efficiency had been another difficult problem in the hepatic differentiation of stem cells. Hepatic progenitor cells were bipotential to differentiate into both functional hepatocytes and bile duct cells with high efficiency in proper conditions [Lazaro et al., 2003; Petkov et al., 2004]. Besides the potential cell source for transplantation, the availability of hepatic progenitor cells at different stages of differentiation was critical for the investigations of hepatic lineage differentiation and liver development. Moreover, isolation and identification of hepatic progenitor populations from ES cells will facilitate the understanding of the relationship between hepatic progenitor cells and ES cell populations. But until now, little is known about the differentiation of hepatic progenitor cells directly from embryonic stem cells.

It was recently reported that hepatocyte-like cells could be differentiated from human ES cells using sodium butyrate, a specific and potent histone deacetylation inhibitor. The hepatocyte-like cells expressed ALB and AAT, exhibited glycogen storage, and inducible cytochrome P450 activity, but lack of the expression of AFP. These results suggested that a population of mature hepatocyte-like cells was differentiated from human ES cells. Moreover, it was assumed that a proliferating hepatic progenitor population existed in the cultures before 4 days' treatment of sodium butyrate, although the expression of the markers was not detected with immunocytochemistry [Rambhatla et al., 2003]. Here we cultured the mouse ES cells in the medium supplemented with 0.8% DMSO for 4 days and then 2.5 mM sodium butyrate for 6–7 days, allowing the differentiation of a hepatic lineage-like population (the granular-rich large cells), which resembles the protocol described by Rambhatla et al. [2003]. However, the subsequent protocols including the withdrawal of sodium butyrate for hepatic progenitor cell generation was different from that described before. By morphological, biochemical, and functional analyses, the acquired ES-derived progenitor cells exhibited a number of characteristics the same as hepatic progenitor cell population, including specific gene and protein expression, and specific potentials of differentiation. However, the phenotypes of the acquired cells were different from the undifferentiated ES cells in many aspects. First, mouse ES cells expressed SSEA-1, while the ES-derived progenitor cells expressed AFP, *AAT*, *GGT*, *HNF3β*, and CK19, but not SSEA-1. Second, when cultured in type-I collagen coated dishes, the ES-derived progenitor cells behaved typical epithelial morphology, while ES cells were still proliferating in the form of colonies. When taken altogether, these results demonstrated that the effects of sodium butyrate on the differentiation of mouse ES cells were partially reversible. This is inconsistent with the observation that reversible effects of sodium butyrate on the differentiation of F9 embryonal carcinoma cells [Kosaka et al., 1991].

Histone deacetylase inhibitors, such as suberoylanilide hydroxamic acid (SAHA), valproic acid (VPA), trichostatin A (TSA), sodium butyrate (NaB) and so on, have recently emerged as a new group of therapeutic agents, effective in some human cancers and in hematologic malignancies [Richon and O'Brien, 2002]. HDACs catalyze the removal of acetyl groups from the histones, thereby mediate condensation of the nucleosomes. While HDAC inhibitors promote histone acetylation, relax the DNA wrapped around the core histones, and allow transcription of genes involved in important cellular processes. Therefore, HDAC inhibitors have been shown to induce growth arrest, terminal differentiation and cell death of a broad variety of transformed cells in vitro and in vivo [Blaheta and Cinatl, 2002]. Much is known about the mechanism of HDAC inhibitors effect on the transformed cells, however, little is known about the functional mechanism that exerts antiproliferative, induction of differentiation or apoptotic effects on the normal cells. Recently, HDAC inhibitors have been reported to inhibit cardiomyocyte differentiation of embryonic stem cells [Na et al., 2003], myofibroblastic differentiation of rat hepatic stellate cells [Niki et al., 1999], mediated neuronal differentiation of multi-potent adult neural progenitor cells [Hsieh et al., 2004], induction of osteogenic differentiation of human mesenchymal stem cells [Cho et al., 2005], and enhances the cytokine-induced expansion of human hematopoietic stem cells [De Felice et al., 2005]. According to our data, the differentiation system with the treatment of sodium butyrate allows ES cells differentiation into hepatic progenitor cells, moreover, VPA, another reported type II HDAC inhibitors, had similar

effects on the process of differentiation (data not shown). According to our data and the previous report [Rambhatla et al., 2003], the majority of granular-rich large cells lost the proliferative capacity in the presence of the revulsant, which could not incorporated BrdU during the DNA synthesis. However, the granular-rich large cells changed into small round ES-derived hepatic progenitor cells possessed rapid proliferation rate after the removal of sodium butyrate. Moreover, in our study, sodium butyrate induced the increasing expression of $p21^{WAF1}$ mRNA (data not shown), which was a cyclin-dependent kinase inhibitor to effect cell-cycle arrest [Archer et al., 1998]. The expression downregulated to the original level when sodium butyrate was removed for 6–12 days. In conclusion, these studies suggested that the increasing expression of $p21^{WAF1}$ may, in part, be responsible for the cell-cycle arrest effects of HDAC inhibitors, and the decreasing expression of $p21^{WAF1}$ may promote the granular-rich large cells reenter the cell cycle.

Previous identified hepatic progenitor/stem cells include hepatoblasts and oval cells, known to have the potential to differentiate into both hepatocytes and biliary epithelial cells. Hepatoblasts are endodermal precursors in the embryonic developing liver bud and express many genes in common with hepatocytes and bile duct cells [Tanimizu et al., 2003; Petkov et al., 2004]. In adult liver, oval cells locate in the canals of Hering and would be activated to proliferate when the regenerative capacity of terminally differentiated hepatocytes was compromised, while others suggest that oval cells could be derived from precursors in the bone marrow [Forbes et al., 2002]. Some studies demonstrated that hepatic progenitor/stem cells expressed both hepatocyte markers, such as AFP, ALB and biliary cell markers, such as CK19, but the results of some articles were different, such as the bipotential mouse embryonic liver (BMEL) cells isolated from E14 embryonic liver of wild-type mice [Weiss and Strick-Marchand, 2003] and the oval cells isolated from Long–Evans Cinnamon rats, were stained positive for AFP, CK18 and CK19, but negative for ALB [Yasui et al., 1997]. These studies may suggest the diversity or different development stages of hepatic stem cells in fetal and adult liver [Zheng and Taniguchi, 2003]. In the present study, we acquired the cell population expressing AFP, CK18 and CK19, but not ALB, which suggests the generation of early hepatic progenitor cells from mouse ES cells. These ALB-negative hepatic progenitor cells could be induced into ALB-positive hepatocytes when treated with HGF, OSM, and Dex in company with the upregulated expression of *G6p* and *TAT*. In addition, these cells were capable of forming bile duct-like structures. The various morphologic and functional characteristics strongly demonstrated that the ES-derived progenitor cells have bipotential to differentiate into hepatocytes and bile duct cells, and indicated that they were a population of hepatic progenitor cells. Interestingly, it was showed that the ES-derived hepatic progenitor cells expressed nestin, which was initially identified as the typical marker of neural stem cells. However, recent studies revealed a wider range of cells expressed nestin than previously thought. Moreover, some articles reported nestin-positive progenitor cells derived from ES cells could differentiate into the neuroectodermal, mesodermal and endodermal lineages, thus demonstrated that nestin-positive cells may represent a population of multi-lineage progenitor cells [Blyszczuk et al., 2003; Kania et al., 2003; Kania et al., 2004].

In summary, we isolated a novel prospective hepatic progenitor cell population differentiated from mouse embryonic stem cells with the induction of sodium butyrate. These cells were defined as the ability of expressing AFP, CK19 and other hepatic progenitor cell-specific markers, high proliferation and potential to differentiate into typical hepatocytes and bile duct structures. The isolation of hepatic progenitor cells from ES cells provides not only a novel cell source for transplantation, but an appropriate model for the study of hepatic linage differentiation, especially for the investigation of early hepatic determination from foregut endoderm. Further studies are needed to elucidate the essential nature of hepatic progenitor cells and their capacity of bipotential differentiation and repopulation of regenerating liver.

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