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Effects of Sodium Butyrate on the Differentiation of Pancreatic and Hepatic Progenitor Cells From Mouse Embryonic Stem Cells

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ABSTRACT

Recently significant progress has been made in differentiating embryonic stem (ES) cells toward pancreatic cells. However, little is known about the generation and identification of pancreatic progenitor cells from ES cells. Here we explored the influence of sodium butyrate on pancreatic progenitor differentiation, and investigated the different effects of sodium butyrate on pancreatic and hepatic progenitor formation. Our results indicated that different concentration and exposure time of sodium butyrate led to different differentiating trends of ES cells. A relatively lower concentration of sodium butyrate with shorter exposure time induced more pancreatic progenitor cell formation. When stimulated by a higher concentration and longer exposure time of sodium butyrate, ES cells differentiated toward hepatic progenitor cells rather than pancreatic progenitor cells. These progenitor cells could further mature into pancreatic and hepatic cells with the supplement of exogenous inducing factors. The resulting pancreatic cells expressed specific markers such as insulin and C-peptide, and were capable of insulin secretion in response to glucose stimulation. The differentiated hepatocytes were characterized by the expression of a number of liver-associated genes and proteins, and had the capability of glycogen storage. Thus, the current study demonstrated that sodium butyrate played different roles in inducing ES cells toward pancreatic or hepatic progenitor cells. These progenitor cells could be further induced into mature pancreatic cells and hepatocytes. This finding may facilitate the understanding of pancreatic and hepatic cell differentiation from ES cells, and provide a potential source of transplantable cells for cell-replacement therapies. J. Cell. Biochem. 109: 236–244, 2010.

KEY WORDS: SODIUM BUTYRATE; EMBRYONIC STEM CELLS; DIFFERENTIATION; PANCREATIC PROGENITOR CELLS; HEPATIC PROGENITOR CELLS

Although islet transplantation has been proved successful in the treatment of diabetes [Guo and Hebrok, 2009], the shortage of tissue donors remains a major obstacle. Similarly, hepatocyte transplantation has been proposed as an alternative to liver transplantation in the treatment of end-stage liver diseases [Hussain and Theise, 2004], but the paucity of cell donors also restricts its widespread availability. To solve the problem of donor shortage, new sources for transplantable pancreatic cells and hepatocytes need to be found. Given the unlimited proliferative and pluripotent differentiation capacity, embryonic stem (ES) cells may potentially supply a sufficient number of pancreatic cells and hepatocytes for cell-transplantation therapy [Jiang et al., 2007b; Navarro-Alvarez et al., 2009].

To date, many studies have been reported regarding the differentiation of ES cells toward pancreatic cells [Strom et al., 1997; Vaca et al., 2006; Jiang et al., 2007a; Li et al., 2009] and hepatocytes [Yamada et al., 2002; Min et al., 2007; Matsumoto et al., 2008; Basma et al., 2009]. However, because of the complicated characteristic of ES cell differentiation, much more researches still need to be carried out to understand and control the differentiation procedure. Although the induction of progenitor cells is a critical step for subsequent definitive cell maturation in vitro, studies about the generation and characteristic of pancreatic and hepatic progenitor cells are still inadequate.

During embryogenesis, the pancreas and liver derive from a common definitive endoderm, and share many similar develop-

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mental features [Deutsch et al., 2001]. Since in vitro ES cell differentiation recapitulate many processes taking place in normal embryonic development, it might be assumed that there existed close relationship between pancreatic and hepatic differentiation from ES cells. Previous studies have shown that sodium butyrate, one kind of histone deacetylase inhibitors, could induce the differentiation of hepatic progenitors and hepatocytes with high efficiency [Rambhatla et al., 2003; Sharma et al., 2006; Zhou et al., 2007]. Considering the close relationship between pancreatic and hepatic development, here we aimed to explore the influence of sodium butyrate on pancreatic progenitor differentiation, and investigated the different effects of sodium butyrate on pancreatic and hepatic progenitor formation. Results demonstrated that different concentration and exposure time of sodium butyrate facilitated different differentiating directions of ES cells toward pancreatic or hepatic progenitor cells. Treatment with 1 mM sodium butyrate for 3 days resulted in the obvious expression of pancreatic progenitor genes such as nestin, pancreatic/duodenal homeobox-1 (PDX-1), and Neurogenin 3 (NgN3), while 3 mM sodium butyrate for 7 days led to increased expression of cytokeratin 19 (CK19), α-fetal protein (AFP), transthyretin (TTR), and α1-antitrypsin (AAT). With the stimulation of exogenous inducing factors, these progenitor cells could be further differentiated into mature pancreatic cells and hepatocytes. The resulting pancreatic cells were identified as they expressed pancreatic specific genes such as PDX-1, insulin, C-peptide, and glucose transporter-2 (Glut-2). The secretion of insulin by these cells was responsive to variations in glucose levels. The differentiated hepatocytes expressed hepatic specific markers such as albumin (ALB), tyrosine aminotransferase (TAT), glucose-6-phophatase (G6P), and cholesterol 7 α-hydroxylase (CYP7A1). Periodic acid-Schiff (PAS) reaction showed the differentiated cells had the capacity of glycogen storage.

MATERIALS AND METHODS

CELL CULTURE

Undifferentiated E14 mouse ES cells (ATCC, Manassas, VA) were maintained on gelatin-coated dishes in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY), supplemented with 15% fetal bovine serum (Hyclone, Rockville, MD), 1,000 U/ml recombinant mouse leukemia inhibitory factor (LIF; Chemicon, Temecula, CA), 1% non-essential amino acids, 1 mM glutamine, and 0.1 mM β-mercaptoethanol (Sigma–Aldrich, St. Louis, MO). The above culture medium, excluding LIF, was defined as the differentiating medium. To induce differentiation, ES cells were incubated using the suspension culture methods. Briefly, the cells were suspended in the differentiating medium and cultured to develop into embryoid bodies (EBs) for 4 days.

ANNEXIN V-FITC/PI STAINED FLUORESCENCE-ACTIVATED CELL SORTER (FACS)

To quantify the survival rate of ES cells after exposure to sodium butyrate, annexin V, and propidium iodide (PI) staining were performed, followed by flow cytometry. Briefly, cells were exposed to different concentrations of sodium butyrate ranging from 1 to 10 mM for 3 days and then were harvested, washed twice with cold PBS, and finally subjected to annexin V and PI staining. After staining, the samples were analyzed by FACS (Becton Dickinson) using Cell Quest Research Software (Becton Dickinson).

IN VITRO DIFFERENTIATION OF ES CELLS INTO PANCREATIC AND HEPATIC PROGENITOR CELLS

To study the effect of different concentrations of sodium butyrate on progenitor cell differentiation, EBs were exposed to 1, 2, or 3 mM sodium butyrate for 5 days, respectively. Then cells were collected for an analysis of the gene expression of pancreatic and hepatic progenitor cells.

To investigate the influence of different exposure time of sodium butyrate on progenitor cell differentiation, EBs were cultured in differentiating medium supplemented with corresponding concentration of sodium butyrate for about 1 week. Every 2 days cells were collected for specific gene detection of pancreatic and hepatic progenitor cells.

FLOW CYTOMETRIC ANALYSIS

Pancreatic or hepatic progenitor cells were washed with PBS and dissociated with 0.25% trypsin–EDTA for 10–15 min. Then cells were centrifuged, washed, fixed, and permeabilized with FIX&-PREM reagents at room temperature for about 30 min (Invitrogen, CA). Then cells were incubated with primary antibody, and further stained with appropriate secondary antibody conjugated to Alexa Fluor 488 at room temperature for 15 min. Finally the cells were washed, resuspended in PBS and used for analysis. The total volume of stained cells was 100 μl. Flow cytometric analysis was performed by FACS (Becton Dickinson) using Cell Quest Research Software (Becton Dickinson). Spontaneously differentiated cells were used as control.

DIFFERENTIATION OF PROGENITORS CELLS INTO MATURE PANCREATIC CELLS AND HEPATOCYTES

For the differentiation of pancreatic cells, EBs were cultured in the differentiating medium containing 25 ng/ml activin A (Sigma–Aldrich) for 48 h. Then cells were switched to the differentiating medium supplemented with 1 mM sodium butyrate. After 3 days of sodium butyrate treatment, the differentiated cells were further maturated in medium containing 10 ng/ml bFGF and 10 ng/ml HGF for 7 days, and then 10 mM nicotinamide (Sigma–Aldrich) for another 7 days.

For the differentiation of hepatocytes, EBs were firstly cultured in the differentiating medium supplemented with 3 mM sodium butyrate for 7 days. Then cells were cultured in the medium withdrew sodium butyrate and supplemented with 20 ng/ml HGF and 10^{-7} M dexamethasone (Dex) for another 2 weeks.

At different differentiation times, cells were collected for an analysis of the mRNA expression of the following genes: PDX-1, p48, NgN3, nestin, Paired Box Gene 6 (PAX-6), insulin1, insulin2, glucagon, Glut-2, and somatostatin as pancreatic markers, CK19, AFP, ALB, AAT, TTR, TAT, G6P, and CYP7A1 as hepatic markers. The insulinoma cell line NIT-1 and liver of mouse were used as positive control.

RT-PCR AND REAL-TIME PCR

Total RNA was extracted from cells using a standard Trizol RNA isolation method. Reverse transcription of 1 mg RNA was carried out according to the instructions of a commercial TaKaRa RT kit. Primers against mouse β-actin were used as an internal control. The primer sequences and the expected sizes of the RT-PCR products were shown in Table I. PCR products were visualized with gel electrophoresis to confirm a single product of the correct size. The ratios of pancreatic and hepatic markers to β-actin were calculated. Semi-quantitative analysis of PCR products was performed using an Alpha Imager TM 2000 instrument (Alpha Innotech, San Leandro, CA) Real-time PCR was performed with a kit (Quantitect SYBR Green; Qiagen, Inc.) by following the manufacturer's instructions. The total reaction volume was 25 μl, and 100 ng cDNA was used as the template. Fluorescence was detected using Prism 7700 detection system (ABI, CA). The relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta C_t}$ method, and the results were expressed as extent of change with respect to control values. Primer sequences used for real-time PCR were available on request.

IMMUNOFLUORESCENCE

The location and expression of pancreatic and hepatic markers such as PDX-1, insulin, C-peptide, and ALB were examined by immunofluorescence. Induced cells were fixed in 4% paraformaldehyde and washed three times with PBS, then incubated with PBS containing 0.3% Triton X-100 (Sigma–Aldrich) and 1% normal serum for 40 min at room temperature. The primary antibodies were obtained and diluted as follows: Rabbit anti-mouse PDX-1 antibody (1:100; Millipore, MA), Rabbit anti-mouse insulin and C-peptide antibody (1:100; Cell Signaling Technology, MA), sheep anti-mouse ALB antibody (1:50; Biodesign, Saco, ME), and relative FITC- and Cy3-conjugated secondary antibodies (Sigma–Aldrich). Nuclear DNA was dyed with Hoechst 33258 (Sigma–Aldrich). In all immunochemistry assays, negative staining controls were carried out by omitting the primary antibody. Images were captured using Olympus microscope IX-71, LEICA microscope.

INSULIN SECRETION TESTS BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Differentiated pancreatic cells were washed and preincubated for 30 min in Krebs-Ringer bicarbonate buffer (KRB) containing 3 mM glucose. After preincubation, the buffer was replaced with fresh KRB containing 3 or 20 mM glucose. After 30 min incubation, the media were collected and the insulin content within the media was measured using an insulin ELISA kit (Millipore). Intracellular protein concentration was determined using a BCA protein assay kit (Bio-Rad, CA). Insulin levels within the media (insulin secretion) were normalized based on the respective intracellular protein content. Each experiment was repeated four times.

GLYCOGEN DETECTION (PAS REACTION)

The PAS staining system was purchased from Sigma–Aldrich. Cells cultured on plates were dried in air and fixed with 95% alcohol for 10 min. After rinsing three times with distilled water, the cells were oxidized in 1% periodic acid for 10 min, rinsed in distilled water again, then exposed to the Schiff reagent for 10 min at 37°C. A third water wash to remove the reagent was followed by an inspection of the cells with a light microscope. Spontaneously differentiated cells were used as controls.

STATISTICAL ANALYSIS

Each experiment was performed at least three times. All values were given as mean ± SD. Data were analyzed using SPSS 11.0 software and ANOVA was used to identify significant differences.

RESULTS

EFFECTS OF SODIUM BUTYRATE ON GENE EXPRESSION OF PANCREATIC PROGENITOR CELLS

In order to investigate the precise effect of sodium butyrate on the survival rate of EBs, cells were exposed to different concentrations of sodium butyrate ranging from 1 to 10 mM for 3 days. Flow cytometry results showed that 1–3 mM sodium butyrate was suitable for cell proliferation and differentiation (Fig. 1A). Light microscopy

TABLE I. Sequence Information on the Primers Used for RT-PCR

Gene	Sense (5′–3′)	Antisense (5′–3′)	Product size (bp)
Nestin	GGAGAGTCGCTTAGAGGTGC	TCAGGAAAGCCAAGAGAAGC	327
PDX-1	GGTGGAGCTGGCAGTGATGTT	ACCGCCCCCACTCGGGGTCCCGC	125
P48	TGCAGTCCATCAACGACGC	GGACAGAGTTCTTCCAGTTC	708
NgN3	TGGCACTCAGCAAACAGCGA	ACCCAGAGCCAGACAGGTCT	444
PAX-6	AAGAGTGGCGACTCCAGAAGT	ACCATACCTGTATTCTTGCTTCAGG	545
Insulin1	CCAGCTATAATCAGAGACCA	GTGTAGAAGAAGCCACGCT	197
Insulin2	TCCGCTACAATCAAAAACCAT	GCTGGGTAGTGGTGGGTCTA	411
Somatostatin	TCGCTGCTGCCTGAGGACCT	GCCAAGAAGTACTTGGCCAGTTC	232
Glut-2	CGGTGGGACTTGTGCTGCTGG	CTCTGAAGACGCCAGGAATTCCAT	416
Glucagon	ACTCACAGGGCACATTCACC	CCAGTTGATGAAGTCCCTGG	353
CK19	GTGCCACCATTGACAACTCC	AATCCACCTCCACACTGACC	288
AFP	CACTGCTGCAACTCTTCGTA	CTTTGGACCCTCTTCTGTGA	300
ALB	GTCTTAGTGAGGTGGAGCAT	ACTACAGCACTTGGTAACAT	568
AAT	AATGGAAGAAGCCATTCGAT	AAGACTGTAGCTGCTGCAGC	483
TTR	AGTCCTGGATGCTGTCCGAG	TTCCTGAGCTGCTAACACGG	441
TAT	ACCTTCAATCCCATCCGA	TCCCGACTGGATAGGTAG	206
G6P	CAGGACTGGTTCATCCTT	GTTGCTGTAGTAGTCGGT	211
CYP7A1	AGGACTTCACTCTACACC	GCAGTCGTTACATCATCC	453
β-Actin	GTTGGTTGGAGCAAACATCC	AAGCAATGCTGTCACCTTCC	232

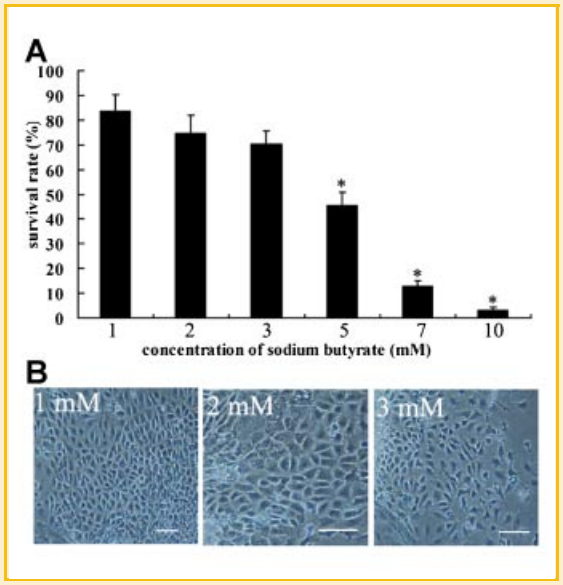

Fig. 1. Morphological changes and survival rate of EBs after the treatment of different concentrations of sodium butyrate. EBs were exposed to different concentrations of sodium butyrate (1, 2, 3, 5, 7, 10 mM) for 3 days. A: Annexin V and PI staining was performed, followed by flow cytometry analysis to detect the survival rate of cells. A representative example of three experiments was shown. Values were given as the mean ± SD. $^{*}P < 0.05$ versus 3 mM sodium butyrate-treated cells. B: Cell morphology was observed through light microscope. Representative images of three experiments were shown. Scale bar: 50 μm.

results showed that sodium butyrate had an immediate effect on EBs. Cell death was observed in the cultures as early as 1 day after plating. Then with the continuous treatment of sodium butyrate, most of the remaining cells spread gradually from EBs with a homogeneous shape of large, round, and center-situated nuclei (Fig. 1B).

To further explore the effects of sodium butyrate on gene expression of pancreatic progenitor cells, EBs were cultured in media supplemented with 1, 2, or 3 mM sodium butyrate for 5 days. As shown in Figure 2A, the specific gene expression levels of pancreatic progenitor cells, such as nestin, PDX-1, p48, and NgN3 were all increased in sodium butyrate treated cells. Importantly, exposure to 1 mM sodium butyrate resulted in maximal mRNA expression, as compared with supplementation of 2 or 3 mM sodium butyrate. This indicated that 1 mM sodium butyrate was more suitable for the differentiation of pancreatic progenitor cells from EBs.

EFFECTS OF SODIUM BUTYRATE ON GENE EXPRESSION OF HEPATIC PROGENITORS

To investigate gene expression of hepatic progenitors, EBs were differentiated with different concentrations of sodium butyrate for 5 days. CK19, AFP, AAT, and TTR were used to evaluate the differentiated characteristics for early hepatic markers. Results showed that with the increase of the concentration of sodium butyrate, the mRNA levels of CK19, AFP, AAT, and TTR were all evaluated accordingly. Exposure of 1 mM sodium butyrate promoted mRNA levels of these specific genes, however, the expression levels were relatively lower than that of 3 mM sodium butyrate treatment. So from the standpoint of generating hepatic progenitors, 3 mM sodium butyrate was more efficient (Fig. 2B).

TIME COURSE OF PANCREATIC AND HEPATIC PROGENITOR GENE EXPRESSION AFTER EXPOSURE TO SODIUM BUTYRATE

To study the effect of sodium butyrate with different exposure time on the differentiation of pancreatic progenitor cells, EBs were treated with 1 mM sodium butyrate for about 1 week. The mRNA levels of early pancreatic markers, such as nestin, PDX-1, p48, and NgN3 were measured at day 1, 3, 5, 7 of sodium butyrate treatment. The gene expression of nestin was detected at day 1, peaked at day 3 and then decreased slightly. PDX-1 and p48, two important transcription factors involved in early stages of pancreatic development [McKinnon and Docherty, 2001; Rose et al., 2001], showed significantly higher expression levels at day 3. NgN3, an important transcription factor for the development of all endocrine cells [Gradwohl et al., 2000], began to express at day 3, and the level lasted until the later time points (Fig. 3A).

To investigate the early hepatic differentiation from EBs, EBs were treated with 3 mM sodium butyrate for about 1 week. After the treatment with sodium butyrate, the mRNA levels of CK19, AFP, AAT, and TTR were all expressed at day 3, and increased over the time course (1 week) of the experiment (Fig. 3B). The expression of ALB, which starts in early fetal hepatocytes and reaches the maximal level in adult hepatocytes [Sellem et al., 1984], was not detectable until 5 days of differentiation, and then gradually increased by 7 days.

ES-DERIVED PANCREATIC PROGENITOR CELLS COULD MATURE INTO PANCREATIC CELLS

To determine whether the pancreatic progenitor population generated with sodium butyrate could be further differentiated to the pancreatic lineage, exogenous factors, including activin A, bFGF, HGF, and nicotinamide, were consecutively added into the differentiating medium. To identify the pancreatic progenitor population, the cells were examined for PDX-1 protein expression by flow cytometric analysis. RT-PCR and real-time PCR for genes involved in pancreas development were performed at various stages throughout the cell differentiation. As was shown in Figure 4A, the gene expression of nestin was induced after the induction of activin A and sodium butyrate, and then decreased with the addition of HGF and nicotinamide. mRNA levels of PDX-1 and p48 were both increased during pancreatic differentiation. The endocrine progenitor-specific gene, NgN3, was also induced, and the expression persisted throughout the differentiation process. Other islet related genes, such as PAX-6, were also detected in the differentiated cells, and their levels peaked at later stages of pancreatic differentiation. After the treatment of activin A and sodium butyrate, the gene expressions of insulin1, insulin2, and Glut-2 were all detected with limited expression levels. With the process of differentiation, the gene expression of these mature pancreatic markers showed maximal expression at the final stage of the protocol. Meanwhile, after the treatment of activin A and sodium butyrate, about 20% of cells expressed PDX-1, which was significantly higher than activin A alone treated cells (Fig. 4B).

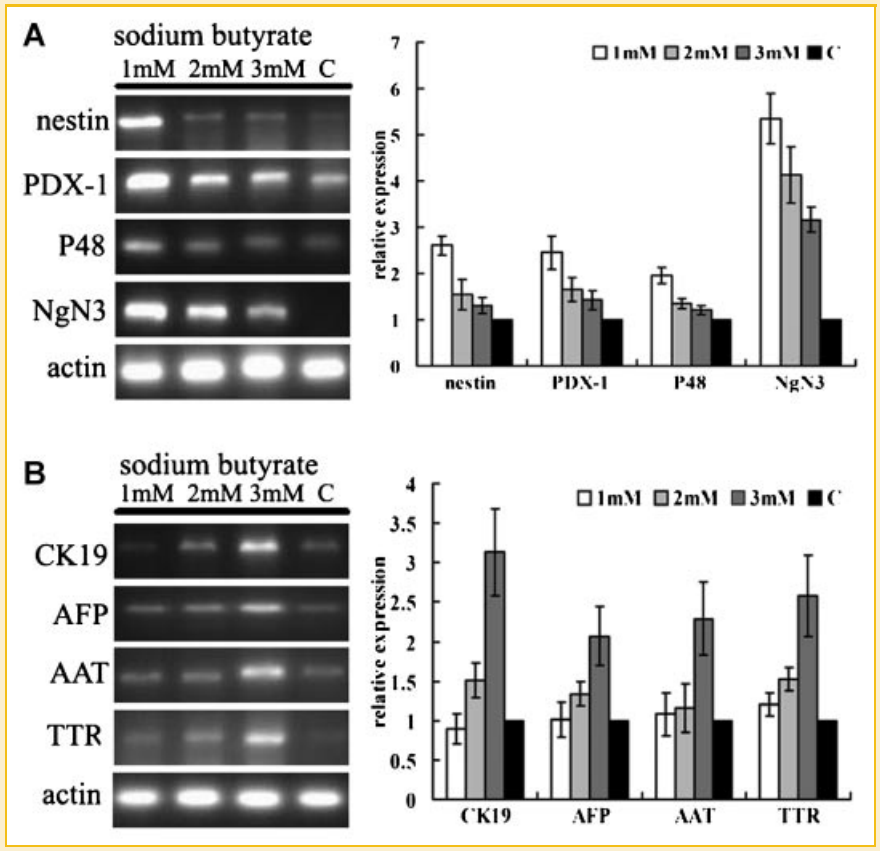

Fig. 2. Effects of different concentrations of sodium butyrate on gene expression of pancreatic and hepatic progenitors. EBs were cultured in media supplemented with 1, 2, or 3 mM sodium butyrate for 5 days. Spontaneously differentiated cells were used as control (c). Total RNA was isolated from cells and the gene expression of pancreatic progenitor cells (A) and hepatic progenitor cells (B) were analyzed by RT-PCR. β-Actin served as a control of equal RNA amount. The experiment was repeated three times. Histogram plots represented the densitometric analysis corresponding to the mean ± SD of three independent experiments.

To determine whether these final differentiated cells were insulin-positive and C-peptide-positive, immunofluorescence was performed to detect PDX-1, insulin, and C-peptide expression in the induced cells. Results showed that the differentiated cells expressed PDX-1, insulin, and C-peptide (Fig. 4C). The expression of C-peptide in these cells suggests that proinsulin was newly synthesized during the later stages of differentiation.

To investigate the functional status of these differentiated cells, we further analyzed glucose-dependent insulin release, and found that the insulin release in the high-glucose medium was nearly six times higher than that in the low-glucose medium. In the presence of 3 mM glucose, insulin content of differentiated cells was 229 ± 32.5 pg/mg protein per 30 min. However, in the presence of 22 mM glucose, differentiated cells secreted insulin at 1,373 ± 116 pg/mg protein per 30 min (Fig. 4D). These results suggested that the ES-derived pancreatic cells secreted insulin in a glucose-dependent manner, just as normal pancreatic β-cells do.

ES-DERIVED HEPATIC PROGENITOR CELLS COULD MATURE INTO HEPATOCYTES

To further induce maturation of hepatocytes from ES cells, EBs were treated with sodium butyrate for 7 days, then HGF for 7 days, and in the later stage Dex was added for another 7 days. After 7 days' induction of hepatic differentiation with sodium butyrate, a number of early hepatocyte markers, such as CK19, AFP, AAT, TTR were detected. Further induction with HGF and Dex enhanced the expression of ALB mRNA, which is an indicator of hepatocyte maturation. Interestingly, most of the final differentiated cells strongly expressed mature hepatic markers such as TAT, G6P, and CYP7A1, whereas the expression of AFP was barely detectable (Fig. 5A). After the treatment of sodium butyrate for 7 days, flow cytometric analysis results showed that the percentage of ALB positive cells was about 50% (Fig. 5B). To further analyze the properties of the final differentiated cells, protein expression of ALB was examined by immunofluorescence (Fig. 5C). Results indicated that ALB was observed in the cytoplasm of final differentiated hepatocytes.

Furthermore, PAS reaction was performed to evaluate the capability of glycogen storage in the differentiated hepatocytes. Glycogen storage is an important metabolic function of hepatocytes, which was manifested as the accumulation of magenta staining in the cytoplasm of cells. Our results demonstrated that at the final stage of differentiation, ES-derived hepatocytes had an accumulation of cytoplasmic deposits with a magenta color (Fig. 5D, right panel), as compared with that of spontaneously differentiated cells

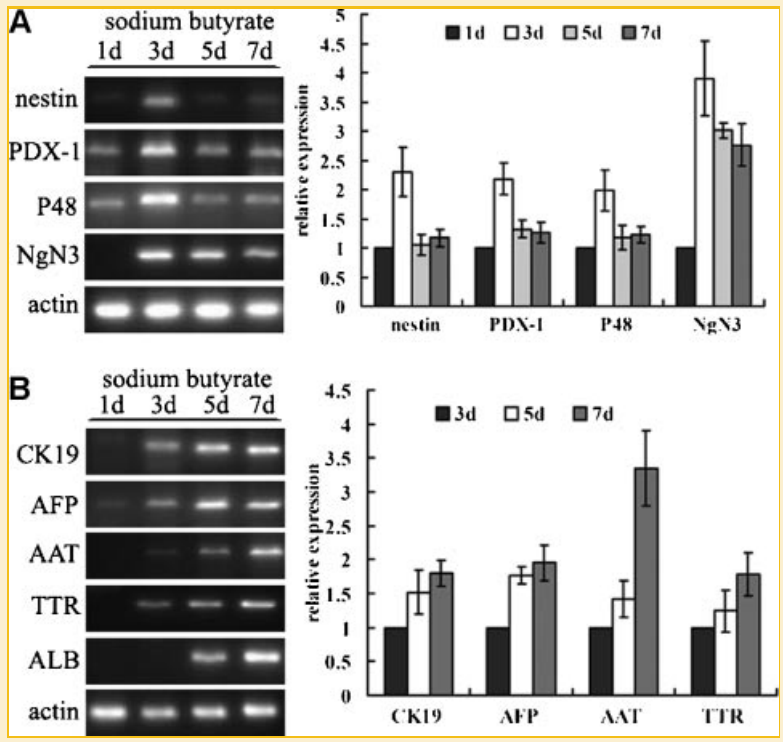

Fig. 3. Time course of pancreatic and hepatic gene expression after exposure to sodium butyrate. EBs were cultured in 1 mM (A) or 3 mM (B) sodium butyrate for 1 week, and at day 1, 3, 5, 7 of differentiation, RT-PCR was performed to analyze the time course of gene expression of pancreatic progenitor cells (A) and hepatic progenitor cells (B). In each experiment, a representative PCR result was shown. Values were given as the mean ± SD. Histogram plots represented the densitometric analysis corresponding to the mean ± SD of three independent experiments.

(Fig. 5D, left panel), indicating the hepatocyte-like cells have the ability of accumulating glycogen.

DISCUSSION

To date, many studies have reported the differentiation of ES cells toward pancreatic cells and hepatocytes. However, little is known about the generation and identification of pancreatic or hepatic progenitor cells from ES cells. During embryogenesis, the pancreas and liver derive from a common definitive endoderm, and share many similar developmental features. So here we studied together the generation and identification of pancreatic and hepatic progenitor cells from ES cells. Our research showed that sodium butyrate could prompt the differentiation of pancreatic and hepatic progenitor cells, and played different roles in pancreatic and hepatic lineage differentiation from ES cells.

Sodium butyrate, a known inhibitor of histone deacetylation [Perry et al., 1979], has been reported to induce the differentiation of hepatocytes from ES cells with a high yield [Rambhatla et al., 2003; Zhou et al., 2007]. Here we found that sodium butyrate also prompted the gene expression of early pancreatic markers. In a group of immature hormone-negative pancreatic cells, nestin was once identified. Cells expressing nestin are thought to represent progenitor cells of the pancreas [Hunziker and Stein, 2000; Zulewski et al., 2001]. NgN3 is expressed in endocrine progenitors and is critical for the development of all endocrine cells [Gu et al., 2002]. Our results showed that the gene expression of nestin and NgN3 was obviously upregulated by the treatment of sodium butyrate. PDX-1 and p48, two critical transcription factors involved in early stage of pancreatic development, were also activated even after 1-day treatment of sodium butyrate. On the other hand, considering the common embryonic origin of liver and pancreas, we also testified the effect of sodium butyrate on the differentiation of hepatic progenitor cells. AFP is a marker of the endodermal differentiation as well as an early fetal hepatic marker, and its expression decreases as the liver develops into adult phenotype [Hamazaki et al., 2001]. TTR and AAT represent endodermal or yolk-sac-like differentiation and are expressed throughout liver maturation [Meehan et al., 1984; Coakley et al., 2001]. Here we found AFP, AAT, and TTR were all induced by sodium butyrate, which was consistent with other studies [Rambhatla et al., 2003; Zhou et al., 2007]. However, the expression of ALB in hepatic progenitor cells remains controversial. ALB is the most abundant protein synthesized by mature hepatocytes. Its expression starts in early fetal hepatocytes and reaches the maximal level in adult hepatocytes [McKinnon and Docherty, 2001]. Some studies demonstrated that early hepatic cells expressed ALB [Hamazaki et al., 2001; Cai et al., 2007], while others reported ALB was not a specific marker of hepatic progenitor cells [Weiss and Strick-Marchand, 2003; Zhou et al., 2007]. Here in our study, we obtained a cell population expressing CK19, AFP, AAT, TTR, and ALB through the treatment of sodium butyrate, which indicates the generation of early hepatic progenitor cells from mouse ES cells. We considered the conflicting results may be due to the differences in cell lines and detailed culture protocol.

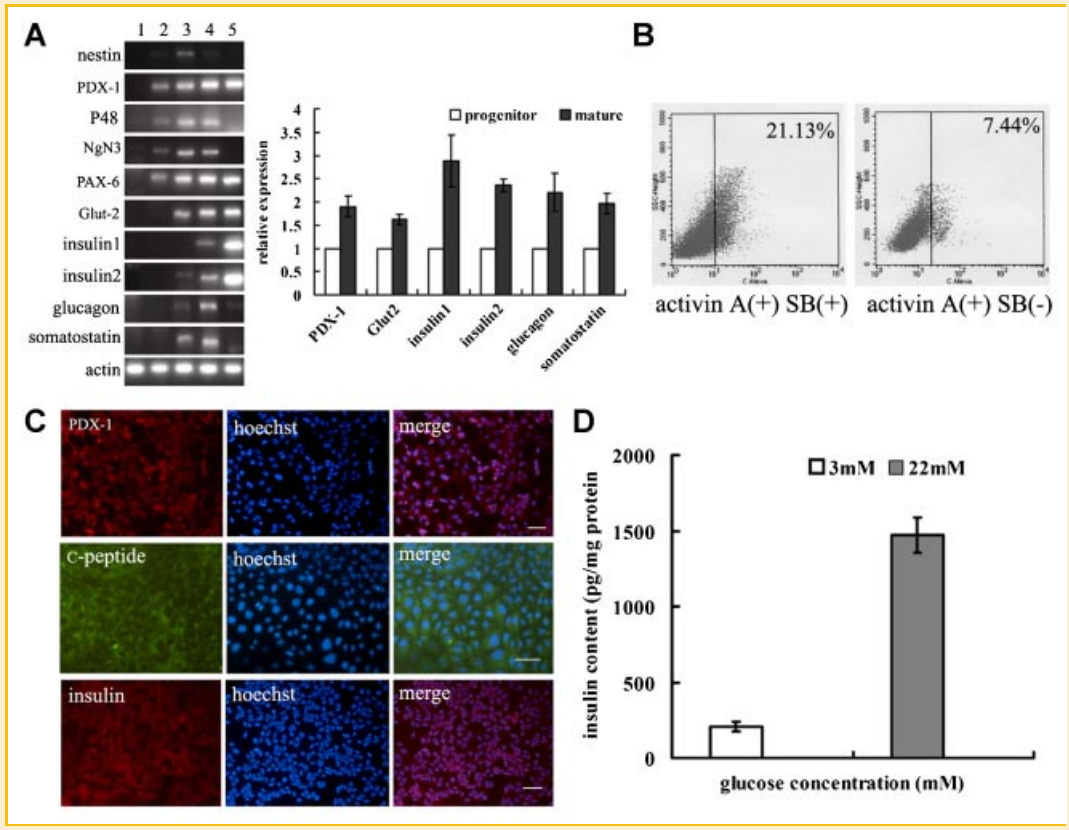

Fig. 4. Characterization of differentiated pancreatic cells by gene and protein expression analysis. A: RT-PCR and real-time PCR were performed to analyze the gene expression of pancreatic markers during the process of differentiation. Lane 1 represents undifferentiated ES cells. Lane 2 represents spontaneously differentiated EBs. Lane 3 represents cells treated with a combination of activin A and sodium butyrate for 5 days (25 ng/ml activin A for 2 days and then 1 mM sodium butyrate for 3 days). Lane 4 represents differentiated pancreatic cells at the final stage (the addition of 10 ng/ml bFGF and 10 ng/ml HGF for 7 days, and then 10 mM nicotinamide for another 7 days). NIT-1 insulinoma cells were used as positive control (lane 5). Histogram plots represented the real-time PCR results of three independent experiments. B: Cells were exposed to activin A for 2 days, then treated with (left panel) or without (right panel) sodium butyrate for another 3 days. Flow cytometric analysis was performed to detect the percentage of PDX-1 positive cells. C: At the final stage of differentiation, cells were immunostained with antibodies against PDX-1, insulin, and C-peptide. The blue color in the images depicts the nucleus stained by Hoechst 33258. Scale bar: 50 μm. D: To determine glucose-dependent release of insulin, cells were incubated with low-glucose (3 mM) or high glucose (22 mM) for 0.5 h. Cell supernatants were collected for insulin release analysis. The amount of insulin released in the supernatants was measured by enzyme-linked immunosorbent assay and normalized to protein content. Values were given as the mean ± SD.

Importantly, our experiments demonstrated that sodium butyrate had different effects on the differentiation of pancreatic and hepatic progenitor cells. The maximal gene expression of these two progenitor lineages relied on the concentration and exposure time of sodium butyrate. A relatively lower concentration of sodium butyrate (1 mM) with a shorter time treatment (3 days) induced the expression of pancreatic progenitor genes more efficiently. As for the differentiation of hepatic progenitor cells, treatment with 3 mM sodium butyrate for 7 days was more suitable. As one kind of histone deacetylase inhibitors, sodium butyrate could lead to chromatin rearrangements and terminal differentiation of many cell types through inhibition of histone deacetylation [Shimada et al., 2001; Kim et al., 2009]. In certain cell types, sodium butyrate has been shown to upregulate target genes for growth factors, signaling molecules, and transcription factors [McCue et al., 1984]. Although the exact mechanism by which sodium butyrate treatment resulted in different differentiation of pancreatic and hepatic progenitors is unclear, one possible reason maybe that sodium butyrate triggered epigenetic changes and influenced several key gene expressions for pancreatic and hepatic differentiation, thus led to the enrichment of these two different kinds of progenitor lineages. The molecular mechanism needs further investigation in future.

Previous studies have confirmed that cytokines involved in the mammalian organ development take part in the cell differentiation of ES cells [Jung et al., 1999; Jorgensen et al., 2007]. To verify the potential of pancreatic progenitor cells to differentiate into mature pancreatic cells, a combination of exogenous inducing factors was used in our differentiation protocol. With the consecutive induction by activin A, sodium butyrate, FGF, HGF, and nicotinamide, the differentiated ES cells sequentially expressed pancreatic specific gene, PDX-1, P48, NgN3, PAX-6, insulin, Glut-2, glucagon, and somatostatin. This suggests a progression similar to that of in vivo pancreas development, that is, the emergence of pancreatic progenitors formation followed by further differentiation to endocrine and exocrine pancreatic cells. Moreover, there exist two nonallelic insulin genes expressed in rodents, insulin1 and insulin2 [Deltour et al., 1993]. The insulin2 gene is expressed in the developing brain and yolk sac as well as in the pancreatic β-cells

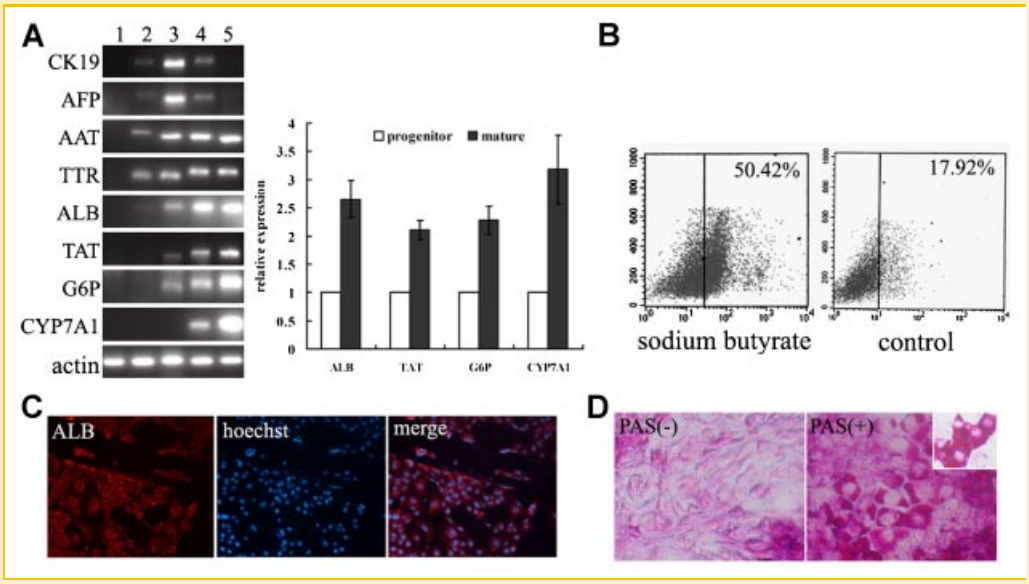

Fig. 5. Characterization of differentiated hepatocytes by gene and protein expression analysis. A: The gene expressions of hepatic markers were measured by RT-PCR and real-time PCR in each differentiation stage. Lane 1 represents undifferentiated ES cells. Lane 2 represents spontaneously differentiated EBs. Lane 3 represents cells treated with sodium butyrate for 7 days. Lane 4 represents differentiated hepatic cells at the final stage. Liver tissue of mouse was used as positive control (lane 5). Histogram plots represented the real-time PCR results of three independent experiments. B: After the treatment of sodium butyrate for 7 days, flow cytometric analysis was performed using an anti-ALB antibody with hepatic progenitor cells (left panel). Spontaneously differentiated cells (right panel) were used as control. C: Immunofluorescence was performed to analyze the protein expression of hepatocytes. Cells were immunostained with antibodies against ALB. The blue color in the images depicts the nucleus stained by Hoechst 33258. Scale bar: 50 μm. D: PAS reaction was performed to evaluate the capability of glycogen storage in the differentiated hepatocytes (right panel). Spontaneously differentiated cells were used as control (left panel).

[Giddings and Carnaghi, 1989; Slack, 1995], whereas insulin1 gene expression is restricted to the pancreatic β-cells [Deltour et al., 1993]. So the expression of insulin1 in the final stage of our protocol indicated a relative mature product. C-peptide, an important indicator to confirm derivation of insulin-producing cells from ES cells, was also clearly detected in differentiated cells in our cultures. It is known that the fundamental role of pancreatic β-cells is the secretion of insulin in response to glucose. The final differentiated ES cells in our protocol possessed the ability of insulin releasing in response to glucose stimulation in vitro, suggesting that some of the differentiated cells had acquired glucose sensing machinery and excitation properties.

It was generally believed that HGF and Dex were essential to the maturation of hepatocytes. HGF is critical to the signaling pathway that controls the proliferation of fetal liver cells [Schmidt et al., 1995]. During embryos development, the proliferation and outgrowth of the liver bud cells require the interaction of HGF [Shelly et al., 1989]. Meanwhile, glucocorticoids was reported to modulate the proliferation and function of adult hepatocytes [Kinoshita and Miyajima, 2002] and found to be involved in hepatic maturation. In the fetal liver, physiological concentrations of Dex, a synthetic glucocorticoid, suppress AFP production and DNA synthesis and upregulate ALB production [Machin et al., 2001]. In the present study, upon consecutive induction with HGF and Dex, the ES-derived hepatic progenitor cells induced by sodium butyrate differentiated into hepatocyte-like cells, expressing mature hepatice markers such as G6P, TAT, and CYP7A1 at the final stage. Functional tests demonstrated the resulting differentiated cells had the capability of glycogen storage. Taken together, the positive expression of ALB, G6P, TAT, and CYP7A1, and the ability to accumulate glycogen suggest that a relatively more mature population of hepatocyte-like cells were isolated using the current culture protocol.

In summary, we have demonstrated that sodium butyrate played different roles in the differentiation of both hepatic and pancreatic progenitor cells from ES cells. These progenitor cells have the potential to be induced into maturation. Investigation of the differentiation of these progenitor cells may not only provide a potential transplantable cell source, but also facilitate the understanding of pancreatic and hepatic lineage differentiation and development. Exact understanding of the developmental processes that lead to a specific cell fate might help us to recapitulate the events in vitro, and engineer artificial pancreatic cells and heaptocytes to cure diabetes and liver diseases.

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