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Evidence for Hepatocyte Differentiation From Embryonic Stem Cells In Vitro

Hitoshi Miyashita,* Atsushi Suzuki,* Katashi Fukao,* Hiromitsu Nakauchi,† and Hideki Taniguchi*

*Department of Surgery, Institute of Clinical Medicine, and †Department of Immunology, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Ten-nodai, Tsukuba, Ibaraki 305-8575, Japan

We confirmed hepatocyte differentiation from embryonic stem (ES) cells in vitro. RT-PCR analysis revealed that a broad range of hepatic gene expression was observed in ES cells differentiated through formation of embryoid bodies (EBs) and its attachment culture. Quantitative PCR analysis revealed that hepatic gene expression related to early and late-stage liver development were enhanced through in vitro differentiation of ES cells. The presence of albumin-producing cells in the peripheral region of attached EBs was confirmed by immunocytochemical analysis. Future experiments will reveal the molecules that induce hepatocyte differentiation from ES cells in vitro. This research will provide systems for the investigation of mechanisms in liver development and establish a method of ES cell-based therapy for liver diseases.

Key words: Embryonic stem cell; Embryoid bodies; Hepatocyte; Definitive endoderm

INTRODUCTION

Embryonic stem (ES) cells derived from blastocysts have the potential to differentiate into many types of cells in vivo through production of chimera animals or carcinomas. ES cells are capable of being maintained in an undifferentiated state in vitro in the presence of leukemia inhibitory factor (LIF). In the absence of LIF, ES cells can differentiate into many types of cells by the formation of embryoid bodies (EB) or induction of differentiation agents. These in vitro ES cell differentiations were used as models for early development in the past two decades. ES cells were recently established in several organisms including humans, and many studies were done on the establishment of ES cell-based transplantation. These studies revealed that differentiation of ectoderm or mesoderm derivatives is highly inducible from ES cells in vitro. For example, differentiation of ES cell-derived neurons was efficiently induced by cytokines (2) or stromal cell-derived factors (3). These cells were capable of surviving and differentiating after transplantation (2,3,5). Moreover, these cells also enhance the recovery of damaged neural function in host animals after transplantation (5). Mesoderm derivatives (e.g., cardiomyocytes) could differentiate from ES cells at relatively high efficiency in vitro, and these cells were capable of surviving and cooperating with other cells in the host heart after transplantation (4). In contrast to ectoderm or mesoderm derivatives, differentiation of endoderm derivatives from ES cells in vitro is less frequently reported. Classically, endoderm derivatives from ES cells have been considered to be visceral endoderm (1) that appear in the extraembryonic region in early development and have similar characteristics to hepatocytes in functions and gene expressions. But recently it was reported that insulin-secreting cells similar to pancreatic beta cells could differentiate from ES cells in vitro (6), suggesting that gut-derived endoderm could differentiate from ES cells in vitro.

Recent studies have made much progress in understanding the mechanisms of development of gut-derived organs including the liver. In liver development, for example, signals from the nascent heart play an important role in acquiring liver-specific gene expression in gut endoderm. Molecular understanding of early development of cells in gut endoderm could be applied to induce in vitro ES cell differentiation to gut-derived endoderm cells. Here we report the evidence for spontaneous hepatocyte differentiation from ES cell in vitro by PCR and immunocytochemical analysis. This article provides a system for establishment of a more efficient method to induce hepatocyte differentiation from ES cells in vitro.

MATERIALS AND METHODS

ES Cell Cultures

An ES cell line TT2 was cultured on mitomycin-treated embryonic fibroblast feeder cells. The culture medium was Dulbecco's modified Eagle's medium

Address correspondence to Hideki Taniguchi, M.D., Ph.D., Department of Surgery, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan. Tel: 81-298-53-3221; Fax: 81-298-53-3222; E-mail: rtanigu@md.tsukuba.ac.jp

(DMEM) containing 15% fetal calf serum (FCS) (Hyclone), nonessential amino acid (ICN Biomedicals, Inc.), and 0.1 mM 2-mercaptoethanol (Sigma), supplemented with 1000 U/ml leukemia inhibitory factor (LIF) (Chemicon International, Inc.).

In Vitro ES Cell Differentiation

To induce differentiation, 6×10^6 dissociated ES cells were plated on gelatinized tissue culture dishes and incubated for 3 days (1 day with LIF and 2 days without LIF). Dissociated cells (5×10^6) were then plated in 60-mm petri dishes to form aggregates called embryoid bodies (EBs). The culture medium used in the differentiation of ES cells was DMEM containing 10% FCS (Hyclone). Days in suspension were numbered after the first day of aggregation. To induce attachment culture, EBs were plated onto cell culture dishes and allowed to attach after 3 days of suspension. Days in attachment were numbered after the initiation of attachment. The culture medium was changed every third day.

RT-PCR Analysis

Total RNA extraction and cDNA synthesis were performed following the protocol previously reported (7). RT-PCR analysis was performed following the protocol previously reported (7). Forward and reverse primer sequences from 5′ to 3′ direction were as follows: hepatocyte nuclear factor (HNF)-3α (5′-GTC GCA AGG ACC CCT CAG G-3′ and 5′-CTT GAA GTC CAG CTT GTG CTG-3′), HNF-3β (5′-CTT CTC CGT GTC AGG AGC AC-3′ and 5′-CTG GGT AGT GCA TGA CCT G-3′), HNF-3γ (5′-TCT GCC ACC ACT ACA GCT GC-3′ and 5′-CGC TGC TAG GAT GCT GCA TTA AGC-3′), HNF-4 (5′-CTT CCA AGA GCT GCA GAT TG-3′ and 5′-CTT GTA GGA TTC AGA TCC CG-3′), transthyretin (TTR) (5′-TGG TAT TTG TGT CTG AAG CTG-3′ and 5′-TTA ATA AGA ATG CTT CAC GGC-3′), asialoglycoprotein receptor major subunit (Asgr1) (5′-ACA ATG ATA ATG ACC ATC ATC-3′ and 5′-TAC TTG TCA GCT TCA GTC CAA-3′), glutathione *S*-transferase (GST) (5′-AAG TGA TGG GAG TCT GAT GTT-3′

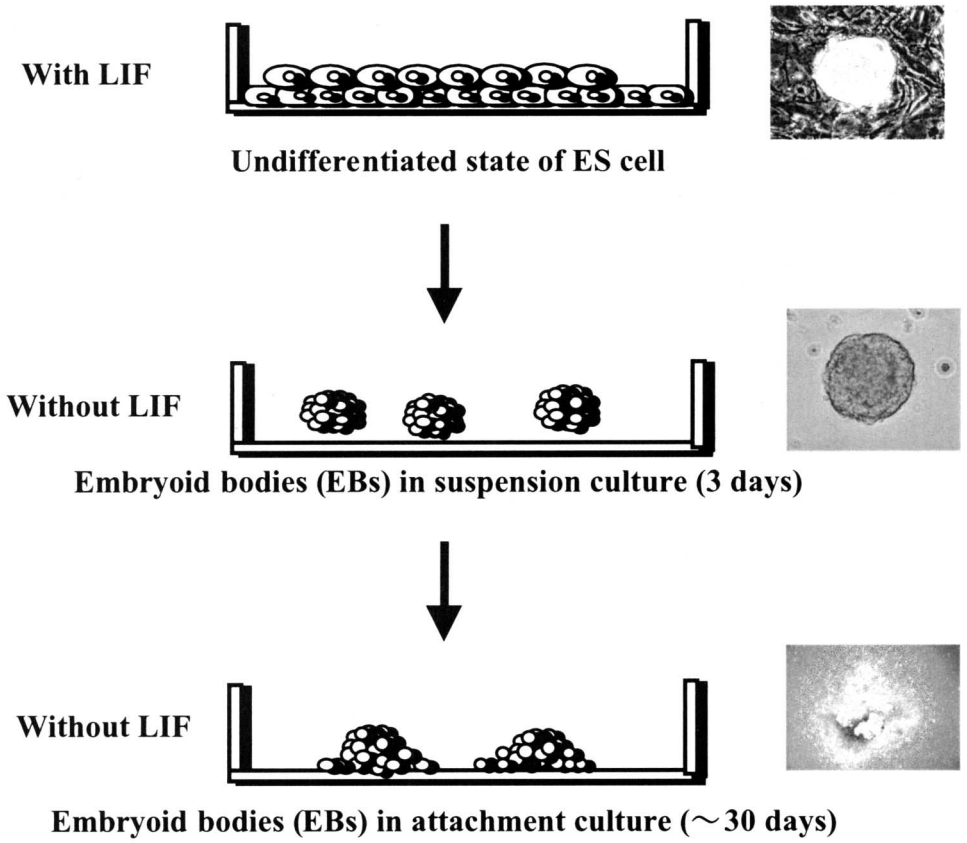

Figure 1. Schematic representation of experimental procedure. Maintenance of undifferentiated state of ES cells was done in the presence of a feeder layer and LIF. To induce differentiation, ES cells were allowed to form EBs in the suspension culture. Then EBs were allowed to attach to dishes after 3 days in suspension.

and 5′-TTC TTT GCT GAC TCA ACA CAT-3′), albumin (ALB) (5′-CAT GAC ACC ATG CCT GCT GAT-3′ and 5′-CTC TGA TCT TCA GGA AGT GTA C-3′), alpha-fetoprotein (AFP) (5′-ACT CAC CCC AAC CTT CCT GTC-3′ and 5′-CAG CAG TGG CTG ATA CCA GAG-3′), alpha-1-antitrypsin (αAT) (5′-TCG ATC CTA AGC ACA CTG AGG-3′ and 5′-CGG CTT GTA AGA CTG TAG C-3′), glucose-6-phosphatase (G6P) (5′-AAC CCA TTG TGA GGC CAG AGG-3′ and 5′-TAC TCA TTA CAC TAG TTG GTC-3′), hypoxanthine phosphoribosyltransferase (HPRT) as a positive control (5′-CTG TAA TGA TCA GTC AAC GGC-3′ and 5′-GGC CTA TAG GCT CAT AGT GCA-3′).

PCR cycles were as follows: initial denaturation at 95°C for 4 min, followed by 60 cycles at 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were separated in 1.7% agarose gel.

Production of RNA Standard in Quantitative PCR Analysis

We isolated total RNA from neonatal liver following the protocol previously described (7). Isolated RNA was diluted and used as a standard for all primers in quantitative PCR.

Sequence of Primer and TaqMan Probe Used in Quantitative PCR Analysis

Sequences from 5′ to 3′ direction of forward and reverse primers used in quantitative PCR analysis were as follows: AFP (5′-CCT GTC AAC TCT GGT ATC AGC CA-3′ and 5′-CTC AGA AAACTG GTG ATG CAT AGC-3′), ALB (5′-TGT CCC CAA AGA GTT TAA AGC TG-3′ and 5′-TCT TAA TCT GCT TCT CCT TCT CTG G-3′), αAT (5′- TCG GAG GCT GAC ATC CAC AA-3′ and 5′-TCA ACT GCA GCT CAC TGT CTG G-3′), G6P (5′-GTT CAA CCT CGT CTT CAA GTG GAT-3′ and 5′-TGC TGT AGT AGT CGG T GT CCA GGA-3′). Sequences from 5′ to 3′ direction of TaqMan probes used in this experiment were as follows: AFP (5′-TGC TGC AAC TCT TCG TAT TCC AAC AGG A-3′), ALB (5′-ACC TTC ACC TTC CAC TCT GAT ATC TGC ACA CT-3′), αAT (5′-TTC CAA CAC CTC CTC CAA ACC CTC AA-3′), G6P (5′-TTT GGA CAA CGC CCG TAT TGG TGG-3′). All TaqMan probes used in this experiment carried a 5′ FAM reporter dye. The PCR primers and the TaqMan probes used in quantitative PCR were designed with the Primer Express software program (Applied Biosystems Version 1.0).

Quantitative PCR Conditions

The reaction mixture (25 μl final volume) contained 500 ng total RNA, 5× TaqMan EZ buffer, 3 mM $Mn(OAc)_2$, 12.5 units rTth DNA polymerase, 300 μM dATP, dCTP, dGTP, dUTP, 200 nM forward and reverse primers, and 100 nM probe. Reverse transcription was performed at 60°C for 30 min. The activation, 5 min at 95°C, of the rTth DNA polymerase was followed by 60 cycles of 15 s at 95°C and 60 s at 60°C. A nontemplate control was included in each primer. All of the nontemplate controls, the standard RNA dilutions, and the EBs samples were assayed in triplicate.

Analysis and Expression of the Real-Time RT-PCR Data

The quantification of the starting quantity of a specific mRNA in an EBs sample was performed by preparing a standard curve with known dilutions of standard RNA from neonatal livers. For each dilution, the Abi-Prism 7700 software (Applied Biosystems) generated a real-time amplification curve constructed by relating the fluorescence signal intensity (ΔRn) to the cycle number. The ΔRn value corresponded to the variation in the reporter fluorescence intensity before and after PCR, normalized to the fluorescence of an internal passive reference present in the buffer solution. The standard curve was then generated on the basis of the linear relationship existing between the Ct value (cycle threshold; corresponding to the cycle number at which a significant increase in the fluorescence signal was first detected) and the logarithm of the starting quantity. The starting quantity of mRNA in EBs was quantified by blotting the Ct in this standard curve.

Immunocytochemical Analysis

Day 24 EBs in the attachment culture were washed three times with PBS, fixed by methanol at −20°C for 10 min, and washed in PBS including 0.05% polyoxyethylene[20] sorbitan mono-laurate (Tween 20) (Wako). Nonspecific binding was blocked with 10% nonimmune serum of a species from which the secondary antibody had been obtained. Fixed EBs were incubated with primary antibody rabbit anti-albumin (Biogenesis, Poole, UK) in a moist chamber for 16 h at 4°C. After washing in PBS-Tween 20 and blocking, the cells were incubated with Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 3 h at 4°C. After final washing, the cells were viewed under a Zeiss LSM510 laser scanning microscope.

RESULTS

When ES cells were cultured in the absence of a feeder layer and LIF, they formed EBs (Fig. 1). Then they were allowed to attach to dishes after 3 days in suspension (Fig. 1). Total RNA was extracted from whole cells and RT-PCR analysis was performed. Around day 10 of differentiation, hepatic gene expression was initiated in EBs. On day 21 of the attachment

culture, a broad range of hepatic gene expression was observed in EBs but it was not observed in undifferentiated ES cells (Fig. 2A). Transcription factors involved in early hepatocyte differentiation such as HNF and TTR, and serum proteins such as AFP, ALB, and αAT were expressed in this culture as Abe et al. reported (1). Moreover, mature hepatocyte markers such as G6P, GST, and Asgr1 were also expressed in our culture. G6P expression was observed in liver from the perinatal period and its proteins play a role in gluconeogenesis. GST catalyzes the conjugation of reduced glutathione to a multitude of electrophilic substrates and endogenous and exogenous toxins and binds passively to hydrophobic ligands. Asgrs play a role in the physiological function in mature hepatocytes such as the removal of desialylated serum glycoproteins and apoptotic cells, and clearance of lipoproteins. These gene expressions were related to the functions of mature hepatocytes in vivo. Quantitative PCR analysis revealed that gene expressions of serum proteins such as AFP and ALB were enhanced through in vitro differentiation of ES cells (Fig. 2B). These gene expressions were quantified by comparison to expression in neonatal livers. AFP and ALB were first expressed in liver during development and these gene expressions were also observed in visceral endoderm. In addition to these early hepatocyte marker genes, mature hepatocyte marker genes such as αAT and G6P were expressed, and this expression was also enhanced through in vitro differentiation of ES cells (Fig. 2B). These gene expressions are involved in the functions of mature hepatocytes and G6P expression is greatly restricted in hepatocytes. All gene expressions examined in this experiment reached a peak at day 21 of attachment and were reduced after that day. Immunocytochemical analysis revealed the presence of albumin-producing cells in EBs at day 24 of attachment culture

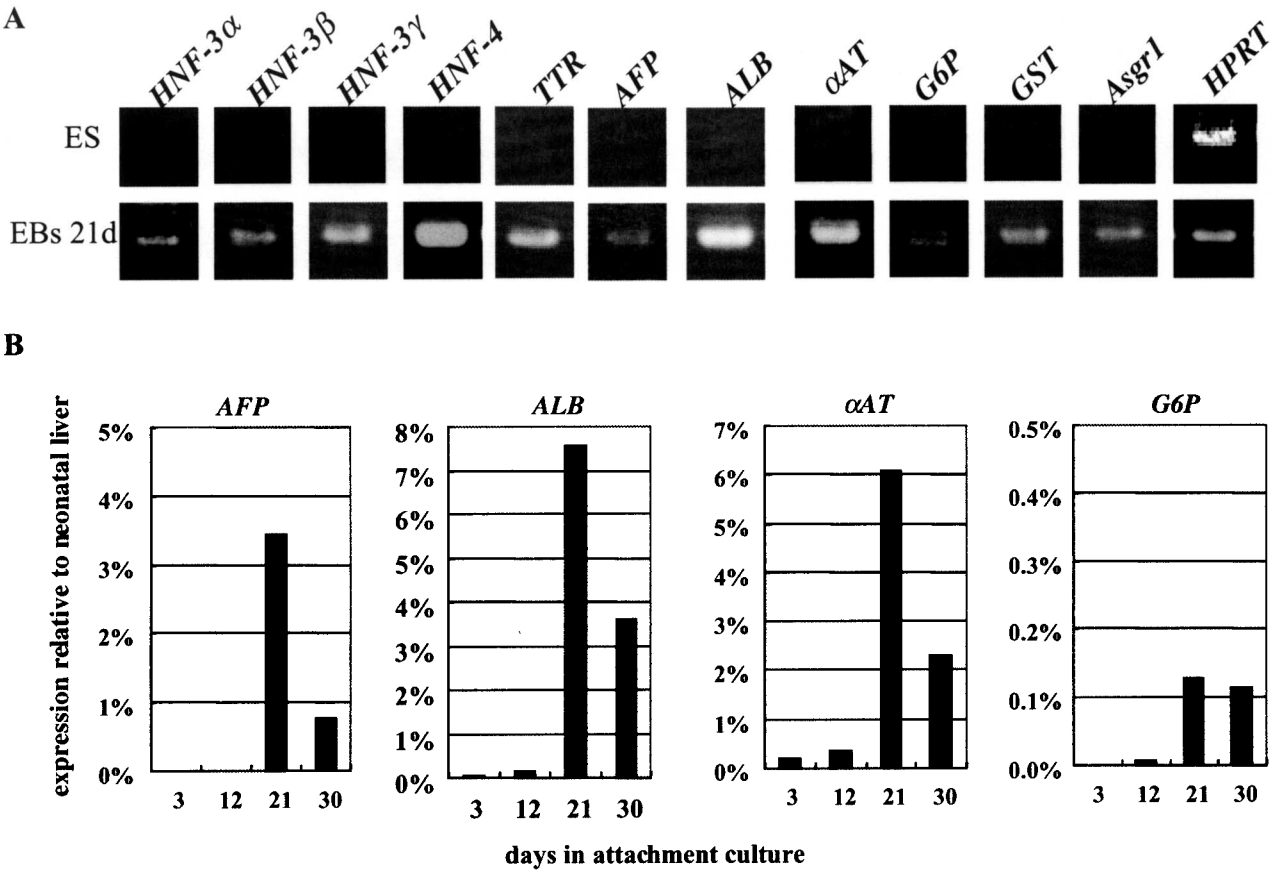

Figure 2. (A) RT-PCR analysis of endodermal or hepatic gene expression in EBs. RNA was extracted from ES cells and EBs at day 21 of attachment and RT-PCR analysis was performed. A broad range of gene expressions related to endodermal/hepatic development in vivo was observed in EBs but not in ES cells. (B) Quantitative PCR analysis was performed in EBs at days 3, 12, 21, and 30 of attachment. Hepatic gene expression in EBs was quantified by comparison with expression in neonatal livers. Hepatic genes including mature hepatocyte markers were expressed and enhanced through in vitro differentiation. All gene expression examined in this experiment reached a peak at day 21 of the attachment culture.

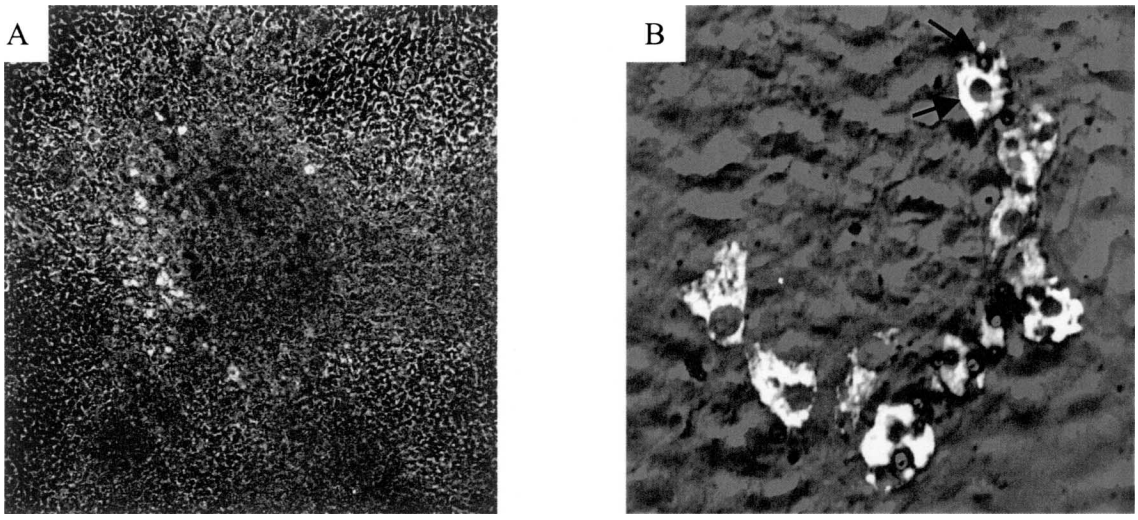

Figure 3. Immunocytochemical analysis of albumin expression in EBs at day 24 of the attachment. (A) Lower magnification of EBs. Albumin-positive cells existed in the peripheral region of EBs. (B) Higher magnification of EBs. Some albumin-positive cells had binuclear morphology characteristic of mature hepatocytes (arrows).

(Fig. 3A). These albumin-positive cells existed in the peripheral region of EBs. Among these cells, there were binuclear cells that are characteristic of mature hepatocytes (Fig. 3B).

DISCUSSION

Previous reports suggested that endoderm-specific gene expression was derived from visceral endoderm in EBs (1), but in our experiments, in addition to early endoderm or hepatocyte markers, mature hepatocyte makers were expressed. This suggests the differentiation of functional mature hepatocytes from ES cells through the formation of EBs and its attachment culture. Moreover, quantitative PCR analysis revealed that hepatic gene expressions, including that of mature hepatocytes, were enhanced through in vitro differentiation. Although G6P expression was enhanced at relatively lower efficiency than early marker genes, the expression of G6P suggests that mature hepatocytes could differentiate from ES cells in a late stage of in vitro differentiation. Immunocytochemical analysis revealed that albumin-producing cells, including binuclear cells, were present in the peripheral region of attached EBs. This shows that the ES cell-derived hepatocytes could synthesize serum proteins, but so far we cannot induce the hepatocyte differentiation and maturation from ES cells in vitro. The efficiency of spontaneous differentiation of hepatocytes from ES cells was very low. This makes it difficult to determine the functions of these cells, such as their albumin-secreting ability and ammonium metabolism. Further experiments are needed to identify the factor(s) inducing hepatocyte differentiation from ES cells. Specific induction and enrichment of ES cell-derived hepatocytes help to determine the functions of these cells in vitro and in vivo.

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