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Characterization of genetically engineered mouse hepatoma cells with inducible liver functions by overexpression of liver-enriched transcription factors

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New cell sources for the research and therapy of organ failure could significantly alleviate the shortage of donor livers that are available to patients who suffer from liver disease. Liver carcinoma derived cells, or hepatoma cells, are the ideal cells for developing bioartificial liver systems. Such cancerous liver cells are easy to prepare in large quantities and can be maintained over long periods under standard culture conditions, unlike primary hepatocytes. However, hepatoma cells possess only a fraction of the functions of primary hepatocytes. In a previous study, by transducing cells with liver-enriched transcription factors that could be inducibly overexpressed—hepatocyte nuclear factor (HNF)1α, HNF1β, HNF3β [FOXA2], HNF4α, HNF6, CCAAT/enhancer binding protein (C/EBP)α, C/EBPβ and C/EBPγ—we created mouse hepatoma cells with high liver-specific gene expression called the Hepa/8F5 cell line. In the present study, we performed functional and genetic analyses to characterize the Hepa/8F5 cell line. Further, in three-dimensional cultures, the function of these cells improved significantly compared to parental cells. Ultimately, these cells might become a new resource that can be used in basic and applied hepatic research.

[**Key words:** Hepatoma; Gene expression system; Liver function; Three-dimensional culture; Liver-enriched transcription factor]

The liver is a vital multifunctional organ. In cases in which liver function is compromised, often the only treatment is transplantation of a donor liver. However, the severe shortage of donor organs complicates the treatment of liver failure, prompting interest in alternative approaches to transplantation. One such option for liver failure is the application of extracorporeal support devices, such as a bioartificial liver device. Bioartificial devices usually contain a bioreactor that is embedded with isolated cells, providing support and essential substances to the patient. Therefore, the type of cell that is incorporated is an important and actively researched subject (1).

Parenchymal hepatocytes, which constitute 70%–80% of the liver, are responsible for most hepatic functions (2). However, primary hepatocytes are difficult to culture *in vitro* and lose their functions rapidly under normal culture conditions. Thus, many recent studies have attempted to generate functional hepatocytes *in vitro* from cells that can proliferate and differentiate under certain culture conditions—for example, differentiation from embryonic stem (ES) cells and bone marrow cells that have been converted into hepatic cells by controlling the culture environment, such as the culture medium (2,3). Further, several methods that promote hepatic differentiation from ES or induced pluripotent stem (iPS) cells by transduction with transcription factor genes have been reported (4–8). Even fibroblasts have been converted into hepatocyte-like cells for research (9,10). However, culturing to differentiate such cells is an expensive and time-consuming endeavor.

Hepatoma cells are often considered a source of functional hepatic cells. For example, methods that improve ammonia metabolism in hepatoma cells by transduction of the gene encoding glutamine synthetase have been reported (11). There have also been established hepatoma cells lines, such as HepG2, C3A and HepaRG, which are considered alternative hepatic cell sources in bioartificial livers (12,13). Methods that can enhance the function of these cells more efficiently are needed, because most activities are lost in hepatoma cells.

Thus, in a previous study (14), we developed genetically engineered Hepa/8F5 hepatoma cells (available from RIKEN BioResource Center [RCB4661]), with high inducibility of typical liver functions, by overexpressing liver-enriched transcription factors (LETFs) that are connected to the transcription of genes for liver-specific functions. The genes of eight LETFs—hepatocyte nuclear factor (HNF)-1α, HNF-1β, HNF-3β [FOXA2], HNF-4α, HNF-6, CCAAT/enhancer binding protein (C/EBP)-α, C/EBP-β and C/EBP-γ—were transduced into Hepa/8F5 cells as drug-inducible expression cassettes. In this study, we further characterized Hepa/8F5 cells with regard to their functions and gene expression profiles before and after induction, using two-dimensional monolayer and three-dimensional spheroid cultures.

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MATERIALS AND METHODS

Cell culture Hepatoma (Hepa1-6 and Hepa/8F5) cells were cultured in 10 mL high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich, St. Louis, MO, USA), added with 10% fetal bovine serum (FBS), 100 U/mL penicillin G potassium and 0.1 mg/mL streptomycin sulfate (Wako Pure Chemical Industries, Osaka, Japan) in 100 mm cell culture dishes (Thermo Fisher Scientific, Rochester, NY, USA). Cells were incubated at 37°C in a 5% (v/v) CO_2 incubator. All cells used for analyses were passaged within 6–8 times from the original Hepa/8F5 stock.

Immunofluorescence staining Hepatoma cells were seeded 6-well plates (Thermo Fisher Scientific) at a cellular density of 2.0×10^5 cells/well with 3 mL-medium/well in the absence or presence of 0.1 μg/mL doxycycline (Dox; Sigma–Aldrich). On Day 5, the cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with phosphate-buffered saline (PBS) containing 0.2% Triton X-100 for 15 min. After being blocked with PBS containing 1% bovine serum albumin, the samples were incubated with goat anti-mouse albumin (Bethyl, Montgomery, TX, USA), followed by incubation with rabbit anti-goat immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA, USA) that was conjugated to rhodamine. 4′,6-diamidino-2-phenylindole (DAPI; Roche Applied Science, Mannheim, Germany) was used for nuclear counterstaining. After washing the cells three times with PBS, they were observed under a BZ-9000 fluorescence microscope (Keyence, Tokyo, Japan).

Periodic acid-Schiff (PAS) staining Hepatoma cells were seeded at a density of 2.0×10^5 cells/well in 6-well plates with 3 mL-medium/well with or without Dox (0.1 μg/mL). On Day 5, cells were washed with 3 mL-PBS/well and fixed in 4% paraformaldehyde for 15 min. Samples were then oxidized in 1% periodic acid (Merck, Darmstadt, Germany) for 5 min, rinsed three times with 1 mL pure water and treated with Schiff's reagent (Wako Pure Chemical Industries) for 15 min. After washing three times with PBS, the cells were observed under a microscope.

Real-time RT-PCR analyses On Day 5, total RNA was extracted from the cells using a commercially available kit (RNAiso plus; Takara, Otsu, Japan) following to the manufacturer's protocol. Isolated RNA was treated with DNase I (Takara) to remove contaminated genomic DNA and reverse-transcribed with an oligo-dT primer using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). For determining the mRNA expression levels, real-time PCR was performed using a commercially available real-time PCR reagent (Thunderbird SYBR qPCR Mix; Toyobo) at 95°C for 30 s, followed by 40–45 cycles of amplification at 95°C for 5 s and 60°C for 30 s. To measure expression of liver-specific genes, specific primers for albumin (ALB), transthyretin (TTR), α1-antitrypsin (AAT), tyrosine aminotransferase (TAT), glucose-6-phosphatase (G6P), carbamoyl phosphate synthetase 1 (Cps1), apolipoprotein A-I (ApoAI), apolipoprotein B (ApoB), asialoglycoprotein receptor 1 (ASGPR1), peroxisome proliferator-activated receptor α (PPARα), GATA-binding protein 4 (GATA4) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used. All used primer sequences for ALB, TTR, TAT, G6P and GAPDH have been described in a previous study (14), and those for AAT, Cps1, ApoAI, ApoB, ASGPR1, PPARα and GATA4 are listed in Table S1. To measure the expression of LETF genes, specific primers for HNF-1α, HNF-1β, HNF-3β, HNF-4α, HNF-6, CEBP-α, CEBP-β and CEBP-γ were used; these primer sequences are listed in Table S2. For analysis of expression of exogenous LETF genes, specific primers described in a previous study (14) were used. These mRNA expression levels were normalized to GAPDH. Amplification from contaminated genomic DNA was not detected by PCR using non-reverse-transcribed samples and primers for GAPDH.

Formation of spheroids For the formation of uniformly sized spheroids, hepatoma cells were seeded into 96-well low-attachment tissue culture plates (PrimeSurface; Sumitomo Bakelite, Tokyo, Japan) at a density of 250 cells/well and statically cultured for 4 days in 100 μL-medium/well with or without Dox (0.1 μg/mL). About 25 spheroids were then transferred into 24-well low-attachment tissue culture plates (Corning, NY, USA) and cultured for 1 additional day in 1 mL-medium/well. Formed spheroids were observed, and diameters were measured on Day 5. The diameter of the spheroid was calculated as the average between the minor and major axes of 10 randomly selected spheroids. For observation of cells in spheroids, spheroids were washed thrice with 1 mL PBS and subsequently incubated in PBS containing 0.5 μM propidium iodide (PI; Dojindo, Kumamoto, Japan) at 37°C for 15 min. After three washes with PBS, spheroids were observed under a BZ-9000 fluorescence microscope (Keyence). Live cells express green fluorescence derived from transgene expression, and dead cells express red fluorescence by PI-staining.

Liver function analyses Hepatoma cells were cultured in 1 mL/well medium with or without Dox (0.1 μg/mL) at 2.0×10^4 cells/well in 24-well plates for monolayer culture or at 25 spheroids/well in 24-well low-attachment tissue culture plates for spheroid culture. The medium was changed every other day. Culture medium was collected on Day 5 in 1.5 mL microtubes. For ammonia removal and urea synthesis assays, the medium was changed to fresh medium containing 2 mM NH_4Cl (Wako Pure Chemical Industries) on Day 5 and the cells were incubated for a further 6 h. The culture medium was then collected. The medium samples were stored at −80°C and thawed for analysis of liver functions (14). The albumin concentration was measured by ELISA using a commercially available kit (Mouse Albumin ELISA Quantitation Set; Bethyl). The ammonia and urea concentrations in the medium were determined using kits (Ammonia-Test Wako and Urea Nitrogen-Test, respectively; Wako Pure Chemical Industries) according to the manufacturer's protocols. Cytochrome P450 activity (CYP3A) was measured using a luminescence-based assay kit (P450-Glo, CYP3A4 assay with luciferin-IPA; Promega, Madison, WI, USA). These functions were normalized to the number of cells, calculated based on DNA content. Amounts of DNA were measured in lysate of sonicated cells using a commercially available DNA measurement kit (QuantiFluor dsDNA system; Promega).

Microarray gene expression analyses Hepatoma cells were cultured in 1 mL medium with or without Dox (0.1 μg/mL) at 2.0×10^4 cells/well in 24-well plates for monolayer culture or at 25 spheroids/well in 24-well low-attachment culture plates for spheroid culture. The medium was changed every other day. On Day 5, cell-spheroids or monolayer cells were harvested, and RNA was extracted from the cells using a commercially available kit (RNeasy Mini Kit; Qiagen, Hilden, Germany) and treated with DNase I (Qiagen) to remove contaminating genomic DNA. DNA microarray analyses were performed for genes in the entire mouse genome using a commercially available DNA chip (1 color 4 × 44K; Agilent Technologies, Santa Clara, CA, USA). Microarray data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5770. The data were analyzed using GeneSpring software (TOMY Digital Biology, Tokyo, Japan).

Statistical analysis Statistical comparisons were evaluated using the student's *t*-test, and the values of $P < 0.05$ were considered significantly different.

RESULTS

Liver functions in Hepa/8F5 cells The expression of eight exogenous LETF genes in Hepa/8F5 cells was induced by Dox addition to the medium, based on transgene transduction using a tetracycline-inducible gene expression system (14). The effective concentration of Dox for the induction of Hepa/8F5 function was 0.1 μg/mL (Fig. S1). After 5 days of culture of Hepa/8F5 cells with or without Dox, albumin expression was recorded as a measure of liver function by immunocytochemistry (Fig. 1A). Albumin expression was low in parental Hepa1-6 cells and Hepa/8F5 cells that were cultured in medium without Dox. In contrast, strong fluorescence was observed in Hepa/8F5 cells that were cultured with Dox. This result shows that albumin expression was enhanced by the overexpression of LETFs via the addition of Dox.

Glycogen storage, which is a typical liver-specific function, was also measured (Fig. 1B). By PAS staining, a robust signal was seen in Hepa/8F5 cells that were cultured with Dox for 5 days compared with Hepa1-6 and Hepa/8F5 cell cultures that lacked Dox. Based on this result, glycogen storage was enhanced in Hepa/8F5 cells by Dox.

For a more detailed analysis, other liver functions in Hepa/8F5 cells were investigated by quantitative RT-PCR (Fig. 1C). After 5 days of culture, the expression of liver-enriched genes, such as ALB, TTR, AAT, TAT, G6P, Cps1, ApoAI, ApoB, ASGPR1, PPARα and GATA4, were evaluated. By real-time RT-PCR, the expression of all 11 genes was high in Hepa/8F5 cells that were cultured with Dox versus Hepa1-6 and Hepa/8F5 cells without Dox. Specifically, although AAT, G6P, Cps1 and ASGPR1 were undetectable in Hepa1-6 and normal Hepa/8F5 cells, their expressions were confirmed in Hepa/8F5 cells that were cultured with Dox. These results show that various liver functions that are closely related to these liver-enriched genes are activated in Hepa/8F5 cells by overexpression of LETFs through the addition of Dox.

Expression of LETF genes in Hepa/8F5 cells To examine the expression profile of LETF genes in Hepa/8F5 cells, expression levels of eight transduced genes was measured by quantitative RT-PCR. First, the total levels of endogenous and exogenous genes were measured in Hepa1-6 and Hepa/8F5 cells on Day 5 of the culture without or with Dox (Fig. 2A). Although expression leaks were observed in Dox-free cultures, all eight LETF genes were significantly induced to various levels in Hepa/8F5 cells after the addition of Dox.

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