miR-214 expression in a mouse model of liver fibrosis

Liver fibrosis was induced by feeding mice a MCDD for 5 or 15 weeks and then compared with mice fed a methionine- and choline-control diet (MCCD). Sirius red staining and α-SMA immunostaining confirmed the time-dependent induction of fibrosis in the liver of MCDD-fed mice, especially around the central vein area (Figure 2A). The mRNAs of liver fibrosis factors, such as α-SMA, Col1a1, platelet-derived growth factor receptor (PDGFR)-β, TGF-β1, fibronectin (FN) 1, discoidin domain receptor (DDR) 2, and β1 integrin (ITGB1), were upregulated in the livers of MCDD-fed mice compared to MCCD-fed mice (Figure 2B). miR-214-5p expression was significantly higher in the livers of MCDD-fed mice than in control mice (2.1-fold, $P < 0.01$ at 5 weeks; and 4.8-fold, $P < 0.01$ at 15 weeks) (Figure 2C).

miR-214 expression in a rat resolution model of liver fibrosis

We previously demonstrated the resolution of liver fibrosis with steatohepatitis in a rat model induced by giving MCDD; that is, rats received either MCCD for 10 weeks, MCDD for 10 weeks, or MCDD for 8 weeks followed by MCCD for the last 2 weeks (the last of the these being the recovery group) [26]. miR-214-5p expression was significantly greater in the livers of rats that received MCDD for 10 weeks than in those that received MCCD for 10 weeks. However, these levels returned to control levels in the livers of rats that received the MCDD diet for 8 weeks followed by the MCCD diet for 2 weeks, consistent with recovery from the fibrosis (Figure 3). These results clearly suggest a close correlation between miR-214-5p expression in the liver, fibrosis development, and fibrosis-related mRNA expression.

miR-214-5p expression in hepatic stellate cells

We assessed the contribution of activated hepatic stellate cells to the increase in miR-214-5p in fibrotic mouse livers. miR-214-5p expression increased during the culture-dependent activation process in mouse stellate cells (2.7-fold increase at day 7 compared to day 1, $P < 0.05$) (Figure 4A). As expected, the induction of miR-214-5p was accompanied by an increase in the expression of α-SMA, Col1a1, PDGFR-β, and FN1 mRNA (Figure 4B). In addition, miR-214-5p expression was markedly higher in LX-2, a widely used human hepatic stellate cell line, than in human liver cancer cell lines such as HepG2 and Huh7 (108- and 39-fold, respectively) (Figure 4C).

We next isolated individual hepatocytes, non-paren chymal cells, and hepatic stellate cells from intact mouse livers to verify the cellular source of miR-214-5p. miR-214-5p was localized to non-parenchymal cells and hepatic stellate cells but expressed at negligible levels in hepatocytes (Figure 4D). These results suggest that miR-214 induction in fibrotic livers reflects the number and activation status of hepatic stellate cells.

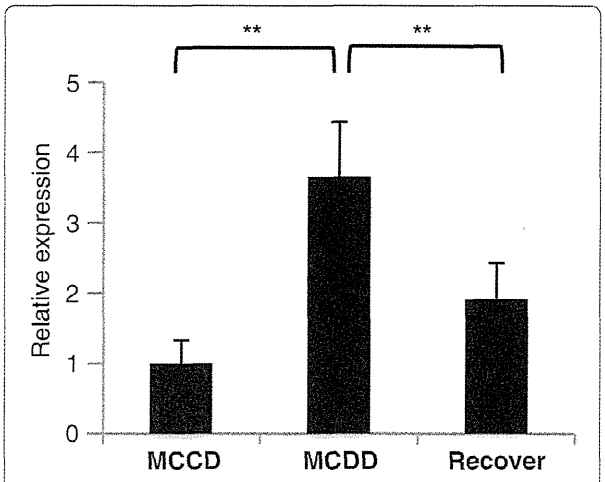

Figure 3 miR-214-5p expression in rat livers with fibrosis induced by a methionine- and choline-deficient diet. Expression of miR-214-5p in methionine- and choline-deficient diet (MCDD)-induced liver fibrosis and recovery phase in rats. The results are expressed relative to the expression of miR-214-5p in the livers of rats fed the methionine- and choline-control diet (MCCD) for 10 weeks. MCCD; rats fed MCCD for 10 weeks. MCDD; rats fed MCDD for 10 weeks. Recover; rats fed MCDD for 8 weeks followed by MCCD for 2 weeks. $^{**}P < 0.01$.

The effect of miR-214 overexpression on gene expression in stellate cells

We investigated the effect of miR-214-5p overexpression on fibrosis-related gene expression in stellate cells to clarify the role of this miRNA in stellate cell activation. miR-214-5p was overexpressed in LX-2 cells by transfection with an miR-214 precursor. The overexpression of miR-214 significantly increased the expression of matrix metalloproteinase-2 (MMP-2), MMP-9, α-SMA, and TGF-β1 compared to cells transfected with control microRNA (1.7-, 2.8-, 1.7- and 2.0-fold, respectively; $P < 0.01$) (Figure 5). These results indicate the strong participation of miR-214 in the activation of stellate cells.

Induction of miR-214 expression by TGF-β1

TGF-β_1 induces miR-214 expression in rat tubular epithelial cells and mesangial cells [23]. TGF-β_1 is essential for hepatic stellate cell activation. We assessed the stimulatory effect of TGF-β on miR-214-5p expression in LX-2 cells. TGF-β_1 (3 and 10 ng/ml) significantly stimulated miR-214-5p expression in LX-2 cells after 24 hours (1.75-fold, $P < 0.05$)(Figure 6A). In contrast, the expression of the miR-214/199a cluster is controlled by the transcription factor Twist-1 [22]. Real-time PCR analysis revealed that Twist-1 expression increased in the livers of mice that received MCDD compared to those of MCCD-fed mice

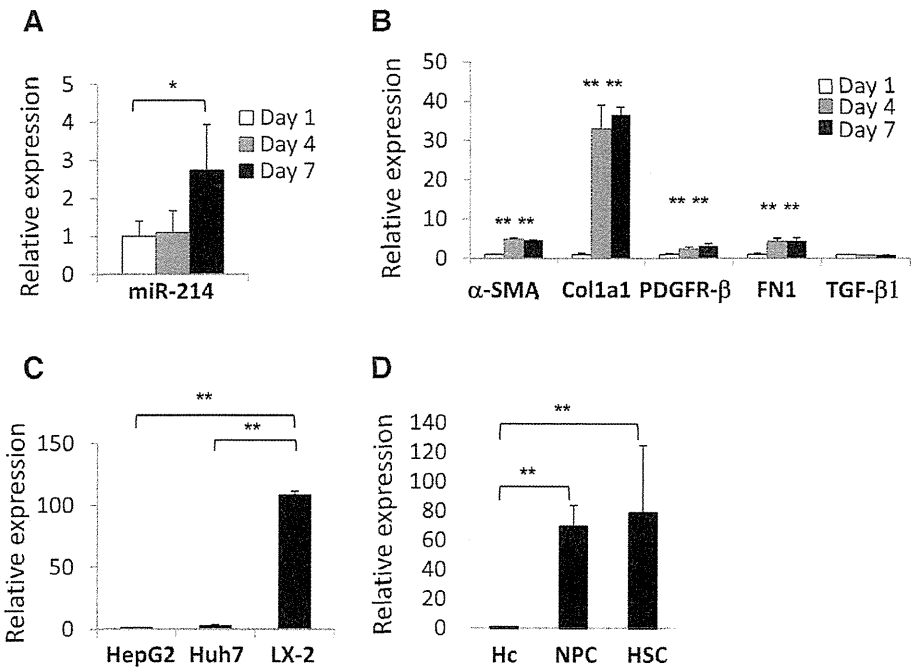

Figure 4 miR-214-5p expression in liver cells, including stellate cells. (A) miR-214-5p expression in mouse stellate cells during primary culture was analyzed using real-time PCR. Stellate cells were isolated from mouse livers and cultured for 1, 4 or 7 days. The results are expressed relative to the expression of miR-214-5p at day 1. $^{*}P < 0.05$. **(B)** The expression of α- smooth muscle actin (α-SMA), the type 1 collagen alpha 1 chain (Col1a1), platelet-derived growth factor receptor (PDGFR)-β, fibronectin (FN)1 and transforming growth factor (TGF)-β1 mRNA in primary-cultured mouse stellate cells was analyzed using real-time PCR. The results are expressed relative to the expression of the same mRNA at day 1. $^{**}P < 0.01$. **(C)** miR-214-5p expression in HepG2, Huh7 and LX-2 cells was analyzed using real-time PCR. The results are expressed relative to the expression of miR-214-5p in HepG2. $^{**}P < 0.01$. **(D)** miR-214-5p expression in hepatocytes (Hc), the non-parenchymal cell (NPC) fraction, and the hepatic stellate cell (HSC) fraction. The cells were isolated from intact mouse livers. The miR-214-5p expression in Hc, the NPC fraction, and the HSC fraction was analyzed using real-time PCR. The results are expressed relative to the expression of miR-214-5p in hepatocytes. $^{**}P < 0.01$.

(2.2-fold at 5 weeks, $P < 0.05$; and 3.6-fold at 15 weeks, $P < 0.05$) (Figure 6B). Twist-1 mRNA expression was also induced in a time-dependent manner after culture initiation in primary-cultured mouse stellate cells (Figure 6C).

Discussion

This is the first report to show that miR-214-5p is involved in organ fibrogenesis, specifically in the liver. miR-214 has previously been predicted to be a key molecule in proliferation in breast [27] and ovarian cancer cells [28], tumor progression in melanoma [29], and growth in HeLa cells [30]. miR-214 and miR-199a are encoded in a region that contains an E-box DNA promoter sequence [22]. A transcription factor, Twist-1, binds to the E-box region, regulating miR-214 and miR-199a expression [22]. The present study showed that miR-214 expression is upregulated in a fibrosis progression-dependent manner in the livers of patients with chronic HCV infection and in mice with diet-induced steatohepatitis (Figures 1 and 2). We previously reported an increase in miR-199a in the fibrotic livers of patients with chronic HCV infection [25], and similar findings have been reported by others [31-33]. These data and the upregulation of Twist-1 in MCDD-induced mouse liver fibrosis (Figure 4) suggest that Twist-1 controls the expression of the miR-214/199a cluster in the liver. Further studies will be needed to clarify the possible involvement of Twist-1 in the expression of miR-214-5p in LX-2 cells.

The present study revealed that miR-214-5p overexpression in LX-2 cells significantly increased MMP-2, MMP-9, α-SMA, and TGF-β1 mRNA expression. The overexpression of miR-199a in LX-2 cells triggers the upregulation of tissue inhibitor of metalloproteinase (TIMP)-1, Col1a1, and MMP-13 mRNA [34]. These results suggest that the miR-214/199a cluster plays a primary role in stellate cell activation. However, an understanding of the precise molecular events involved requires further research.

Conversely, the overexpression of miR-214-5p in LX-2 cells did not alter the expression of MAPK/Erk kinase 3 (MEK3), transcription factor AP-2 gamma (TFAP2C) [29], Plenxin-B1 [30], c-Jun N-terminal kinase 1 (Jnk1) [34], phosphatase and tensin homolog (PTEN) [35], enhancer of zeste homolog 2 (Ezh2) [36], and Quaking mRNA [24], which had been reported to be targets of miR-214 (MEK3: 0.72- to 0.77-fold, Jnk1: 1.05- to 1.20-fold,

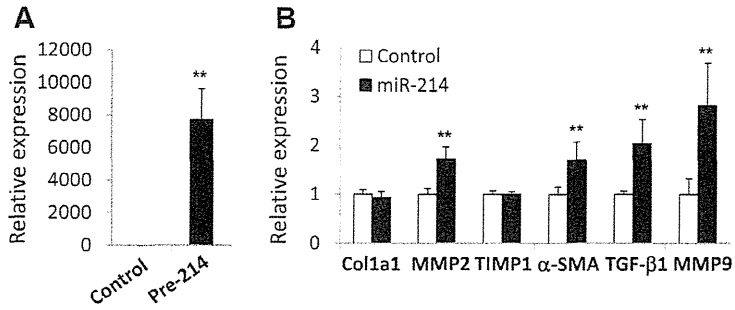

Figure 5 Effect of miR-214 overexpression on mRNA expression in LX-2 cells. (A) LX-2 cells were transfected with a miR-214 precursor or a negative control (control) at a final concentration of 50 nM and incubated for 24 hours. miR-214 expression was quantitated using real-time PCR. **(B)** The expression of fibrosis-related genes in LX-2 cells transfected with miR-214 precursors was analyzed using real-time PCR. The results are expressed as the expression relative to that in cells transfected with the control. $^{**}P < 0.01$.

PTEN: 0.97- to 1.12-fold, Plenxin-B1: 0.99-fold, Ezh2: 0.96-fold, TFAP2C: 0.94-fold, and Quaking: 0.88- to 1.18-fold change compared with cells transfected with control miRNA). The PTEN 3'-UTR did not interact with miR-214-5p in a luciferase reporter assay in LX-2 cells (data not shown). We also found that miR-214-5p overexpression had a negligible effect on LX-2 proliferation and migration. Therefore, the mRNA targets of miR-214-5p in LX-2 cells are not identical to those in previous reports.

Conclusions

We report an increase in miR-214-5p in liver fibrosis in humans and mice and the possible association of miR-214-5p with stellate cell activation. miR-214 expression in

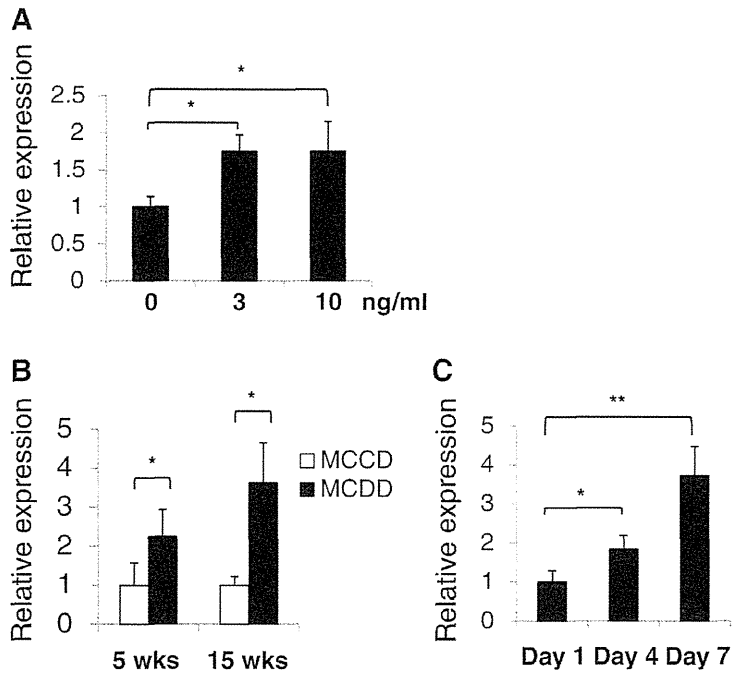

Figure 6 Regulation of miR-214-5p expression. (A) The effect of transforming growth factor (TGF)-β1 on miR-214-5p expression. LX-2 cells were treated with recombinant human TGF-β1 (3 or 10 ng/ml) for 24 hours in DMEM containing 0.1% fetal bovine serum (FBS). The results are expressed relative to miR-214 expression in cells that did not receive TGF-β1 treatment. $^{*}P < 0.05$. **(B)** Twist-1 expression in the fibrotic livers of mice fed a methionine- and choline-deficient diet (MCDD). Twist-1 expression was analyzed using real-time PCR. The results are expressed relative to Twist-1 expression in methionine- and choline-control diet (MCCD) mice. $^{*}P < 0.05$. **(C)** Twist-1 expression in primary-cultured mouse stellate cells. Twist-1 expression was analyzed using real-time PCR. The results are expressed relative to Twist-1 expression in cells on day 1. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1 List of primers

Gene name		Sequence from 5' to 3'
Mouse GAPDH	F	TGCACCACCAACTGCTTAG
	R	GGATGCAGGGATGATGTTC
Mouse α-SMA	F	TCCCTGGAGAAGAGCTACGAACT
	R	AAGCGTTCGTTTCCAATGGT
Mouse Col1a1	F	CCTGGCAAAGACGGACTCAAC
	R	GCTGAAGTCATAACCGCCACTG
Mouse PDGFR-β	F	GCGTATCTATATCTTTGTGCCAGA
	R	ACAGGTCCTCGGAGTCCAT
Mouse TGF-β1	F	GCAACATGTGGAACTCTACCAGAA
	R	GACGTCAAAAGACAGCCACTC
Mouse FN1	F	GATGCCGATCAGAAGTTTGG
	R	GGTTGTGCAGATCTCCTCGT
Mouse DDR2	F	CGAAAGCTTCCAGAGTTTGC
	R	GCTTCACAACACCACTGCAC
Mouse ITGB1	F	CAACCACAACAGCTGCTTCTAA
	R	TCAGCCCTCTTGAATTTTAATGT
Mouse Twist-1	F	AGCTACGCCTTCTCCGTCT
	R	TCCTTCTCTGGAAACAATGACA
Human GAPDH	F	GCACCGTCAAGGCTGAGAAC
	R	TGGTGAAGACGCCAGTGGA
Human Col1a1	F	CCCGGGTTTCAGAGACAACTTC
	R	TCCACATGCTTTATTCCAGCAATC
Human MMP2	F	TGACATCAAGGGCATTCAGGAG
	R	TCTGAGCGATGCCATCAAATACA
Human TIMP1	F	GGATACTTCCACAGGTCCCACAA
	R	CTGCAGGTAGTGATGTGCAAGAGTC
Human α-SMA	F	GACAATGGCTCTGGGCTCTGTAA
	R	CTGTGCTTCGTCACCCACGTA
Human TGF-β1	F	AGCGACTCGCCAGAGTGGTTA
	R	GCAGTGTGTTATCCCTGCTGTCA
Human MMP9	F	TCGAACTTTGACAGCGACAAGAA
	R	TCAGTGAAGCGGTACATAGGGTACA

α-SMA, α-smooth muscle actin; Col1a1, the type 1 collagen alpha 1 chain; DDR, discoidin domain receptor; F, forward primer; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ITGB1, β1 integrin; MMP, matrix metalloproteinase; PDGFR, platelet-derived growth factor receptor; R, reverse primer; TIMP, tissue inhibitor of metalloproteinase; TGF, transforming growth factor.

stellate cells is regulated by TGF-β and possibly by the transcription factor Twist-1. These results should be pursued further to identify the role of miR-214-5p in liver fibrogenesis and to develop a biomarker that reflects the stage of liver fibrosis more accurately than a pathological staging score.

Methods

Ethics Statement

The Ethics Committee of the Osaka City University Graduate School of Medicine approved this study (Approval No. 1358), which complied with the principles of the Declaration of Helsinki (2008 revision). All of the patients provided written, informed consent.

Liver biopsy specimens

Liver biopsy specimens were obtained from 35 patients with chronic HCV (genotype 1) infection as described previously [25]. The stage of liver fibrosis was evaluated using the METAVIR scoring system [37]. Normal liver tissues were taken as control samples from four patients who underwent resection for metastatic liver tumors.

Animals

Eight- to 12-week-old male C57BL/6N mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animal procedures were performed according to the guidelines of the Osaka City University and Faculty of Medicine Animal Research Committee and were approved by the committee. The mice received either a MCDD (n = 7, MP Biomedicals, Solon, OH, USA) or a MCCD (n = 7, MP Biomedicals) for 5 or 15 weeks, as previously described [26]. A similar protocol was followed in rats purchased from Japan SLC, Inc. Rats received MCCD for 10 weeks, MCDD for 10 weeks, or MCDD for 8 weeks followed by MCCD for the last 2 weeks (the last of the these being the recovery group) [26].

Cells

LX-2 cells (donated by Dr Scott Friedman [38]) and Huh7 cells were maintained in plastic culture plates in DMEM (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). HepG2 cells (JCRB1054) were obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in plastic culture plates in Minimum Essential Medium (Invitrogen) supplemented with 10% FBS, 1 mM sodium pyruvate (Invitrogen), and 1% non-essential amino acids (Invitrogen). Primary hepatic stellate cells and hepatocyte-rich and Kupffer cell-rich fractions were prepared from mouse livers according to the previously reported method [39].

Histochemistry and immunohistochemistry

The sections were stained with 0.1% (w/v) Sirius red in a saturated aqueous solution of picric acid (Direct Red 80; Aldrich, Milwaukee, WI, USA) for 1 hour at room temperature to visualize collagen fibers. Immunostaining for α-SMA was performed as previously described [25]. Mouse liver tissue was fixed in 10% formaldehyde, embedded in paraffin, and cut into 4 μm thick sections.

Quantitative real-time PCR

Gene expression was measured by real-time PCR using cDNA, real-time PCR Master Mix Reagents (Toyobo, Osaka, Japan), and gene-specific oligonucleotide primers

(Table 1) in an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), as previously described [25].

Transforming growth factor-β1 stimulation of LX-2 cells

LX-2 cells were seeded on 24-well plates in DMEM supplemented with 10% FBS at a density of 2×10^5 cells/ml. The cells were cultured for 14 hours, and the medium was changed to DMEM supplemented with 0.1% FBS plus TGF-β1 (3 or 10 ng/ml). The culture was continued for an additional 24 hours.

Transient transfection of miRNA precursors

miR-214-5p precursors and negative control miRNA were transfected into LX-2 cells using Lipofectamine 2000 (Invitrogen) at a final concentration of 50 nM, as described previously [20,25]. The cells were collected after 24 hours, and total RNA was extracted.

Statistical analysis

The data shown in the bar graphs represent the means ± SD of at least three independent experiments. Statistical analysis was performed using the Student's *t*-test. The Jonckheere-Terpstra test was used to compare differences between the four groups in the progressive stages of liver fibrosis. $P < 0.05$ was considered statistically significant.

Abbreviations

bp: base pair; Col1a1: the type 1 collagen alpha 1 chain; DDR: discoidin domain receptor; DMEM: Dulbecco's modified Eagle's medium; ECM: extracellular matrix; FBS: fetal bovine serum; FN: fibronectin; HCV: hepatitis C virus; ITGB1: β1 integrin; MCCD: methionine- and choline-control diet; MCDD: methionine- and choline-deficient diet; miRNA: microRNA; MMP: matrix metalloproteinase; PCR: polymerase chain reaction; PDGFR: platelet-derived growth factor receptor; α-SMA: α-smooth muscle actin; TGF: transforming growth factor; UTR: untranslated region.

Competing interests

KY is an employee of PhoenixBio Co. Ltd as an academic advisor. There is no direct financial benefit to KY for the publication of this manuscript. All other authors declare that they have no competing interests.

Authors' contributions

Conception and design (MI, TO, KI, NK); data acquisition (TO, ME, YM), data analysis and interpretation (MI, TO, KY); writing and review of the manuscript (MI, KY, KI, NK). All authors read and approved the final manuscript.

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Human Hepatocyte Propagation System in the Mouse Livers: Functional Maintenance of the Production of Coagulation and Anticoagulation Factors

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We previously reported that cell-based therapies using isolated hepatocytes including hepatocyte transplantation and liver tissue engineering approaches provide therapeutic benefits to hemophilia. For clinical application of these approaches, it is important to establish an active hepatocyte proliferation system that enables providing a sufficient number of hepatocytes. We also reported that human hepatocytes, which were transplanted into the liver of urokinase-type plasminogen activator transgenic severe combined immunodeficiency (uPA/SCID) mice, were able to proliferate while retaining their ability to produce coagulation factor IX. The objective of this study was to explore the functionalities of other coagulation and anticoagulation factors of the propagated human hepatocytes in uPA/SCID mice. Human hepatocytes were transplanted into the liver of uPA/SCID mice, and the propagation status of human hepatocytes in the mice was monitored by the increase in serum human albumin levels and immunohistochemical evaluation on the liver sections. Using uPA/SCID livers with various stages of human hepatocyte propagation, we analyzed the gene expression levels of coagulation factors (prothrombin, factor VII, factor X, and factor VIII) and anticoagulation factors (protein C and protein S) by real-time polymerase chain reaction (PCR) using human-specific primers. As a result, the total amount of raw messenger RNA expression levels increased in all genes analyzed according to the progress of hepatocyte propagation and proliferation. Except for factor VIII, the gene expression levels of the highly repopulated uPA/SCID mouse livers with human hepatocyte showed higher levels than those of normal human livers, indicating that propagated human hepatocytes in the uPA/SCID system possess full functions to produce most of the coagulation-related factors. The current work demonstrated that human hepatocytes can be propagated in experimental animals while maintaining normal gene expression levels of coagulation-related factors. It could be speculated that the propagated cells serve as a cell source for the treatment of various types of coagulation factor deficiencies.

Key words: Hepatocyte; Cell therapy; Hepatocyte transplantation; Coagulation factor; Urokinase-type plasminogen activator transgenic severe combined immunodeficiency (uPA/SCID) mouse; Anticoagulation factor

INTRODUCTION

Production of coagulation and anticoagulation factors is one of the important functions of the liver, and most of these coagulation-related factors are produced by hepatocytes (4,6,30,31,33). There are various types of congenital bleeding disorders that lack a production of coagulation factor in the liver, showing a symptomatic bleeding tendency. Hemophilia A or B is well known as a representative bleeding disorder, which is caused by a failure in the production of functional coagulation factor VIII (FVIII) or factor IX (FIX) from the liver. Although the ultimate cure for hemophilia patients could be obtained by liver transplantation (10,13,14), world-wide donor organ shortage is the most critical obstacle. For patients with hemophilia and other congenital coagulation factor deficiencies, the elevation of the responsible factor level to 1–2% of normal levels can provide a phenotypic change from severe to moderate form, resulting in a marked improvement in the symptomology and the

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quality of life (5). Cell-based approaches using isolated hepatocytes could be a feasible therapeutic option toward these coagulation factor deficiencies (18,20,21,24).

Proof-of-concept studies for hepatocyte-based approaches have been accomplished in both laboratory animals and humans. We recently reported that hepatocyte transplantation provided an increase of 1–2% of coagulation activities in a mouse model of hemophilia B, FIX-knockout mice (31). We also reported that engineering functional liver tissues beneath the kidney capsule were able to provide therapeutic effects in the mouse model of hemophilia A as well as hemophilia B (20,21). As reported by Dhawan et al. (7), hepatocyte transplantation was also successful in the clinic for the treatment of congenital factor VII deficiency. In utero liver cell transplantation was also investigated by Rosen et al. (25). They described phenotypic improvements in the mouse model of factor X deficiency. It is important to note that these hepatocyte-based approaches could be employed with a simple procedure and in a less invasive manner compared with organ transplantation (18,22).

One of the major hurdles in establishing hepatocyte-based approaches is the limited availability of biologically functional hepatocytes. At present, the number of donor livers for hepatocyte isolation remains severely limited. In most of cases, donor livers are of marginal quality that makes it difficult to obtain functional hepatocytes (23). An additional issue is that current technology for hepatocyte primary culture appears to be unable to support extensive cell proliferation (19). Under these circumstances, we previously proposed urokinase-type plasminogen activator transgenic severe combined immunodeficiency (uPA/SCID) mice as a feasible in-mouse hepatocyte propagation tool. uPA/SCID mice have a feature to develop an active damage of their own hepatic parenchymal cell and subsequent occurrence of continuous release of regenerative stimulus. Because of this nature, uPA/SCID mice provide a hepatic environment that is more conducive to the engraftment of human hepatocytes and a selective advantage for transplanted cells to proliferate (29). An important property of uPA/SCID mice was recently reported to allow human hepatocytes transplanted into the liver of uPA/SCID mice to actively propagate while retaining their ability to produce and secrete human FIX (30). From these data, it was reasonably speculated that the propagated human hepatocytes can serve as a cell source for future hepatocyte-based therapies toward hemophilia B. The functional preservation of FIX production of the propagated human hepatocytes encourages us to further assess functionalities for the production of other coagulation or anticoagulation factors.

We hypothesized that propagated human hepatocytes in the uPA/SCID mouse livers retained a normal gene expression of other coagulation and anticoagulation factors including prothrombin, factor VII, factor VIII, factor X, protein C, and protein. This report documents the first comprehensive analyses of coagulation factor-related gene expressions during in-mouse propagation status of human hepatocytes.

MATERIALS AND METHODS

Animals

Recipient uPA/SCID mice were generated at Phoenix Bio (Higashihiroshima, Hiroshima, Japan) as described previously (29). Genotyping for the presence of uPA transgene in SCID mice was confirmed by polymerase chain reaction assay of isolated genomic DNA as described previously (11,29). Experimental protocols were developed in accordance with the guidelines of the local animal committees located at both PhoenixBio and Nara Medical University.

Transplantation of Human Hepatocytes for Propagation in the uPA/SCID Livers

Human hepatocytes, isolated from a 1-year-old white male and a 6-year-old Afro-American female were purchased from In Vitro Technologies (Baltimore, MD). The cryopreserved hepatocytes were thawed and suspended in transplant medium (9,29). The cell viability of the human hepatocytes was determined to be 64.4% and 49.2% by trypan blue exclusion test, respectively. One day prior to the transplantation and 1 week after the transplantation, uPA/SCID mice, 20–30 days old, received intraperitoneal injections of 0.1 mg of anti-asialo GM1 rabbit serum (Wako Pure Chemical Industries, Osaka, Japan) to inhibit recipient natural killer cell activity against the transplanted hepatocytes. Viable human hepatocytes (0.75×10^6) were transplanted using an infusion technique into the inferior splenic pole in which the transplanted cells flow from the spleen into the liver via the portal system ($n = 18$). After the transplantation, uPA/SCID mice were treated with nafamostat mesilate to inhibit complement factors activated by human hepatocytes as described elsewhere (29).

Determination of Replacement Ratio

Blood samples were collected periodically from the tail vein, and the levels of human albumin were determined with a Human Albumin ELISA Quantitation kit (Bethyl Laboratories, Montgomery, TX) to estimate the status of proliferation and propagation of the transplanted human hepatocytes as previously described (29). For accurate determination of the ratio that transplanted human hepatocytes occupied in the recipient mouse livers (the replacement ratio), the harvested liver section were stained with antibodies against human-specific

cytokeratins 8 and 18 (hCK8/18), as described elsewhere (29). The replacement ratios of the mouse liver that received human hepatocytes were calculated as the ratio of area occupied by hCK8/18-positive hepatocytes to the entire area examined immunohistochemical sections of six lobes.

RNA Isolation and Quality Controls

Total RNA was extracted from the liver of the recipient mice with various stages of the replacement and normal human liver tissue samples by a RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Normal human liver tissue portions were obtained from surgical specimens during liver surgery for metastatic liver tumors after acquiring a written informed consent for the experimental use of harvested liver samples. DNase I was used to eliminate genomic DNA contamination, and the concentration of the RNA was determined by UV spectrometry. All of the RNA samples used in this study had an absorbance ratio (260/280 nm) between 1.9 and 2.1, and the integrity of RNA samples was confirmed by electrophoresis on a 1% agarose gel.

Reverse Transcription Coupled to Quantitative Real-Time PCR (Real-Time RT-PCR)

Total RNA (1 μg) was reverse-transcribed using oligo d(T)16 primers as described by the manufacturer (Omniscript RT Kit; QIAGEN). First-strand cDNA samples were subjected to quantitative PCR amplification using a StepOne Real-time PCR system (Applied Biosystems, Tokyo). For this experiment, we examined the following gene groups: 1) seven housekeeping reference genes, including glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), β-actin (*Actb*), peptidylprolyl isomerase A (*Ppia*), ribosomal protein L4 (*Rpl4*), transferrin receptor (*Tfrc*), β-glucuronidase (*Gusb*), and hypoxanthine phosphoribosyltransferase (*Hprt1*); 2) the genes of five vitamin K-dependent coagulation factors, including prothrombin (*F2*), factor VII (*F7*), factor X (*F10*), protein C (*Prosc*), and protein S (*Pros1*); and 3) factor VIII (*F8*) gene. TaqMan probes and primers for these genes were chosen from a TaqMan Gene Expression Assay (Applied Biosystems), and the information regarding these primer sets are listed in Table 1. All PCR analyses were performed using the following cycling conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. The specificity of the primers was verified by 2% agarose gel electrophoresis of the amplicons derived from naive human liver cDNAs. The PCR primers used in this study were confirmed to be human specific and to have no cross-reactions with mouse-derived genes. For quantification of gene expression, the cDNAs derived from total RNA extracted from normal human liver tissues were serially diluted and used to generate calibrations.

Statistical Analysis

Correlation coefficients between the repopulation rate and each gene expression were determined using Excel (Microsoft).

RESULTS

Propagation of Human Hepatocytes in uPA/SCID Mouse Liver

Human hepatocytes were transplanted to uPA/SCID mice, which were sacrificed to excise the liver tissues at various time periods after the transplantation with monitoring the levels of blood human albumin. Using the collected liver samples, hCK8/18 immunostaing on the liver sections was performed to accurately assess the replacement ratios with human hepatocytes as described in Materials and Methods. As a result, the repopulation ratios ranged from 0% to 98%, and the number of mice in each repopulation category was 2, 4, 4, 4, and 4 in 0–20%, 21–40%, 41–60%, 61–80%, and 81–100%, respectively.

Selection of an Appropriate Reference Gene

The expressions of seven commonly used housekeeping genes (HKG) specific to human cells were evaluated in the recipient uPA/SCID mouse livers. Figure 1 shows that the raw expression levels of all seven HKGs increased in parallel to the replacement ratios. The correlation coefficiency with the replacement ratios of each gene was 0.68 for h*Gapdh*; 0.86 for h*Actb*; 0.72 for h*Ppia*; 0.82 for h*Rpl4*; 0.68 for h*Tfrc*; 0.75 for h*Gusb*; and 0.78 for h*Hprt1*. When the replacement ratios exceeded 80%, the expression levels of all HKGs but *Hprt1* became higher than those of normal human liver samples. h*Gapdh* expression levels in the repopulated uPA/SCID livers were also observed to be beyond the levels of normal human livers at the repopulation ratio as low as 40%. Eventually, h*Gapdh* expression levels reached approximately sixfold of normal human liver levels when the repopulation ratios exceeded 80%. In contrast, gene expression levels of h*Actb* failed to reach to comparable levels with normal human liver until the repopulation ratios were close to 100%. Under the condition of varied gene expression levels of HKGs, it is important to select the most stably expressed HKG to assess the expression of target human genes in the uPA/SCID livers. For achieving this, *Actb* gene, which demonstrated the best correlation coefficient with the replacement ratios, was selected as a reference normalizing gene in the following gene expression analyses.

Table 1. Primers Used in This Study

Symbol	Gene Name	Assay ID
Housekeeping genes		
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
Actb	actin, beta	Hs99999903_m1
Ppi	peptidylprolyl isomerase A	Hs99999904_m1
Rpl4	ribosomal protein L4	Hs03044647_g1
Tfrc	transferrin receptor	Hs00174608_m1
Gusb	glucuronidase, beta	Hs99999908_m1
Hprt1	hypoxanthine phosphoribosyltransferase	Hs99999909_m1
Target genes		
F2	coagulation factor II (prothrombin)	Hs01011995_g1
F7	coagulation factor VII	Hs00173398_m1
F9	coagulation factor IX	Hs00609168_m1
F10	coagulation factor X	Hs00173450_m1
Prosc	protein C	Hs00165584_m1
Pros1	protein S, alpha	Hs00165590_m1
F8	coagulation factor VIII	Hs00240767_m1

Expression of Coagulation Factor Genes

Human-specific coagulation-related gene expression levels were assessed on human hepatocyte-repopulated uPA/SCID mouse livers. The genes analyzed were: vitamin K-dependent coagulation factors (prothrombin, factor VII, and factor X) and anticoagulation factors (protein C and protein S), in addition to factor VIII. Raw expression levels of all vitamin K-dependent coagulation and anticoagulation factor genes showed a positive correlation with the repopulation ratios (Fig. 2). The correlation coefficient between the gene expression levels and the repopulation ratios were 0.78, 0.74, 0.80, 0.80, and 0.82 in *F2*, *F7*, *F10*, *Prosc*, and *Pros1*, respectively. The raw gene expression levels of all but *F8* were beyond the levels of the normal human liver samples (defined as 1.0) as the repopulation ratios increased. In marked contrast, *F8* gene expression levels were less than 40% of normal human liver tissues even though the repopulation ratios reached approximately 100%. The low levels of *F8* gene expression failed to show a significant correlation with the repopulation ratios ($R = 0.66$).

In order to evaluate the gene expression levels per human hepatocytes, the gene expression levels were normalized by *ACTB* gene expression levels in each sample (Fig. 3). As a result, normalized gene expression values of all the analyzed coagulation-related factor genes showed constant expression levels regardless of the repopulation ratios, demonstrating that the human hepatocytes in the uPA/SCID livers stably express coagulation-related factor genes throughout the repopulation stages.

DISCUSSION

Propagation of primary human hepatocytes that possess hepatocyte-specific functionalities including blood clotting factor production has been one of the major paradigms in liver regenerative medicine (18,24). In the present study, we transplanted primary human hepatocytes to the liver of uPA/SCID mice and succeeded in propagating the human hepatocytes in the mouse livers. We then investigated mRNA expression levels of human-specific vitamin K-dependent coagulation factors (prothrombin, factor VII, and factor X), anticoagulation factors (protein C and protein S), and factor VIII of the propagated hepatocytes at various stages of propagation. The results showed that mRNA expression levels per human hepatocyte of all the analyzed genes were maintained through the propagation stage, indicating that the uPA/SCID in-mouse hepatocyte propagation system is a viable method to propagate hepatocytes that are intact in coagulation factor productions.

Coagulation factors are produced mainly by hepatocytes, and the long-term synthesis of these factors from primary human hepatocytes in vitro have been recently achieved by plating cells inside a 3D collagen gel matrix together with hormonally enriched culture medium under chemically defined conditions (6), indicating the pivotal role of the extracellular environment for coagulation factor production. However, the current procedure for the culture of primary hepatocytes appears to be difficult to support extensive cell proliferation (19), still remaining the problem of donor cell shortage unresolved. It is true that isolated hepatocytes could obtain proliferating ability and long-term survival in vitro by immortalization (26,34,35) or by selective culture of small hepatocyte population (27), but there is no report for studying the gene expression and production of coagulation factors including factor IX in these cell types. On the other hand, embryonic stem (ES) cells and