The drug-metabolizing enzyme CYP is mainly involved in the metabolism of various substances in the liver, such as drugs, endogenous substances, and carcinogens. Recent reports have also revealed that CYP1B1 plays a major role in the developmental process. Because the level of CYP expression is markedly high in the liver, we hypothesize that CYP plays a role in the developmental process of the liver. To verify this hypothesis, we analyzed the expression patterns of various CYP molecular species and their functions during the differentiation of embryonic stem cells (ES cells) into hepatocytes and the developmental process in mice. The results demonstrated that CYP2R1 and CYP26A1 are expressed at an earlier stage of the differentiation of ES cells into hepatocytes than hepatoblast-specific markers. Additionally, during the development of the mouse liver, CYP2R1 and CYP26A1 were mostly up-regulated during the stage when hepatoblasts appeared. In addition, when CYP2R1 and CYP26A1 expressions were forced in ES cells and liver of adult mice, they differentiated into hepatoblast marker positive cells. These results suggest that CYP2R1 and CYP26A1 may play a major role in hepatoblast cell differentiation during the development of the liver.

Key words embryonic stem cell; liver; CYP; hepatoblast

The mature liver contains a variety of cell types, such as hepatocytes, sinusoidal endothelial cells, bile duct epithelial cells, stellate cells and Kupffer cells. The hepatocyte account for 80% of the volume of the liver and play a major role in liver functions, such as metabolism and excretion of drugs. Additionally, the fetal liver is known to act as a hematopoietic organ.

The expression of CYP is significantly up-regulated in the liver, and CYP converts fat-soluble drugs into a water-soluble state so that they can be easily excreted. In addition to drug metabolism, CYP is also known to be involved in the oxidative metabolism of endogenous substances, including steroids, bile acid, hormones, and eicosanoids. To date, 58 types of CYP molecular species have been identified in humans, and 108 types have been identified in mice.1-3) As the CYP molecular species share high amino acid sequence homology in several substrate-recognition sites, in addition to the above-mentioned functions of CYP, it may also play a major role in development and differentiation in the body. Because CYP begins to be expressed close to the time of hematopoiesis initiation during the fetal stage and CYP requires heme for its structure, CYP is considered to be involved in the development of the liver during the fetal stage. Moreover, it has been reported that the metabolites of endogenous substances and the intermediate metabolites of chemical substances have an effect on the development of individuals and on homeostasis.4-6) Therefore, CYP, which transiently appears during the process of development, is thought to possibly play an important role in the development of the liver.

It has been reported that the knockout of either the CYP26A1 gene in mice causes abnormal embryony result-
RIKEN BRC CELL BANK. Culture of the mouse ES cells was performed using a known methodology \(^1\) (Fig. 1). Frozen cells were thawed, dispersed at a density of \(1 \times 10^6\) cells/mL in culture medium, and seeded at 10 mL per 100 mm in a gelatin-coated dish.

The induction of the differentiation of ES cells into hepatocytes was performed using a known methodology. The ES cells were seeded at \(2 \times 10^7\) cells per 35 mm gelatin-coated dish or at \(4 \times 10^4\) cells per well on a Lab-Tek™ II Chamber Slide (Nunc, CA, U.S.A.) and were incubated at 37°C in 5% CO\(_2\) for 72 h in stem medium (DS farmabiomedical, Osaka, Japan) (final conc. LIF: 1000 units/mL). The culture medium was then changed to stem medium containing LIF and \(10^{-8}\) mol/L all-trans-retinoic acid (RA) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and the cells were incubated for 3 d.

After 3 d, the cells were cultured with LIF (−) culture medium for 5 d in the presence of fibroblast growth factor (FGF)-1, 100 ng/mL; FGF4, 20 ng/mL; hepatocyte growth factor (HGF), 50 ng/mL (Wako) (at \(10^7\) cells per 100 mm gelatin-coated dishes).

After 5 d, the cells were subcultured from the gelatin-coated dishes to collagen-coated dishes. At this point, the cells were washed gently three times with ES Cell Qualified Dulbecco’s phosphate buffered saline (D-PBS) and incubated with 1 mL of PBS for subculture at 37°C in 5% CO\(_2\) for 2 min. The reaction was stopped by adding serum-free ES Cell Qualified Dulbecco’s modified Eagle’s medium (DMEM) (DS farmabiomedical), and the cells were collected and centrifuged at 100 \(\times g\) for 3 min. After removing the supernatant, the cells were resuspended in ES Cell Qualified Dulbecco’s modified Eagle’s medium (DMEM) (DS farmabiomedical), and the cells were collected and centrifuged at 100 \(\times g\) for 3 min, and the supernatant was removed. The cells were then resuspended in stem medium, seeded at \(2 \times 10^5\) cells per 35 mm collagen-coated dish and incubated in stem medium containing oncostatin M (OsM) (Wako) (10 ng/mL) for 2 d. After 2 days, the culture medium was changed from stem medium to hepatocyte medium (William’s E medium (Wako) containing insulin (Wako) 5 \(\mu\)g/mL, transferrin (Wako) 5 \(\mu\)g/mL, Bovine Serum Albumin (Wako) 0.5 mg/mL, ascorbic acid (Wako) 2 \(\mu\)mol/L, hydrocortisone-21-hemisuccinate (Wako) \(10^{-7}\) M and the cells were incubated for 8 d.

**Extraction of Total RNA** Total RNA was extracted from the cells during the induction of the differentiation of ES cells into hepatocytes using TRI reagent (Sigma-Aldrich, St. Louis, MO, U.S.A.).

cDNA was synthesized from 1 \(\mu\)g purified total RNA using a High Capacity cDNA synthesis Kit (Applied Biosystems, Foster City, CA, U.S.A.). The concentration of the total RNA (\(\mu\)g/mL) was calculated, and the purity of the total RNA was evaluated by measuring its absorbance at 260 and 280 nm.

For each sample, 2.0 \(\mu\)L of 10×RT buffer, 0.8 \(\mu\)L of 25×deoxyribonucleotide (dNTP) Mix, 2.0 \(\mu\)L of 10×RT Random Primer, 1.0 \(\mu\)L MultiScribe™ Reverse Transcriptase, 1.0 \(\mu\)L ribonuclease (RNase) Inhibitor, and 3.2 \(\mu\)L ultrapure water, which were all included in the High Capacity cDNA synthesis Kit, were mixed gently on ice to prepare the 2×Reverse Transcription (RT) Master Mix.

**RT-PCR** The following reagents were added to each well of the PCR 8 Strip Tube: 0.1 \(\mu\)L TaKaRa Ex Taq (TaKaRa-Bio, Shiga, Japan), 2.5 \(\mu\)L of 10×Ex Taq buffer, 2.0 \(\mu\)L dNTP mixture, 1.25 \(\mu\)L dimethyl sulf oxide (DMSO), 1 \(\mu\)L cDNA solution, 2.5 \(\mu\)L forward primer (20 pmol/\(\mu\)L), 2.5 \(\mu\)L reverse primer (20 pmol/\(\mu\)L) and 13.15 \(\mu\)L ultrapure water. Using an iQ™ Thermal Cycler (Bio-Rad, Hercules, CA, U.S.A.), samples were first denatured at 94°C for 2 min and then at 98°C for 10 s; then primer annealing was performed at 55 to 58°C for 30 s, followed by elongation at 72°C for 30 s. These steps constituted one cycle. After 30 to 35 cycles, an extension step was performed at 72°C for 1 min and 30 s to amplify the cDNA.

The forward and reverse primers for the hepatocellular differentiation markers, hepatocyte nuclear factor 3-beta (HNF-3\(\beta\)), \(\alpha\)-fetoprotein (AFP), delta-like 1 (DLL1), albumin (ALB), and tryptophan dioxygenase (TDO), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene are listed in Table 1. After the PCR was completed, 2.5 \(\mu\)L of 10× loading buffer was added to the 25 \(\mu\)L PCR products, and the solution was mixed well. Agarose gel electrophoresis was performed with a 1.5% agarose gel and Tris acetate EDTA (TAE) buffer with 15 \(\mu\)L of PCR product/lane at r.t. for 30 min (Mupid-2 plus, TaKaRa-Bio). After electrophoresis, the agarose gel was soaked in EtBr solution (Nippon Gene, Tokyo, Japan), a nucleic acid stain solution, in the dark at r.t. for 15 min. The agarose gel was photographed using a cooled CCD camera (LAS-3000mini, FUJIFILM Corporation, Tokyo, Japan).

**Immunocytochemistry** After washing with PBS(−), ES cells were fixed with 4% paraformaldehyde (PFA)/PBS at r.t.
for 10 min. After washing gently with PBS(−) once, the cells were incubated with 0.05% TritonX-100/5% FCS/PBS at r.t.
for 1 h and then with the primary antibody (Ab) solution at 4°C overnight. After washing gently with PBS(−) three times, the cells were reacted with the secondary Ab solution in the dark at r.t. for 1 h. After washing gently with PBS(−) three times, the cells were encosed with VECTASHIELD (Vector Laboratories, Burlingame, CA, U.S.A.) using MICRO COVER GLASS. The immunostained sections were detected using FV-1200 (Olympus). Anti-mouse CYP2R1 (AP7894c) was purchased from Abcam plc. Alexa Fluor 488 donkey anti-rabbit immunoglobulin G (IgG) (A21206) was purchased from Invitrogen.

**Immunohistochemistry** ICR pregnant mice at 9 and 11 d of gestation were anesthetized with pentobarbital via intraperitoneal injection, and fetuses were removed by cesarean section. The fetuses were fixed in 4% PFA at 4°C for 20 min. After washing gently with PBS(−), the samples were soaked in 10, 20, and 30% sucrose solutions at 4°C in sequence until the tissues sank in each solution. The samples were embedded in O.C.T compound and stored at −80°C. Then, they were sectioned into 12-µm slices using LEICA CM1850 and placed on micro slide glass. Once the sections were sufficiently dry, they were stored at −80°C as frozen sections until use. Immunostaining was then performed in the same manner as immunocytochemistry.

**Plasmid DNA** pEF-BOS were by inserting the EcoRI/SalI mouse CYP2R1 and CYP26A1 cDNA fragment amplified by PCR into the EcoRI/SalI site of pEF-BOS. pEF-BOS-ires-GFP was by constructing the green fluorescent protein (GFP). To estimate the transfected cells, we constructed pEF-BOS-ires-GFP containing a GFP driven by an internal ribosomal entry site (IRES). CYP2R1 Forward Primer: 5′-GACTGACAGCTACTACG-3′, Reverse Primer: 5′-CCCAAGAAGGTCTCCTGTTG-3′, GAPDH Forward: Product size (bp) 177 58 30

### RESULTS

**Expression Patterns of CYPs mRNA during the Induction of the Differentiation of ES Cells into Hepatocytes**

We analyzed the mRNA expression patterns of CYP molecular species that appeared during the induction of the differentiation of ES cells into hepatocytes (Fig. 2). The results showed that hepatocyte nuclear factor 3-beta (HNF-3/β), which indicates differentiation of ES cells into endodermal cells, was first expressed on day 3 of differentiation induction, and its expression was highest on day 7. For α-fetoprotein (AFP) and delta-like-1 (DLK1), which are hepatoblast markers, expression was first observed on day 7 of differentiation induction. Albumin (ALB), which is expressed at the late stage of hepatocyte differentiation, was first expressed on day 11 of differentiation induction. The expression of glucose-6-phosphate (G6Pase), a marker of adult hepatocytes, was first observed on day 7 of differentiation induction, while that of Trp dioxy-
genase (TDO) was only observed on day 18 (Fig. 2). On day 18, the cells were also positive for periodic acid-Schiff (PAS) staining, which is an indicator of the ability of cells to store glycogen (data not shown).

The most important role of CYP3A11 is its drug-metabolizing enzyme function, and CYP3A11 was first expressed on day 11 of differentiation induction. Thereafter, the expression level of CYP3A11 increased with the maturation of the hepatocytes. CYP2R1 was first expressed on day 3 of differentiation induction, and its expression was highest on day 7, after which it decreased with further differentiation induction. In addition, high expression of CYP26A1 was observed on day 3 of hepatocyte differentiation induction; its expression was decreased on day 7 before being highly expressed again on day 18 (Fig. 2). Intriguingly, CYP2R1 and CYP26A1 were both induced prior to AFP and DLK1, which are markers of hepatoblasts.

Expression Patterns of CYP2R1 and CYP26A1 during the Induction of Differentiation of ES Cells into Hepatocytes mRNA expression of CYP2R1 and CYP26A1 was observed prior to the differentiation of ES cells into hepatoblasts. Therefore, to evaluate whether both CYPs were also expressed at the protein level at that time, immunostaining was performed using anti-CYP2R1 Ab or anti-CYP26A1 Ab and an Ab for delta-like 1 (DLK-1), a marker of hepatoblasts. As a result, on day 3 of differentiation induction, when ES cells had differentiated into endoderm, although CYP2R1 and CYP26A1 were already expressed, the expression of DLK1, a marker of hepatoblasts, was not observed (Fig. 3). On day 7, many cells expressing CYP2R1 or CYP26A1 were positive for DLK1 (Fig. 3).

Based on these results, it is possible that CYP2R1 and CYP26A1 are expressed prior to hepatoblast markers during hepatocyte differentiation and that they play a particular roles in the process of differentiation of ES cells into hepatoblasts.

Expression Pattern of CYP2R1 and CYP26A1 in the Fetal Liver During the development of the mouse liver, the development of the hepatic primordium of fetal mice at E8 to E13 days of gestation was considered to correspond to the differentiation of endoderm to hepatoblasts. Therefore, we conducted immunohistochemical staining of the livers of fetal mice at E9 and E11 days of gestation to analyze whether CYP2R1 and CYP26A1 were expressed at the stage of hepatoblast appearance.

The results revealed that CYP2R1 and CYP26A1 were expressed in the hepatic primordium of fetal mice at E9 days of gestation and that some of the CYP2R1-positive cells and CYP26A1-positive cells were also positive for DLK1 (Fig. 4). Furthermore, many cells in the liver of fetal mice at E11 days of gestation were positive for both CYP2R1 and CYP26A1, and a significant number of them also expressed DLK1 (Fig. 4).

Based on these results, the probable involvement of CYP2R1 and CYP26A1 in the differentiation of hepatoblasts was also confirmed in the process of mouse liver development.

Induction of Delta-Like 1-Positive Cells by Forced Expression of CYP2R1 and CYP26A1 in ES Cells Because the involvement of CYP2R1 and CYP26A1 in the differentiation of hepatoblasts was indicated by the results of our above experiments, to further verify this finding, we investigated whether forced expression of CYP2R1 or CYP26A1 in ES cells would produce DLK1-positive cells. Expression plasmids encoding CYP2R1 or CYP26A1 were transfected into ES cells, and differentiated state of the cells were examined at 72 h after transfection. Our results showed that GFP expressing cells (expressing CYP2R1 or CYP26A1) differentiated into hepatoblasts that were positive for DLK1 (Fig. 5).

Forced Expression of CYP2R1 and CYP26A1 in Mouse Liver Induces DLK1-Positive Cells In the in vitro experiments, it was revealed that forced expression of CYP2R1 or

![Fig. 2. Patterns of mRNA Expression of CYP Molecular Species during the Induction of Differentiation of ES Cells into Hepatocytes](image-url)
CYP26A1 caused differentiation of ES cells into hepatoblasts. Thus, in this study, we investigated whether forced expression of CYP2R1 and CYP26A1 would differentiate into hepatoblasts in mouse liver. For gene transfer to the liver, mice received a tail vein injection of plasmid DNA (Vehicle, CYP2R1, CYP26A1) dissolved in a hydrodynamic solution. The results showed that GFP expressing cells differentiated into DLK1-positive cells (Fig. 6).

**DISCUSSION**

To efficiently induce differentiation of ES cells into hepatocytes, it is essential to cause differentiation of ES cells into endodermal cells and then further differentiation and maturation specifically into hepatocytes. It has been reported that when ES cells are incubated with retinoic acid (RA) in the presence of leukemia inhibitory factor (LIF), most cells differentiate into endodermal cells. The factors that were added during the process of differentiation induction from endodermal cells to hepatocytes are the factors secreted during the development of hepatoblast tissues. The budding of hepatoblast tissue is initiated by stimulation by fibroblast growth factor (FGF) secreted by the adjacent cardiac mesoderm and bone morphogenic protein (BMP) secreted by the transverse septum. Thereafter, maturation of hepatocytes is promoted by adding hepatocyte growth factor (HGF) and oncostatin M (OsM), which are involved in the maturation of fetal hepatocytes. ES cells were reported to be efficiently differentiated into hepatocytes by the addition of a combination of FGF-1, FGF-4, and HGF, which are molecules that are increased during hepatic damage, to the culture medium.

This study used the differentiation induction system to differentiate ES cells into hepatocytes to examine CYP molecular species that contribute to the proliferation and differentiation of the fetal liver. In the process of differentiation induction from ES cells into hepatocytes, the CYP molecular species that showed interesting expression patterns were CYP2R1 and CYP26A1. CYP2R1 and CYP26A1 are involved in the metabolic pathways of vitamin A and vitamin D, respectively.
CYP26A1 contributes to the synthesis and metabolism of retinoic acid. β-Carotene and retinyl ether are converted to retinol (alcohol) and subsequently oxidized to retinal (aldehyde) and then retinoic acid, which has physiological action after it undergoes further oxidation. It is known that the metabolism of retinoic acid involves CYP26 family members in the embryonic stage and CYP3A7 in the fetal stage to regulate the concentration of retinoic acid. Retinoic acid contributes to development and differentiation by binding to nuclear receptors, such as retinoic acid receptor (RAR) and retinoid X receptor (RXR). Vitamin A is the collective name for retinal, retinol and retinoic acid and is associated with the differentiation, proliferation, morphogenesis, and apoptosis of cells. Because an excessive intake of vitamin A also causes teratogenicity, regulation of the concentration of retinoic acid is highly important for the living body. In CYP26A1 knockout mice, which show embryonic lethal or death shortly after birth, morphological defects are particularly observed in the tailbud and hindbrain, likely because CYP26A1 expression is highly conserved in various species, and mice and humans share 89% homology in the CYP2R1 sequence. Active vitamin D metabolite 25-hydroxyvitamin D3 (25-OH-D3) and 1,25-dihydroxyvitamin D3 (1,25-(OH)2-D3) has physiological action after it undergoes further oxidation.

In this study, in both the induction of differentiation of ES cells into hepatocytes experiments and the fetal mice experiments, the expressions of CYP2R1 and CYP26A1 were induced prior to the expression of DLK1, a hepatoblast marker. These results suggest the involvement of these CYPs in hepatoblast differentiation through the metabolism of endogenous substances, such as vitamins D and A.

Uncovering the mechanism of CYP2R1 and CYP26A1 expression regulation will likely help clarify the mechanism of early liver development and/or liver regeneration.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Christmas P, Tolentino K, Primo V, Berry KZ, Murphy RC, Chen M, Lee DM, Soberman RJ. Cytochrome P-450 4F18 is the leukotriene B4 omega-1/omega-2 hydroxylase in mouse polymorphonuclear leukocytes: identification as the functional orthologue of human polymorphonuclear leukocyte CYP4F3A in the down-regulation of responses to LTB4. J. Biol. Chem., 281, 7189–7196 (2006).

2) McLaughlin LA, Dickmann LJ, Wolf CR, Henderson CJ. Functional expression and comparative characterization of nine murine cytochromes P450 by fluorescent inhibition screening. Drug Metab. Dispos., 36, 1322–1331 (2008).

3) Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, Nebert
Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics*, 14, 1–18 (2004).

4) Gonzalez FJ. The molecular biology of cytochrome P450s. *Pharmacol. Rev.*, 40, 243–288 (1988).

5) McArthur AG, Hegelund T, Cox RL, Stegeman JJ, Liljenberg M, Olsson U, Sundberg P, Celander MC. Phylogenetic analysis of the cytochrome P450 3 (CYP3) gene family. *J. Mol. Evol.*, 57, 200–211 (2003).

6) Zhao XJ, Ishizaki T. The in vitro hepatic metabolism of quinine in mice, rats and dogs: comparison with human liver microsomes. *J. Pharmacol. Exp. Ther.*, 283, 1168–1176 (1997).

7) Abu-Abed S, Dolle P, Metzger D, Beckett B, Chambon P, Peitcovitch M. The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. *Genes Dev.*, 15, 226–240 (2001).

8) Bidinost C, Hernandez N, Edward DP, Al-Rajhi A, Lewis RA, Lupski JR, Stockton DW, Bejjani BA. Of mice and men: tyrosinase modification of congenital glaucoma in mice but not in humans. *Invest. Ophthalmol. Vis. Sci.*, 47, 1486–1490 (2006).

9) Libby RT, Smith RS, Savinova OV, Zabaleta A, Martin JE, Gonzalez FJ, John SW. Modification of ocular defects in mouse developmental glaucoma models by tyrosinase. *Science*, 299, 1578–1581 (2003).

10) Hrycay EG, Bandiera SM. Expression, function and regulation of mouse cytochrome P450 enzymes: comparison with human P450 enzymes. *Curr. Drug Metab.*, 10, 1151–1183 (2009).

11) Teratani T, Yamamoto H, Aoyagi K, Sasaki H, Asari A, Quinn G, Sasaki H, Terada M, Ochya T. Direct hepatic fate specification from mouse embryonic stem cells. *Hepatology*, 41, 836–846 (2005).

12) Evans MJ, Kaufman MH. Establishment in culture of pluripotent cells from mouse embryos. *Nature*, 292, 154–156 (1981).

13) Mummery CL, Feven A, Freund E, Shen S. Characteristics of embryonic stem cell differentiation: a comparison with two embryonal carcinoma cell lines. *Cell Differ. Dev.*, 30, 195–206 (1990).

14) Fukuda-Taira S. Hepatic induction in the avian embryo: specificity of reactive endoderm and inductive mesoderm. *J. Embryol. Exp. Morphol.*, 63, 111–125 (1981).

15) Mongan NP, Gudas LJ. Diverse actions of retinoid receptors in cancer prevention and treatment. *Differentiation*, 75, 853–870 (2007).

16) Mark M, Ghyselinck NB, Chambon P. Function of retinoic acid receptors during embryonic development. *Nucl. Recept. Signal.*, 7, e002 (2009).

17) Huang J, Bi Y, Zhu GH, He Y, Su Y, He BC, Wang Y, Kang Q, Chen L, Zuo GW, Luo Q, Shi Q, Zhang BQ, Huang A, Zhou L, Feng T, Lau HH, Haydon RC, He TC, Tang N. Retinoic acid signaling induces the differentiation of mouse fetal liver-derived hepatic progenitor cells. *Liver Int.*, 29, 1569–1581 (2009).

18) Peña C, García JM, Silva J, García V, Rodríguez R, Alonso I, Milan I, Salas C, de Herreros AO, Malo A, Bonilla P. E-cadherin and vitamin D receptor regulation by SNAIL and ZEB1 in colon cancer: clinicopathological correlations. *Hum. Mol. Genet.*, 14, 2281–2300 (2005).

19) Monga SP, Monga HK, Tan X, Mule K, Pediatikas P, Michalopoulos GK. Beta-catenin antisense studies in embryonic liver cultures: role in proliferation, apoptosis, and lineage specification. *Gastroenterology*, 124, 202–216 (2003).

20) Tan X, Yuan Y, Zeng G, Apte U, Thompson MD, Cieply B, Stolz DB, Michalopoulos GK, Kaestner KH, Monga SP. Beta-catenin deletion in hepatoblasts disrupts hepatic morphogenesis and survival during mouse development. *Hepatology*, 47, 1667–1679 (2008).