DMD Fast Forward. Published on January 13, 2006 as doi:10.1124/dmd.105.007674

CHARACTERIZATION OF CYTOCHROME P450 EXPRESSION IN MURINE EMBRYONIC STEM CELL-DERIVED HEPATIC TISSUE SYSTEM

MASARU TSUTSUI, SHINICHIRO OGAWA, YOICHI INADA, EISUKE TOMIOKA, AKIKO KAMIYOSHI, SATORU TANAKA, TOMOYUKI KISHIDA, MASAHIKO NISHIYAMA, MAKOTO MURAKAMI, JUNJI KURODA, YASUHIKO HASHIKURA, SHINICHI MIYAGAWA, FUMIYASU SATOH, NOBUO SHIBATA, AND YOH-ICHI TAGAWA

Development Research, R&D, Kissei Pharmaceutical Co., Ltd., Nagano, Japan (M.T., Y.I., S.T., T.K., M.N., M.M., J.K., F.S., N.S.); Department of Surgery, Shinshu University School of Medicine, Nagano, Japan (S.O., Y.H., S.M.); Division of Laboratory Animal Research, Research Center for Human and Environmental Sciences, Shinshu University, Nagano, Japan (S.O., A.K., Y.T.); Department of Organ Regeneration, Shinshu University Graduate School of Medicine, Nagano, Japan (E.T.); and Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Kanagawa, Japan (E.T., Y.T.)

DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.007674
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title

Drug metabolism in ES cell-derived hepatic tissue system

Address correspondence to: Yoh-ichi Tagawa, Ph.D., Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, B-51 4259 Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa 226-8501, Japan. Phone: +81-45-924-5791, Fax: +81-45-924-5815, E-mail: ytagawa@bio.titech.ac.jp

The number of text page: 18

Number of tables: 1

Number of figures: 4

Number of references: 33

The number of words in the Abstract: 234 words

The number of words in Introduction: 356 words

The number of words in Discussion: 809 words

Abbreviations used are: HPRT, hypoxanthine phosphoribosyltransferase; AH, adult hepatocytes; FH, fetal hepatocytes; OHT, hydroxytestosterone

DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.007674
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT:

An *in vitro* system for liver organogenesis from murine embryonic stem (ES) cells has been recently established. This system is expected to be applied to the development of a new drug metabolism assay system that uses ES cells as a substitute for animal experiments. The objective of this study was to elucidate the drug metabolism profiles of the murine ES cell-derived hepatic tissue system compared with those of primary cultures of murine adult and fetal hepatocytes. The expression of the genes of the cytochrome P450 (CYP) family, such as *Cyp2a5, Cyp2b10, Cyp2c29, Cyp2d9, Cyp3a11,* and *Cyp7a1* was observed in the murine ES cell-derived hepatic tissue system at 16 days and 18 days after plating (A16 and A18). To investigate the activities of these Cyp family enzymes in the murine ES cell-derived hepatic tissue system at A16 and A18, testosterone metabolism in this system was analyzed. Testosterone was hydroxylated to 6β-hydroxytestosterone (6β-OHT), 16α-OHT, 2α-OHT, and 2β-OHT in this system, and was not hydroxylated to 15α-OHT, 7α-OHT, and 16β-OHT. This metabolism profile was similar to fetal hepatocytes, and different from adult hepatocytes. Further, pretreatment with phenobarbital resulted in a 2.5 and 2.6-fold increase in the production of 6β-OHT and 16β-OHT. Thus, evidence for drug metabolic activities in relation to Cyps has been demonstrated in this system. These results in this system would be a steppingstone of the research on the development and differentiation to adult liver.

Embryonic stem (ES) cells are pluripotent and can differentiate *in vitro* and *in vivo*. There have been several reports on the differentiation of murine or human ES cells into hepatocyte-like or albumin-producing cells and their isolation (Chinzei et al., 2002; Jochheim et al., 2004; Shirahashi et al., 2004); these cells also differentiate into a variety of other cell lineages. Thus far, in all of the above researches, the ES cells were differentiated into a single cell lineage by the addition of specific growth factors and chemicals to the culture. Limiting these differentiation systems during *in vivo* liver development is considered difficult because of the multiple functions and complex structure of the liver. However, we recently succeeded in establishing an *in vitro* system of liver morphogenesis by using murine ES cells (Ogawa et al., 2005). This system consists of not only hepatocytes but also cell lineages such as cardiomyocytes and endothelial cells that support liver-specific functions and differentiations. The system is more efficient with respect to hepatic functions such as albumin production and ammonia degradation. Further, the expression of the *transthyretin*, *α-fetoprotein*, *α1-antitrypsin*, and *tyrosine aminotransferase* genes is higher in this system than in the cultures of hepatic cell lines and murine primary cultures of adult hepatocytes. This system is expected to have many practical applications. It can be used in the development of new drugs and in drug metabolism assays as an alternative to animal experiments and *in vitro* experiments using primary hepatocytes. Further, it can be used for the development of bioartificial liver systems.

In the present study, we investigated drug metabolism capability of the ES cell-derived hepatic tissue system. First, to identify and quantify the expression of *cytochrome P450 (Cyp)* genes, quantitative

reverse transcription-polymerase chain reaction (RT-PCR) was employed. Second, the basal activities of selective Cyp enzymes were determined by measuring their testosterone hydroxylation activity and comparing with those observed in both murine adult and fetal hepatocytes. Further, the phenobarbital inducibility of selective Cyp enzymes was analyzed. This is the first study that investigates both the expression of the *Cyp* genes and the testosterone hydroxylation activities in a murine ES cell-derived hepatic tissue system.

Materials and Methods

Chemicals

Testosterone (4-androsten-17β-ol-3-on), 15α-hydroxytestosterone (15α-OHT), 6β-OHT, 2α-OHT, 2β-OHT, 11α-hydroxyprogesterone, and phenobarbital were purchased from Sigma (St. Louis, MO, USA). 16α-OHT, 16β-OHT, and 7α-OHT were purchased from Daiichi Pure Chemicals Corp. (Tokyo, Japan). All other chemicals used in this study were of analytical grade.

Differentiation of Murine Embryonic Stem Cells

E14-1 ES cells that were derived from 129/Ola mice (Japan SLC, Inc.) were grown on mitomycin C-treated mouse embryonic fibroblast feeder layers. The murine ES cell-derived hepatic tissue system, adult hepatocytes, and fetal hepatocytes were obtained as described previously (Ogawa et al., 2005). In brief, the E14-1 cells were used for all the following experiments as the parent ES cell line. These cells were dissociated with 0.25% trypsin, 1% chicken serum (Invitrogen Corp., Carlsbad, CA, USA), and 1 mM EDTA in phosphate-buffered saline (PBS) and resuspended in Iscove's modified Dulbecco's medium (IMDM; Invitrogen Corp., Carlsbad, CA, USA) containing 20% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 μM nonessential amino acids, and 100 μM 2-mercaptoethanol without leukemia inhibitory factor (LIF). The cells were cultured in a hanging drop (1000 cells per 50-μL drop) in an atmosphere of 5% CO_2 at 37℃ for 5 days. Twenty 5-day-old embryoid bodies (EBs) were plated on a 6-cm dish coated with gelatin containing the differentiation medium. The day on which the 5-day-old EBs were plated on the dish was denoted as day 0 (A0).

Isolation and Culture of Murine Primary Hepatocytes

DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.007674
This article has not been copyedited and formatted. The final version may differ from this version.

To obtain fetal hepatocytes, 129/sv mouse liver at E15 was minced and dissociated with collagenase II (Sigma, St Louis, MO, USA) in Hanks' buffer (Invitrogen Corp., Carlsbad, CA, USA). The cells were seeded on a gelatin-coated dish (6-well plate) at a density of 2×10^5 cells/dish containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 μM nonessential amino acids, and a mixture of 100 U/mL penicillin, 1000 μg/mL streptomycin, and 292 μg/mL glutamine for a few hours and washed once with the same as the ES cell-derived hepatic tissue system cultured medium. The medium was changed daily.

Primary adult hepatocytes were isolated from 129/sv male mice (Japan SLC, Inc., 6 - 10 weeks old) using the two-step collagenase perfusion method. The hepatocytes were separated from the resulting cell suspension by centrifugation and again centrifuged through a 50% Percoll gradient (Sigma, St Louis, MO, USA). The isolated hepatocytes were plated onto gelatin-coated dishes (6-cm) at a density of 4×10^5 cells/dish. Culture medium was the same as above and was changed a few hours later to remove unattached hepatocytes. After 12 hours of culture, these murine primary hepatocytes were used for the experiment.

The animal experiments were carried out at the Research Center for Human and Environmental Sciences, Shinshu University, and were conducted in accordance with the ethical guidelines of the Shinshu University.

Assay for Albumin Protein

To confirm albumin production, samples of the cultured medium were analyzed using the Lebis ELISA kit (Shibayagi, Gunma, Japan). After daily incubation with the murine ES cell-derived hepatic

tissue system, the media samples were collected at 15, 16, 17, and 18 days after plating (A15, A16, A17, and A18).

Quantitative RT-PCR Analysis for Cytochrome P450 Gene Expression

Total RNA was isolated from the cell pellets using the "SV Total RNA Isolation System" (Promega, Tokyo, Japan) along with the DNase treatment step. The mRNA levels in the ES-derived hepatic tissue system at A16 and A18, 12-h-old and 60-h-old primary cultured adult and fetal hepatocytes were determined by quantitative RT-PCR using SYBR green and GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

The primers used for quantitative RT-PCR are listed in Table 1. All the primers were synthesized by Operon Technologies (Tokyo, Japan). Designed primers included Cyp2b10 (Jackson et al., 2004), Cyp2d9, Cyp3a11 (Jinmel et al., 2000), Cyp2a5, Cyp7a1 (Wang and Seed, 2003), 2c29 (Jackson et al., 2004), and albumin (Miyake et al., 2002). HPRT (hypoxanthine phosphoribosyl transferase) that is a housekeeping gene was used as the internal standard (Ball et al., 2002).

Testosterone Metabolism Assay

Murine ES cell-derived hepatic tissue system at A16 and A18, 12-h-old and 60-h-old primary cultured adult and fetal hepatocytes were washed with Dulbecco's phosphate-buffered saline (Invitrogen Corp., Carlsbad, CA, USA) and incubated for 40 min at 37℃ with the cultured medium containing 0.25 mM testosterone (Sigma, St Louis, MO, USA). Following incubation, the reaction was terminated by aspirating the medium from the plates. The amounts of testosterone metabolite products, i.e., 15α-hydroxytestosterone (15α-OHT), 6β-OHT, 7α-OHT, 16α-OHT, 16β-OHT, 2α-OHT, and 2β-OHT,

were measured according to the method previously described by Arlotto et al. (2002) with some modification. In brief, the cultured medium samples were placed in 5 mL ethyl acetate, and 11α-hydroxyprogesterone was added as an internal standard. Subsequently, it was vigorously mixed in a vortex mixer and centrifuged at 3000 rpm for 5 min. The organic phase was transferred and evaporated to dryness under a stream of nitrogen gas. The residue was reconstituted in 100 μL of methanol-water (1:1 vol/vol) and analyzed by high performance liquid chromatography (HPLC). The HPLC conditions were as follows: column, Cadenza CD-C18 (15 cm × 4.6 mm ID; Intakt, Kyoto, Japan); mobile phases, A: methanol-water-acetonitrile (39:60:1 vol/vol) and B: methanol-water-acetonitrile (80:18:2 vol/vol). The gradient elution system was as follows: 0 min, B = 18%; 12 min, B = 18%; 17 min, B = 80%; 20 min, B = 80%; 23 min, B = 18%; and 35 min, B = 18%. The metabolites were detected by UV absorbance at 254 nm. The retention times of the testosterone metabolites were as follows: 15α-OHT, 14.4 min; 6β-OHT, 14.8 min; 7α-OHT, 15.8 min; 16α-OHT, 20.6 min; 16β-OHT, 23.7 min; 2α-OHT, 25.0 min; 2β-OHT, 25.4 min; 11α-Hydroxyprogesterone, 26.4 min; and testosterone, 28.8 min. The peak of each metabolite was compared with that of the internal standard to determine the amount of metabolites.

Phenobarbital Induction Assay

The murine ES cell-derived hepatic tissue system at A21, 12-h-old primary cultured adult and fetal hepatocytes were exposed to 2 mM phenobarbital (PB) for 48 hours. PB was prepared as an aqueous solution and added directly to the culture daily. The culture was then treated with 0.25 mM testosterone, and the amounts of 6β-OHT and 16β-OHT were measured by HPLC using the method described in the

above section (testosterone metabolism).

Statistical analysis

Each experiment was performed in three different cultures. Results are shown as means ± SE. $p<0.05$ was considered as statistically significant.

Results

Murine Embryonic Stem Cell-derived Hepatic Tissue System

Twenty 5-day-old EBs were placed together on gelatin-coated plastic dishes containing the differentiation medium. Contracting cardiomyocytes appeared in the EB outgrowths. The EB outgrowths were cultured in the differentiation medium for 18 days (A18), and then were formed to hepatic tissue-like morphology having endothelial cell-networks and albumin-producing cell area, as previously reported (Ogawa et al., 2005). The amount of albumin released from the ES cell-derived hepatic tissue system into the medium was measured in each medium at A15–18 by enzyme-linked immunosorbent assay (ELISA). As seen in FIG. 1, the albumin level increased gradually from A15 to A18. This result suggests that the ES cell-derived hepatic tissue system had been differentiated from ES cells.

Cyp Family Genes were Expressed in the Murine Embryonic Stem Cell-Derived Hepatic Tissue System.

The Cyp enzymes catalyze the oxidative metabolism of endogenous and exogenous compounds and play a major role in the biotransformation of xenobiotics. To measure the constitutive expression of the *Cyp family* genes in the murine ES cell-derived hepatic tissue system, quantitative RT-PCR was employed. The expression of the *Cyp family* genes (*Cyp2a5*, *Cyp2b10*, *Cyp2c29*, *Cyp2d9*, and *Cyp3a11*), which regard to the hydroxyl metabolism of testosterone, was detected in the murine ES cell-derived hepatic tissue system at A16 and A18), 12-h-old and 60-h-old primary cultured adult and fetal hepatocytes (FIG. 2). Further, the expression of the liver specific genes, *Cyp7a1* and *Albumin*, was

also observed in the murine ES cell-derived hepatic tissue system (FIG.2), and this system was shown to be actually differentiated ES cells into liver tissue.

Testosterone Metabolism Profiles

Testosterone is metabolized in a regioselective manner by different Cyp enzymes and can be used as a multi-enzymatic substrate to simultaneously investigate the activities of multiple enzymes. Testosterone (0.25 mM) was added into the ES cell-derived hepatic tissue system at A16 and A18) and 12-h-old and 60-h-old primary cultured adult and fetal hepatocytes, and then the cells were exposed for 40 min (FIG. 3). The metabolized testosterone was analyzed as hydroxylated products (15α-OHT, 6β-OHT, 7α-OHT, 16α-OHT, 16β-OHT, 2α-OHT, and 2β-OHT). Specifically, the products (15α-OHT, 6β-OHT, 7α-OHT, 16α-OHT, 16β-OHT, and 2α-OHT) are the indexes of specific cytochrome P450 (Cyp2a4/5, Cyp3a, Cyp2a4/5 and 2d9, Cyp2d9 and 2b, Cyp2c29 and 2b, and Cyp2d) activities. In the murine ES cell-derived hepatic tissue system, 6β-OHT, 16β-OHT, 2α-OHT, and 2β-OHT were observed at A16 and A18; however, 15α-OHT, 7α-OHT, and 16α-OHT were not observed. This hydroxylation pattern of the murine ES cell-derived hepatic tissue system was similar to that of the fetal hepatocytes, and was different from adult hepatocytes. The Cyp functionality in the murine ES cell-derived hepatic tissue system at A16 and A18 was assessed from these results, and it was demonstrated similar to the fetal hepatocytes.

Phenobarbital Induces Cyp Expression in Embryonic Stem Cell-Derived Hepatic Tissue System.

The induction of Cyp expression is a common cellular defense mechanism against the toxicity of

foreign compounds. The ES cell-derived hepatic tissue system, adult hepatocytes, and fetal hepatocytes were incubated for 48 h with 2 mM PB, and the induction potential of Cyp expression was investigated by measuring the amounts of testosterone metabolite products (6β-OHTand 16β-OHT). In the ES cell-derived hepatic tissue system, the hydroxylation of testosterones (i.e., 6β-OHT, and 16β-OHT) was 2.5 and 2.6-fold higher in the presence of PB than in the absence of PB, as shown in FIG. 4A. Further, PB increased the amounts of 6β-OHT and 16β-OHT in the murine adult hepatocytes (by 1.3 and 2.3-fold, FIG. 4B) and fetal hepatocytes (by 1.9 and 1.1-fold, FIG. 4C). These results revealed that the murine ES cell-derived hepatic tissue system shows the same Cyp inducibility as the murine adult hepatocytes and fetal hepatocytes.

Discussion

The purpose of this study was to characterize the metabolic capability of the novel *in vitro* system for liver organogenesis from murine ES cells, namely, the murine ES cell-derived hepatic tissue system. We have detected the expression of not only the *Cyp2b5*, *Cyp2b10*, *Cyp2c29*, *Cyp2d9*, and *Cyp3a11* genes but also that of the *Cyp7a1* gene in the murine ES cell-derived hepatic tissue system. *Cyp7a1* is known as a liver-specific gene; it is not expressed in the yolk sac and is induced in developing embryonic bodies (Asahina et al., 2004). Therefore, our result demonstrated that the murine ES cell-derived hepatic tissue system has differentiated ES cells into hepatic tissue. Further, Cyp enzyme (Cyp2b, Cyp2d, Cyp2c29, and Cyp3a) activities were identified in this system by measuring the testosterone metabolite products. Therefore, it has been demonstrated that drug metabolizing Cyp enzymes are truly present and are functionally active in the murine ES cell-derived hepatic tissue system.

The liver plays a major role in drug metabolism. Primarily, this can be attributed to the relatively high exposure of the liver to chemicals. Recently, cultured hepatocytes are widely being used for drug metabolism and toxicity studies for new drug development (Rodríguez-Antona et al., 2000; Guilouzo, 1998; Tirona et al., 2003). The endpoints for *in vitro* hepatocytes functions such as albumin secretion, mitochondrial function, lactate dehydrogenase leakage, generation of reactive oxygen species, activities of alanine aminotransferase and aspartate aminotransferase, or intracellular glutathione levels have been generally evaluated in these cultured hepatocytes (Riss and Morvec, 2004; Trohalaki S et al., 2002; Viluksela et al., 1996; Smet et al., 2000; Delraso and Channel, 1999).

However, other studies have also reported that the activities of some enzymes and some hepatic functions are modified by certain employed *in vitro* conditions (Khetani et al., 2004; Guillouzo and Guguen-Guillouzo, 1992; Rogiers et al., 1995). For example, cultured hepatocytes expressing parenchymal functions could be maintained only in monolayer cultures for a limited period and showed a rapid decay of many of their differentiated cell properties and functions, particularly of drug metabolizing enzymes, in other cultures (Gómez-Lechón et al. 1990; Guillouzo 1998). Further, it has been reported that *in vitro* hepatic models using immortalized hepatocyte-like cell lines or hepatoma-derived cell lines are not considered to be fully representative of hepatic functions in the view of their drug metabolizing enzymes (Courjault-Gautier et al., 1997; Grant et al, 1988; Donato et al. 1994, 1999, 2003; Rodriguez-Antona et al. 2002).

Our novel *in vitro* system consists of not only hepatocytes but also other cell lineages such as cardiomyocytes and endothelial cells that support liver-specific functions and differentiations; these cell lineages correspond to those involved in liver organogenesis *in vivo* (Ogawa et al., 2005). Further, it also shows higher levels of hepatic functions such as albumin production and ammonia degradation as well as the expression of *transthyretin*, *α-fetoprotein*, *α1-antitrypsin*, and *tyrosine aminotransferase* genes than those observed in the cultures of other hepatic cell lines and murine primary culture of adult hepatocytes. In the present study, the expression of *Cyp* genes and their activities in the murine ES cell-derived hepatic tissue system were characterized and it is suggested that the murine ES cell-derived hepatic tissue system has the capability to maintain drug metabolic functions. In addition, hepatocyte-endothelial cell contact is very important for the generation of full

hepatic function in hepatocytes, liver development, and proliferation of the embryo (Ogawa et al., 2005). Based on these findings, this system is expected to be able to mimic *in vivo* functions of the liver.

PB is the prototype for a large number of structurally diverse chemicals that induce Cyp belonging to the *Cyp2b* and *Cyp3a* gene families (Waxman and Azaroff, 1992; Gonzalez et al., 1993; Denison and Whitlock, 1995) and other xenochemical-metabolizing enzymes (Waxman and Azaroff, 1992; Honkakoshi et al., 1998a). In the present study, PB was used to confirm Cyp inducibility in the murine ES cell-derived hepatic tissue system; in fact, PB increased the production of testosterone metabolites (namely, 6β-OHT and 16β-OHT) in this system. Therefore, it is confirmed that PB-induction of Cyp, a specific metabolic property of primary hepatocytes, is also present in the murine ES cell-derived hepatic tissue system.

A part of testosterone hydroxylation (6β-OHT, 16β-OHT, 2α-OHT, and 2β-OHT) were detected and maintained in ES cell-derived hepatic tissue system at A16 and A18. But, 15α-OHT, 7α-OHT, and 16α-OHT were not detected. This phenomenon was similar to those of fetal hepatocytes and was different from adult hepatocytes that all of the hydroxylated testosterone was detected and its activities were markedly decreased within 60 hours.

Thus, in Cyp enzyme expression, the murine ES cell-derived hepatic tissue system at A16 and A18 is characterized, and it is similar to fetal hepatocytes.

In the future, detailed studies to investigate the mechanisms of this phenomenon would be helpful to characterize the detailed metabolic profile of this system for development and differentiation to intact

liver.

Acknowledgments. We are grateful to Prof. Nobuaki Yoshida and Prof. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo) for providing us the E14.1 ES cells and RIKEN Cell Bank for the STO cell line.

References

Arlotto MP, Trant JM and Estabrook RW (1991) Measurement of steroid hydroxylation reactions by high-performance liquid chromatography as indicator of P450 identity and function. *Methods Enzymol* **206**:454-462.

Asahina K, Fujimori H, Shimizu-Saito K, Kumashiro Y, Okamura K, Tanaka Y, Teramoto K, Arii S and Teraoka H (2004) Expression of the liver-specific gene Cyp7a1 reveals hepatic differentiation in embryoid bodies derived from mouse embryonic stem cells. *Genes Cells* **9**:1297-1308.

Ball HJ, McParland B, Driussi C and Hunt NH (2002) Isolating vessels from the mouse brain for gene expression analysis using laser capture microdissection. *Brain Res Proto* **9**:206-213.

Chinzei R, Tanaka Y, Shimizu-Saito K, Hara Y, Kakinuma S, Watanabe M, Teramoto K, Arii S, Takase K, Sato C, Terada N and Teraoka H (2002) Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. *Hepatology* **36**:22-29.

Courjault-Gautier F, Antoine B, Bens M, Vallet V, Cluzeaud F, Pringault E, Kahn A, Tourain H and Vandewalle (1997) Activity and inducibility of drug-metabolizing enzymes in mmortalized hepatocyte-like cells (mhPKT) derived from a $_{L}$-PK/Tag1 transgenic mouse. *Exp Cell Res* **234**:362-372.

Delraso NJ and Channel SR (1999) Novel *in vitro* method for rapid screening of volatile compounds using primary or immortalized rat hepatocytes. *Toxicol Methods* **9**:137-152.

Denison MS and Whitlock JP Jr (1995) Xenobiotic-inducible transcription of cytochrome P450 genes.

J Biol Chem **270**:18175-18178.

De Smet K, Bruning T, Blaszkewicz M, Bolt HM, Vercruysse A and Rogiers V (2000) Biotransformation of trichloroethylene in collagen gel sandwich culture of rat hepatocytes. *Arch Toxicol* **74**:587-592.

Donato MT, Bassi AM, Gómez-Lechón MJ, Penco S, Herrero E, Adamo D, Castell JV and Ferro M (1994) Evaluation of the xenobiotic transformation capability of six rodent hepatoma cell lines in comparison with rat hepatocytes. *In Vitro Cell Dev Biol* **30**:574-580.

Donato MT, Castell JV and Gómez-Lechón MJ (1999) Characterization of drug metabolizing activities in pig hepatocytes for use in bioartificial liver devices: comparison with other hepatic cellular models. *J Hepatol* **31**:542-549.

Donato MT, Klocke R, Castell JV, Stenzel K, Paul D and Gómez-Lechón MJ (2003) Constitutive and inducible expression of CYP enzymes in immortal hepatocytes derived from SV40 transgenic mice. *Xenobiotica* **33**:459-473.

Gómez-Lechón MJ, López P, Donato MT, Montana A, Larrauri A, Giménez P, Trullenque R, Fabra R, and Castell JV (1990) Culture of human hepatocytes from small surgical biopsies. Biochemical characterization and comparison with *in vivo*. *In Vitro Cell Dev Biol* **26**:67-74.

Gonzalez FJ, Liu SY and Yano M (1993) Regulation of cytochrome P450 genes: Molecular mechanisms. *Pharmacogenetics* **3**:51-57.

Grant MH, Duthie SJ, Gray AG and Burke MD (1988) Mixed function oxidase and UDP-glucronyltransferase activities in human HepG2 hepatoma cell line. *Biochem Pharmacol*

37:4111-4116.

Guillouzo A (1998) Liver cell models in in vitro toxicology hepatocytes. *Environ Health Perspect* **106**:511-531.

Guillouzo A and Guguen-Guillouzo C (1992) *In vitro* approaches to hepatotoxicity studies. In *In Vitro Methods in Toxicology* (Jolles G and Cordier A eds) pp 132-163, Academic Press, London.

Honkakoshi P, Moore R, Washburn K and Negishi M (1998a) Activation by diverse xenochemicals of the 51-base pair phenobarbital-responsive enhancer module in the CYP2B10 gene. *Mol Pharmacol* **53**:597-601.

Jackson JP, Ferguson SS, Moore R, Negishi M and Goldstein JA (2004) The constitutive active/androstane receptor regulates phenytoin induction of Cyp2c29. *Mol Pharmaco*l **65**:1397-1404.

Jochheim A, Hillemann T, Kania G, Scharf J, Attaran M, Manns MP, Wobus AM and Ott M (2004) Quantitative gene expression profiling reveals a fetal hepatic phenotype of murine ES-derived hepatocytes. *Int J Dev Biol* **48**:23-29.

Khetani SR, Szulgit G, Rio JA, Barlow C and Bhatia SN (2004) Exploring interactions between rat hepatocytes and nonparenchymal cells using gene expression profiling. *Hepatology* **40**:545-554.

Miyake K, Ogawa W, Matsumato M, Nakamura T, Sakaue H and Kasuga M (2002) Hyperinsulinemia, glucose intolerance, and dyslipidemia induced by acute inhibition of phosphoinositide 3-kinase signaling in the liver. *J Clin Investig* **110**:1483-1491.

Ogawa S, Tagawa Y, Kamiyoshi A, Suzuki A, Nakayama J, Hashikura Y and Miyagawa S (2005) Crucial roles of mesodermal cell lineages in a murine embryonic stem cell-derived in vitro liver

organogenesis system. *Stem Cells* **23**:903-913.

Pan J, Xiang Q and Ball S (2000) Use of a novel real-time quantitative reverse transcription-polymerase chain reaction method to study the effects of cytokines on cytochrome P450 mRNA expression in mouse liver. *Drug Metab Dispos* **28**:709-713.

Riss TL and Moravec RA (2004) Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay Drug Dev Technol* **2**:51-62.

Rodriguez-Antona C, Donato MT, Boobis A, Edwards RJ, Watts PS, Castell JV and Gómez-Lechón MJ (2002) Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. *Xenobiotica* **32**:505-520.

Rodriguez-Antona C, Jover R, Gomez-Lechon MJ and Castell JV (2000) Quantitative RT-PCR measurement of human cytochrome P-450s: application to drug induction studies. *Arch Biochem Biophys* **376**:109-116.

Rogiers V, Blaauboer B, Maurel P, Phillips I, and Shepards E (1995) Hepatocyte-based in vitro models and their application in pharmacotoxicology. *Toxicol In Vitro* **9**,685-694.

Shirahashi H, Wu J, Yamamoto N, Catana A, Wege H, Wager B, Okita K and Zern MA (2004) Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage. *Cell Transplant* **13**:197-211.

Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez FJ, Schuetz EG and Kim RB (2003) The orphan nuclear receptor HNF4alpha determines PXR- and

CAR-mediated xenobiotic induction of CYP3A4. *Nat Med* **9**:220-224.

Trohalaki S, Zellmer RJ, Pachter R, Hussain M and Frazier JM (2002) Risk assessment of high-energy chemicals by *in vitro* toxicity screening and quantitative structure-activity relationships. *Toxicol Sci* **68**:498-507.

Viluksela M, Vainio PJ and Tuominen RK (1996) Cytotoxicity of macrolide antibiotics in a cultured human liver cell line. *J Antimicrob Chemother* **38**:465-473.

Wang X and Seed B (2003) A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res* **31**:154-161.

Waxman DJ and Azaroff L (1992) Phenobarbital induction of cytochrome P-450 gene expression. *Biochem J* **281**:577-592.

Footnotes

This study was supported by grants from the Ministry of Education, Sports, Science and Technology of Japan (Tokyo, Japan) (15700314; 13470150, Grant-in-Aid for 21st Century COE program), and the Hokuto Foundation of Bioscience (Nagano, Japan).

The name and full address of person to receive reprint requests: Masaru Tsutsui, 2320-1 Maki, Hotaka, Azumino, Nagano, 399-8305, Japan

Legends for figures

FIG. 1. Time-course of albumin production in the murine embryonic stem cell-derived hepatic tissue system

The amount of albumin production is expressed in nanograms. Each value represents the mean ± SE of three different cultures.

FIG. 2. Cyp gene expressions in the murine embryonic stem cell-derived hepatic tissue system, adult hepatocytes, and fetal hepatocytes

The gene expressions are expressed as the hold of gene expression in 60-h-old primary cultured adult hepatocytes (60-h-old AH).

Each value represents the mean ± SE of three different cultures.

12-h AH and 60-h AH, 12-h-old and 60-h-old primary cultured murine adult hepatocytes; A16 ES and A18 ES, murine embryonic stem cell-derived liver tissue at 16 days and 18 days after plating; 12-h FH and 60-h FH, 12-h-old and 60-h-old primary cultured murine fetal hepatocytes; N.S., no significance

*p<0.05 and **p<0.01

FIG. 3. Testosterone hydroxylation in the murine embryonic stem (ES) cell-derived hepatic tissue system (A), fetal hepatocytes (B), and adult hepatocytes (C)

Activities were assayed in the murine ES cell-derived hepatic tissue system at 16 days and 18 days

after plating (A), 12-h-old and 60-h-old primary cultured mouse fetal (B) and adult (C) hepatocytes. The activities are expressed as nmol/min/mg albumin. The values represent the means ± SE of three different cultures.

15α, 6β, 7α, 16α, 16β, 2α, and 2β indicate the formation of the testosterone hydroxylation. 2a4/5, 2b, 2d, 2d9, 2c29, and 3a indicate cytochrome P450 isozymes. The activities are expressed as nmol/min/mg albumin. The values represent the means ± SE of three different cultures.

*p<0.05 and **p<0.01 with respect to 16 days after plating (A), 12-h-old primary cultured fetal hepatocytes (B), 12-h-old primary cultured adult hepatocytes (C) according to Student's *t*-test

FIG. 4. Phenobarbital (PB) inductions on testosterone hydroxylation activities in the murine embryonic stem (ES) cell-derived hepatic tissue system at A21, fetal hepatocytes, and adult hepatocytes

The murine ES cell-derived hepatic tissue system at A21 (A), and 12-h-old primary cultured mouse fetal (B) and adult (C) hepatocytes were exposed to PB, and testosterone hydroxylation activities was assayed 48 hours later as described under *materials and methods*. The activities are expressed as a hold of the control (noninduced).

6β and 16β indicate the formation of the testosterone hydroxylation. 2b, 2d9, 2c29, and 3a indicate cytochrome P450 isozymes.

*p<0.05, **p<0.01 with respect to the control (noninduced) according to Student's *t*-test.

DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.007674
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1

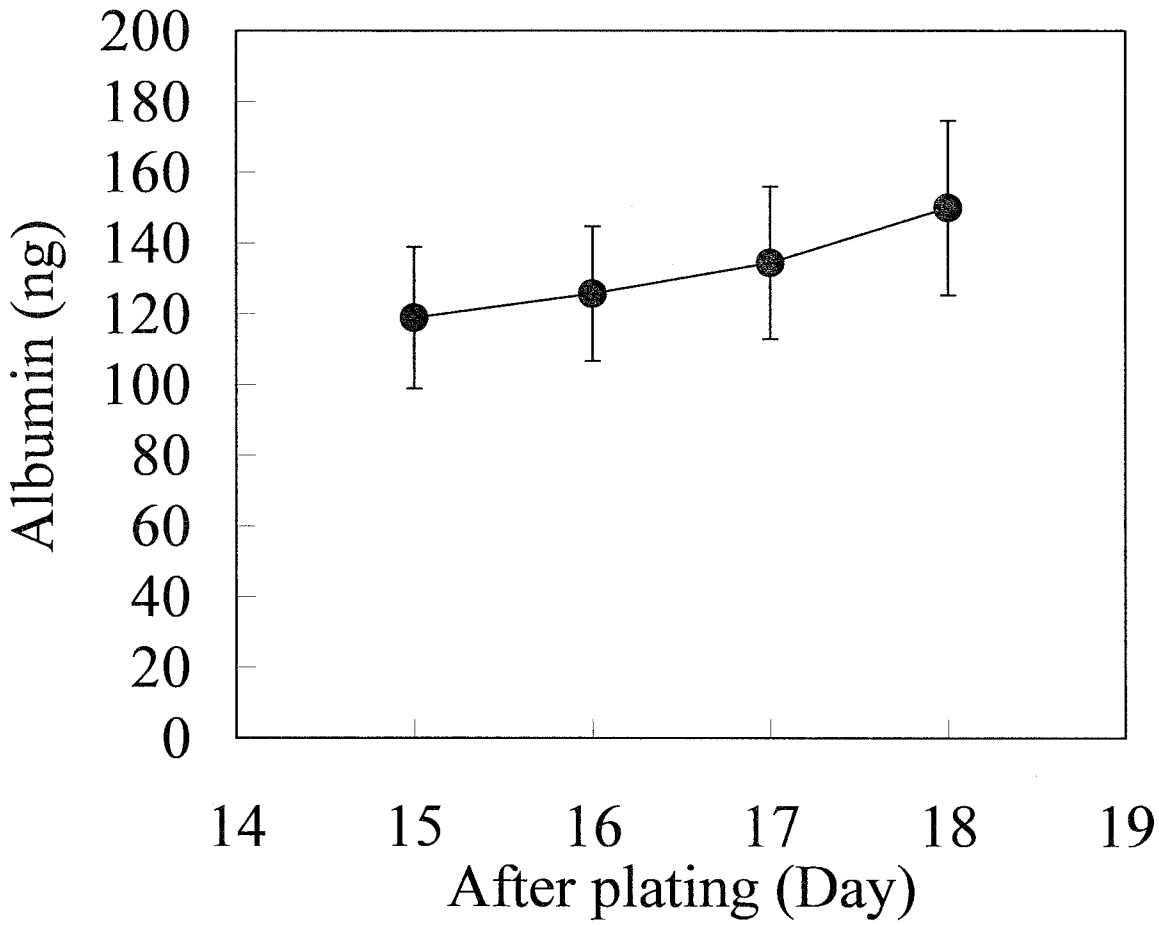
Primer sequences for quantitative RT-PCR

Name	Primers	5' – 3' Sequence	Amplicon (bp)	Accession No.
Cyp2a5	Forward	ATGCTGACCTCAGGACTCCTC	217	NP_031838
	Reverse	GGTAGATGGTGAATACAGGACCA		
Cyp2b10	Forward	ACCCCACGTTCCTCTTCCA	100	NM_009999
	Reverse	CAGCAGGCGCAAGAACTGA		
Cyp2c29	Forward	GCTCAAAGCCTACTGTCA	179	NM_007815
	Reverse	CATGAGTGTAAATCGTCTCA		
Cyp2d9	Forward	TGGCACAGATAGAGAAGGCCA	76	NM_010006
	Reverse	TCACGCACCACCATGAGC		
Cyp3a11	Forward	GGATGAGATCGATGAGGCTCTG	73	NM_007818
	Reverse	CAGGTATTCCATCTCCATCACAGT		
Cyp7a1	Forward	AGCATTTCTTTGATCTGGGGG	185	NP_031850
	Reverse	CCATGTTTCCTTTGCTTTGCTCT		
HPRT	Forward	GCTTTCCCTGGTTAAGCAGTACA	125	MUSHPRT
	Reverse	CAAACTTGTCTGGAATTTCAAATC		
Albumin	Forward	GACTTTGCACAGTTCCTGGATACA	125	MMU457860

Reverse	TTGTGGTTGTGATGTGTTTAGGCTA

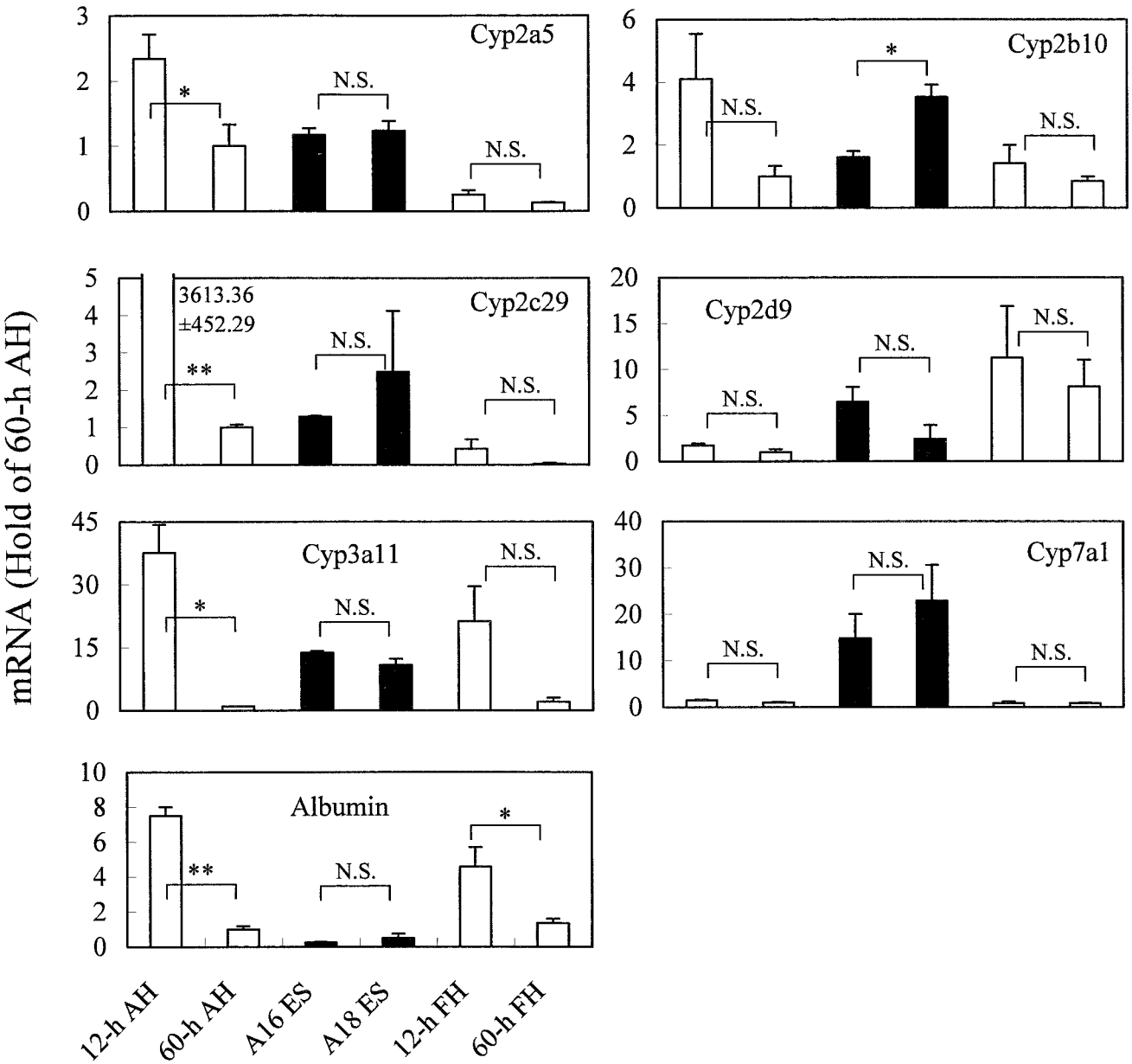
DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.007674

FIG.1



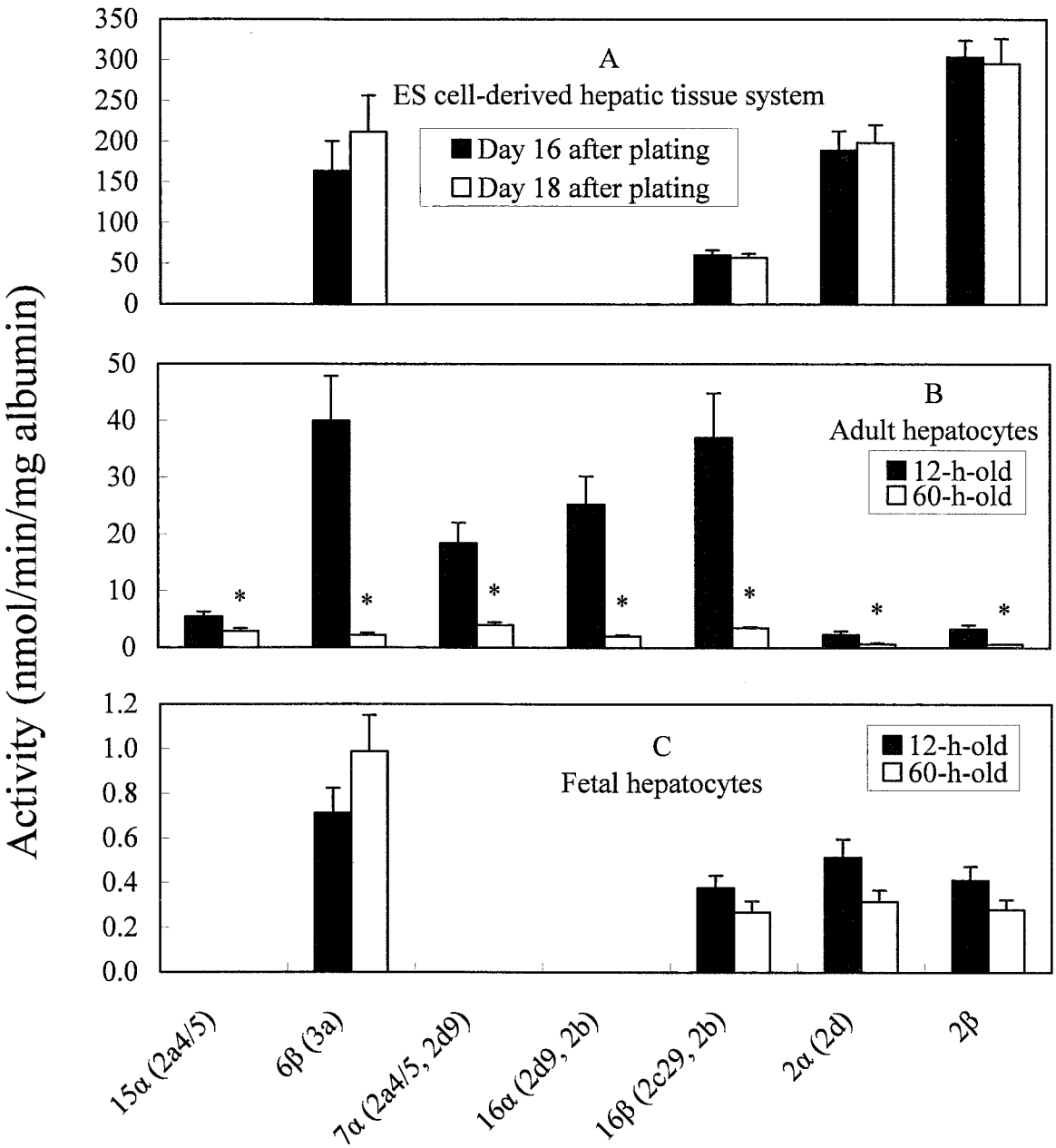
DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.007674
This article has not been copyedited and formatted. The final version may differ from this version.

FIG.2



DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.007674

FIG.3



DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.007674

FIG.4