Expression of the liver-specific gene Cyp7a1 reveals hepatic differentiation in embryoid bodies derived from mouse embryonic stem cells

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Hepatic differentiation from mouse embryonic stem (ES) cells via the formation of embryoid bodies (EBs) has been revealed by the expression of hepatocyte-related genes such as α-fetoprotein and albumin. It is known, however, that the visceral endoderm differentiates in early EBs and expresses these hepatocyte-related genes. Thus, it remains unclear whether ES cells are capable of differentiating into hepatocytes derived from definitive endoderm *in vitro*. In the present study, yolk sac tissues isolated from the foetal mouse were found to express many hepatocyte-related genes. Among the hepatocyte-related genes examined, cytochrome P450 7A1 (Cyp7a1) was identified as a liver-specific gene that was not expressed in the yolk sac. Cyp7a1 was induced in developing EBs, and hepatic differentiation was preferentially observed in the developing EBs in attached culture as compared to those in suspension culture. Leukaemia inhibitory factor permitted the differentiation of visceral endoderm, but inhibited the expression of gastrulation-related genes and the hepatic differentiation in cultured EBs. ES cells expressing green fluorescent protein (GFP) under the control of the Cyp7a1 enhancer/promoter showed that cultured EBs contained GFP-positive epithelial-like cells. These results demonstrate that ES cells can differentiate *in vitro* into hepatocytes derived from definitive endoderm.

Introduction

During mouse embryogenesis, the inner cell mass of blastocysts differentiates into the primitive endoderm and primitive ectoderm on embryonic (E) day 4.5 (Beddington & Robertson 1999). The primitive endoderm gives rise to the visceral and parietal endoderm, and subsequently forms the extraembryonic yolk sac. The yolk sac also contains mesoderm that is derived from the epiblast. The visceral endoderm expresses many genes that are also expressed in hepatocytes including α-fetoprotein (Afp), albumin (Alb), and transthyretin (Ttr), and plays an important role in axis formation during early embryogenesis as well as in foetal development by the formation of the yolk sac (Dziadek & Adamson 1978; Meehan *et al.* 1984; Sellem *et al.* 1984; Makover *et al.* 1989; Beddington & Robertson 1999). It should be noted that the visceral endoderm gives rise to the extraembryonic yolk sac, but not to the liver or pancreas (Gardner & Rossant 1979).

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The primitive ectoderm develops into the three embryonic germ layers of ectoderm, mesoderm, and endoderm through the gastrulation on E6.5. The definitive endoderm migrates along the mid line, intercalates into the visceral endoderm, and develops into the endodermal gut tube in the mouse embryo by E7.5 (Wells & Melton 1999). The ventral foregut region develops into both hepatic and pancreatic anlagen under the influence of cardiac mesoderm and septum transversum on E8.5 (Deutsch *et al.* 2001).

Mouse embryonic stem (ES) cell lines have been established from the inner cell mass of blastocysts (Evans & Kaufman 1981; Martin 1981). ES cells are pluripotent, and remain in an undifferentiated state in a medium containing leukaemia inhibitory factor (LIF) (Williams *et al.* 1988). When ES cells are allowed to aggregate in the absence of LIF, ES cells form embryoid bodies (EBs), which initially consist of an outer layer of primitive endoderm and an inner primitive ectoderm that are similar to the early embryos at the egg cylinder stage (Doetschman *et al.* 1985). EBs develop into many different cell types in culture, indicating that ES cells may be

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a useful source for cell replacement therapy if particular cell types can be induced from ES cells *in vitro*.

Hepatic differentiation from mouse ES cells has been shown by the expression of hepatocyte-related genes such as Afp, Alb, Hnf4 and Ttr in developing EBs (Hamazaki *et al*. 2001; Chinzei *et al*. 2002; Miyashita *et al*. 2002; Yamada *et al*. 2002; Kuai *et al*. 2003). However, these hepatocyte-related genes are expressed in both the liver and visceral endoderm of the yolk sac (Dziadek & Adamson 1978; Meehan *et al*. 1984; Sellem *et al*. 1984; Makover *et al*. 1989; Duncan *et al*. 1997). Therefore, it remains unclear whether ES cells are capable of differentiating into definitive endoderm as well as visceral endoderm *in vitro* (Abe *et al*. 1996). Although Jones *et al*. (2002) demonstrated hepatic differentiation using an ES cell line expressing β-galactosidase only in hepatocytes, hepatic differentiation in commonly used mouse ES cell lines has not been demonstrated due to the lack of a specific marker for hepatocytes.

To address whether hepatocytes can be differentiated in developing EBs *in vitro*, we sought out genes that were specifically expressed in the liver, but not in the yolk sac. Among the hepatocyte-related genes examined, we identified cytochrome P450 (Cyp) 7a1 encoding CYP7A1 (cholesterol 7α-hydroxylase) as a gene expressed in the liver but not in the yolk sac, and found that its expression can be induced in developing EBs *in vitro*. Furthermore, we generated an ES cell line expressing green fluorescent protein (GFP) under the control of the Cyp7a1 enhancer/promoter, and visualized GFP-positive epithelial-like cells in the developing EBs. These results demonstrate that ES cells can differentiate *in vitro* into hepatocytes of definitive endoderm.

Results

Differentiation of EBs in culture

EBs formed from a BL6 mouse ES cell line were cultured on dishes coated with gelatin as previously described (Chinzei *et al*. 2002). Morphologically distinct cell types migrated away from the attached EBs, and a yolk sac-like structure was found on culture day 15 (Fig. 1A). Immunocytochemistry showed that a cluster of ALB-positive cells appeared in a multilayered structure of EBs, but not in the yolk sac-like structure on culture day 15 (Fig. 1B–D). These results imply that hepatocytes can be differentiated in cultured EBs. However, ALB has been shown to be expressed in both hepatocytes and the visceral endoderm of the yolk sac during mouse development (Sellem *et al*. 1984). Therefore, we could not necessarily conclude that these ALB-positive cells exhibited differentiation into hepatocytes in the cultured EBs.

Identification of genes expressed in mouse liver but not in the yolk sac

In order to identify the genes expressed in the liver, which is derived from definitive endoderm, but not in the yolk sac, which is derived from visceral endoderm, we determined the expression of hepatocyte-related genes in the mouse yolk sac and liver by RT-PCR. Many hepatocyte-related genes are significantly expressed in both the yolk sac and the liver as previously reported (Dziadek & Adamson 1978; Meehan *et al*. 1984; Sellem *et al*. 1984; Makover *et al*. 1989; Duncan *et al*. 1997). We

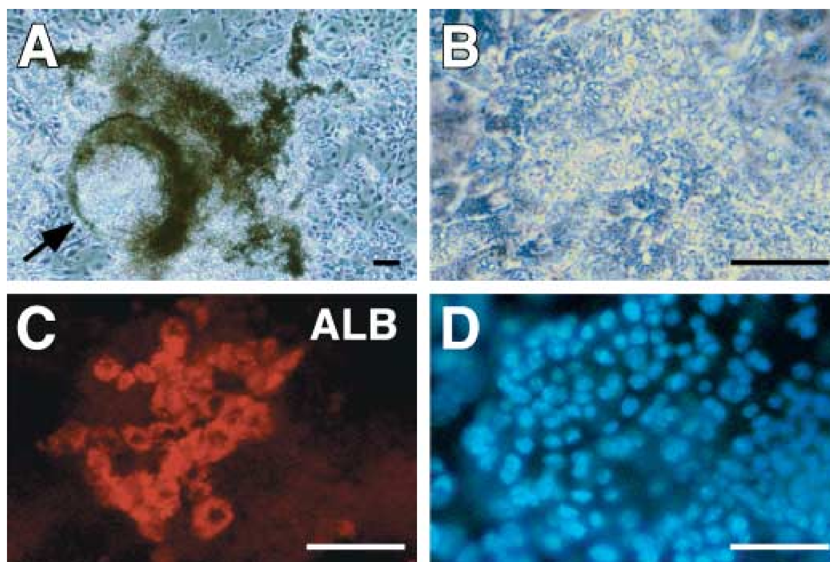

Figure 1 Development of EBs in attached culture on day 15. EBs were cultured on dishes coated with gelatin in the differentiation medium. (A) Developing EBs on day 15. Morphologically distinct cell types migrated away from the attached EBs and a yolk sac-like structure (arrow) was formed. (B) A multilayered structure of developing EBs on day 15. (C) Immunocytochemistry with anti-ALB antibodies. A fluorescent image of the same field shown in (B). A cluster of ALB-positive cells (red) were seen in the multilayered structure. (D) Visualization of nuclei by DAPI staining. A fluorescent image of the same fields shown in (B) and (C). Scale bars, 100 μm.

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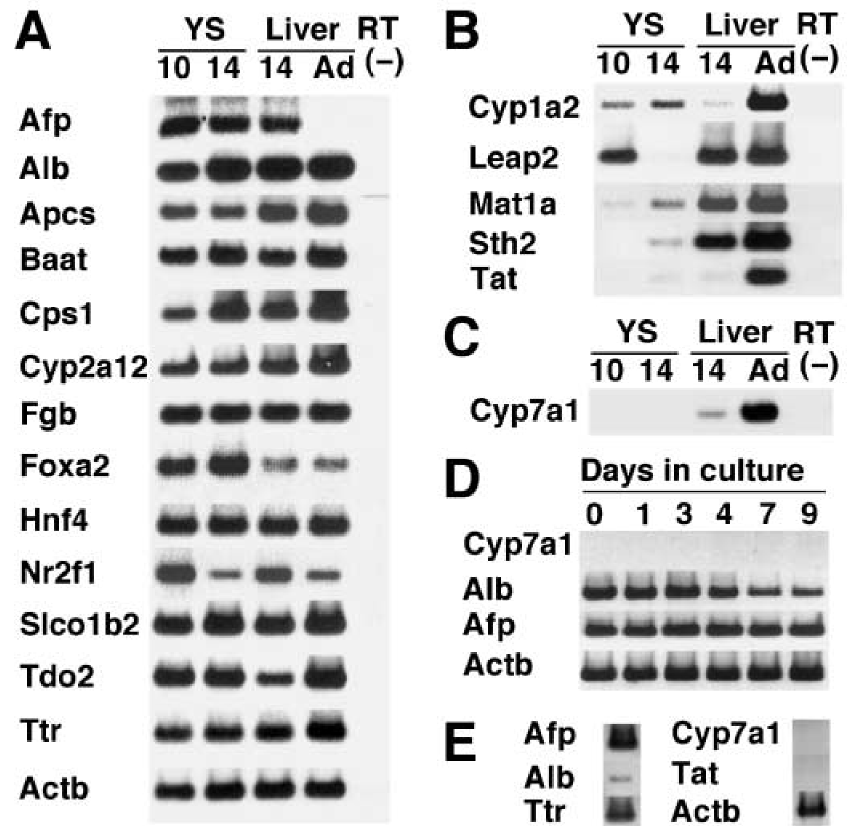

Figure 2 Gene expression in yolk sac and liver. Expression of hepatocyte-related genes was examined in E10 and E14 yolk sac (YS), E14 foetal liver, and adult liver (Ad) by RT-PCR. (A) Genes expressed in both the yolk sac and liver. Actb was used as a positive control, and no amplification was detected in a negative control in which the mRNA was not treated with reverse transcriptase (RT-). (B) Genes expressed at higher levels in liver than in yolk sac. (C) Cyp7a1 gene was specifically expressed in liver. (D) Gene expression in cultured yolk sac tissues. Yolk sac tissues expressed Alb, Afp, and Actb, but not Cyp7a1. (E) Gene expression in yolk sac-like structures formed in the cultured EBs on day 15. The yolk sac-like structures expressed Afp, Alb, Ttr, Actb, but not Cyp7a1.

classified the hepatocyte-related genes into three categories based on their expression patterns:

- genes expressed in both the yolk sac and liver (Fig. 2A);
- genes expressed higher in the liver than in the yolk sac (Fig. 2B);
- a gene specifically expressed in the liver only (Fig. 2C).

Afp, Alb, serum amyloid P-component (Apcs), bile acid-Coenzyme A: amino acid N-acyltransferase (Baat), carbamoyl-phosphate synthetase 1 (Cps1), Cyp2a12, fibrinogen Bβ (Fgb), Hnf3β (Foxa2), Hnf4, COUP-TFI (Nr2f1), organic anion transporter-1 (Slco1b2), tryptophan 2,3-dioxygenase (Tdo2), and Ttr belong to the first category of genes expressed in both the yolk sac and liver (Fig. 2A). β-Actin (Actb) was used as an internal control, and no amplification was detected in a negative control in which the mRNA was not treated with reverse transcriptase (RT-). Cyp1a2, liver-expressed anti-microbial peptide 2 (Leap2), methionine adenosyltransferase 1A (Mat1a), hydroxysteroid sulfotransferase (Sth2), and tyrosine aminotransferase (Tat) are among those genes expressed at higher levels in the liver than in the yolk sac, and of these, Tat was only faintly expressed in the yolk sac (Fig. 2B). These gene expression profiles indicate that many genes expressed in the liver are also significantly expressed in the yolk sac. Nonetheless, we identified Cyp7a1 (cholesterol 7α-hydroxylase) as a gene expressed in the liver but not at all in the yolk sac (Fig. 2C).

Gene expression in yolk sac tissues

To assess the possibility that the culture conditions of the EBs may have a permissive effect to induce the expression of Cyp7a1 in the visceral endoderm, we cultured yolk sac tissues isolated from E13 embryos. The yolk sac tissues gradually degenerated during the organ culture. RT-PCR showed that the yolk sac tissues continue to express Alb and Afp mRNAs (Fig. 2D). No expression of Cyp7a1 was detected throughout the culture period (Fig. 2D). Next, we determined whether a yolk sac-like structure formed in the developing EBs expressed Cyp7a1 mRNA. The yolk sac-like structures formed in the cultured EBs on day 15 (Fig. 1A, arrow) were dissected and subjected to RT-PCR analysis. The yolk sac-like tissues expressed Afp, Ttr and Actb, but not Cyp7a1 and Tat (Fig. 2E). The expression of Alb was low in the yolk sac-like structure. Thus, it seems that our culture conditions do not generally induce the

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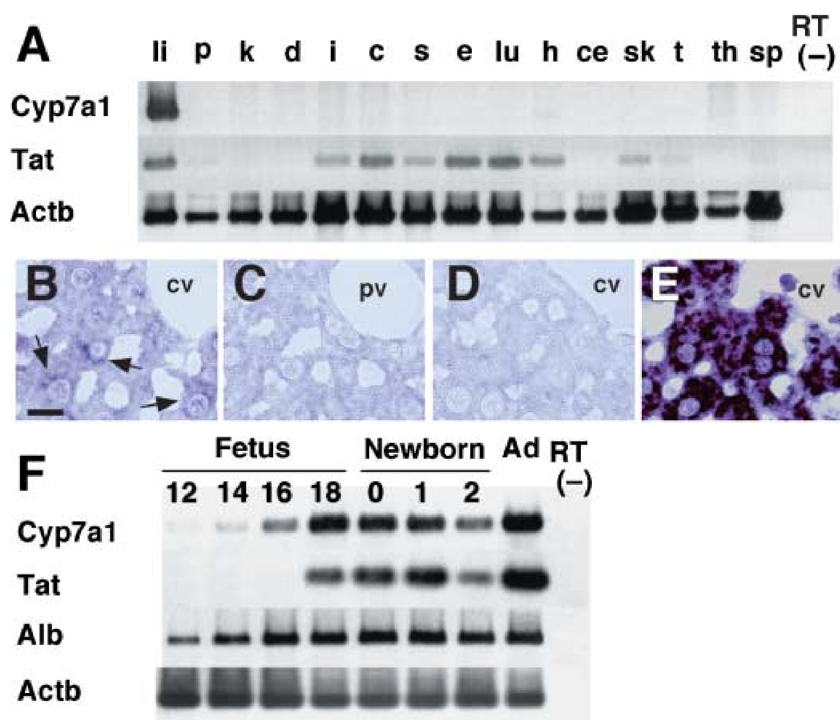

Figure 3 Expression patterns of Cyp7a1 and Tat in mice. (A) RNAs were isolated from liver (li), pancreas (p), kidney (k), duodenum (d), small intestine (i), colon (c), stomach (s), oesophagus (e), lung (lu), heart (h), cerebrum (ce), skeletal muscle (sk), testis (t), thymus (th), and spleen (sp). Expression of Cyp7a1, Tat, and Actb in each tissue of adult mice was determined by RT-PCR. Expression of Cyp7a1 was detectable only in the liver. Tat was strongly expressed in the liver and also expressed in several tissues. Actb was used as a positive control. RT(–), without reverse transcriptase. (B–E) mRNA *in situ* hybridization in adult mouse liver. *In situ* hybridization was performed with Cyp7a1 anti-sense probes (B, C), Cyp7a1 sense probes (D), and Alb anti-sence probes (E). Expression of Cyp7a1 mRNA (arrows) was detected in perivenous hepatocytes (B), but not in periportal hepatocytes (C). cv, central vein; pv, portal vein. No signal was detected in perivenous hepatocytes with Cyp7a1 sense probes (D). Alb mRNA was strongly expressed in hepatocytes (E). Scale bar, 10 μm. (F) Gene expression in developing mouse liver. Expression of Cyp7a1, Tat, Alb, and Actb in foetal (E12-18), newborn (day 0–2), and adult (Ad) livers was determined by RT-PCR. PCR was performed at 28 cycles for Cyp7a1 and Tat, and at 18 cycles for Alb. Cyp7a1 and Tat were expressed in liver from E14 and E18 foetuses, respectively, and these expressions were highest in adult liver. Alb mRNA was detected in the liver from E12 onwards.

expression of Cyp7a1 in the visceral endoderm of developing EBs.

Specific expression of Cyp7a1 gene in mouse liver

Cyp7a1 gene encodes CYP7A1, the rate-limiting enzyme in the conversion of cholesterol to bile acids in the liver (Davis *et al.* 2002). RT-PCR revealed that Cyp7a1 mRNA was exclusively expressed in the adult mouse liver (Fig. 3A). Tat, which is expressed in the adult liver and faintly in both foetal yolk sac and liver (see Fig. 2B), was also detectable in several tissues (Fig. 3A). *In situ* hybridization revealed that Cyp7a1 mRNA was expressed in perivenous hepatocytes (Fig. 3B), but not in periportal hepatocytes of the adult liver (Fig. 3C). The expression level of Cyp7a1 mRNA (Fig. 3B) was lower than that of Alb mRNA (Fig. 3E) in the liver. Next, the expression profiles of Cyp7a1 and Tat during liver development were determined. Cyp7a1 and Tat were expressed from E14 and E18 foetal livers, respectively (Fig. 3F). These results indicate that Cyp7a1 can be used as a specific marker for hepatocytes in mice.

Hepatic differentiation in developing EBs

The definitive endoderm is derived from the primitive ectoderm through the gastrulation in early embryogenesis (Beddington & Robertson 1999). RT-PCR showed that the expression of fibroblast growth factor 5 (Fgf5; primitive ectoderm), goosecoid (Gsc; gastrulation), Wnt3 (gastrulation), and brachyury (T; mesoderm) was transiently up-regulated from days 3–5 in cultured EBs in the absence

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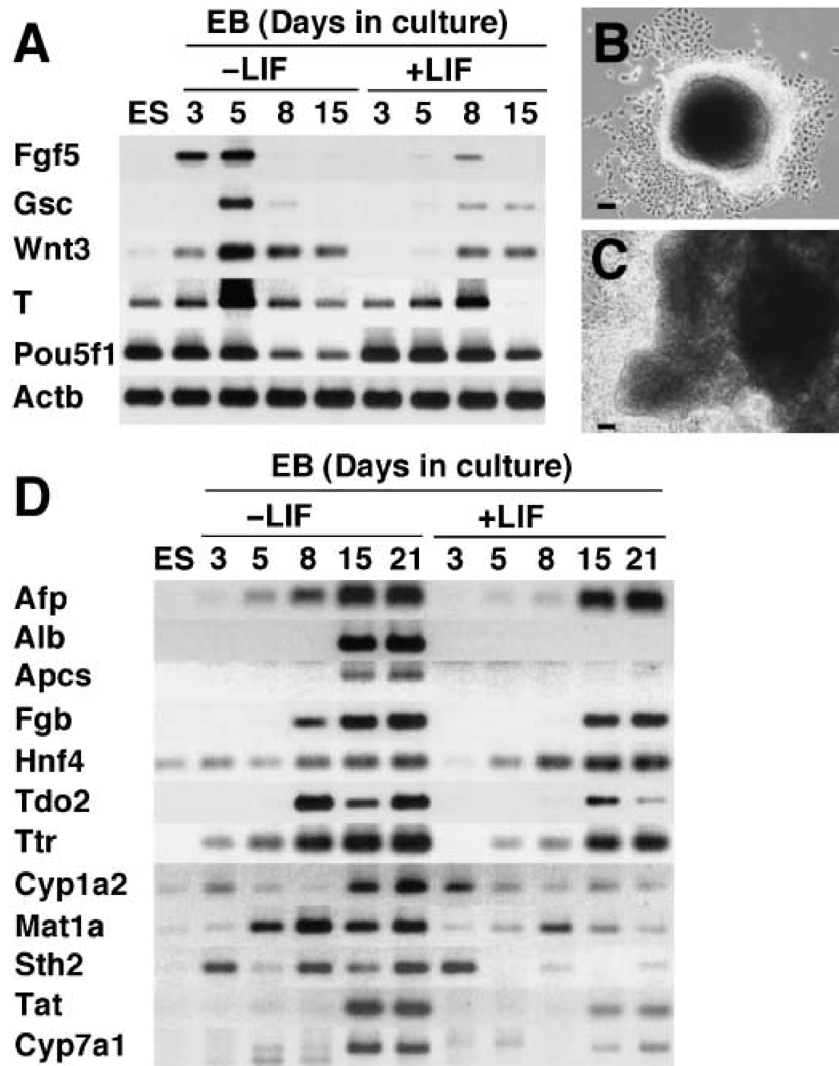

Figure 4 Expression of gastrulation-related genes and hepatic differentiation in developing EBs in attached culture. EBs were cultured on dishes coated with gelatin in the absence (–LIF) or presence of 1000 U/mL LIF (+LIF). (A) RNAs were extracted from ES cells and cultured EBs. RT-PCR was performed using primers for Fgf5, Gsc, Wnt3, T, Pou5f1, and Actb. Expression of Fgf5, Gsc, Wnt3 and T was transiently up-regulated from days 3–5. LIF delayed and decreased the expressions of Fgf5, Gsc, Wnt3, and T. Expression of Pou5f1 was decreased during culture in the absence of LIF, whereas its expression continued in the presence of LIF. Actb was equally expressed in all samples. (B) EBs cultured in the presence of LIF on day 8. The differentiation of EBs was inhibited. (C) EBs cultured in the presence of LIF on day 15. Cell differentiation from EBs was inhibited and no yolk sac-like structure was formed. Scale bars, 100 μm. (D) Gene expression in developing EBs. Genes expressed in yolk sac and liver, which include Afp, Fgb, Hnf4, Tdo2 and Ttr, were up-regulated in cultured EBs, and their expression profiles were maintained in the presence of LIF. Genes expressed at higher levels in the liver than in the yolk sac, which include Cyp1a2, Mat1a, Sth2 and Tat, were expressed in developing EBs. Expression of the liver-specific Cyp7A1 was up-regulated on day 15. Expression of Cyp1a2, Mat1a, Sth2, Tat and Cyp7a1 was reduced in the presence of LIF.

of LIF (Fig. 4A) as previously reported (Blum *et al.* 1992; Leahy *et al.* 1999; Liu *et al.* 1999). The expression of a marker for ES cells (Pou5f1) decreased with the differentiation of the EBs (Fig. 4A). LIF is known to inhibit the gastrulation and differentiation of the primitive ectoderm and mesoderm in developing EBs *in vitro*, while permitting or weakly inhibiting the differentiation of visceral endoderm from primitive endoderm (Shen & Leder 1992; Murray & Edgar 2001). In the presence of LIF, the differentiation of cultured EBs was suppressed (Fig. 4B) and no yolk sac-like structure was observed on day 15 (Fig. 4C) as previously reported (Bader *et al.* 2001). RT-PCR showed that LIF delayed and decreased the expressions of Fgf5, Gsc, Wnt3 and T as compared to the absence of LIF (Fig. 4A), suggesting that LIF inhibited the differentiation of the primitive ectoderm and mesoderm from ES cells, as well as the gastrulation-like process in cultured EBs.

Next, we examined hepatic differentiation in developing EBs by RT-PCR. The expression of Afp, Alb, Apcs, Fgb, Hnf4, Tdo2 and Ttr, which are expressed in both the yolk sac and liver, was induced in differentiating EBs cultured in the absence of LIF (Fig. 4D). Similarly, Cyp1a2, Mat1a, Sth2 and Tat that are expressed predominantly in the liver were also induced in cultured EBs (Fig. 4D). Importantly, the expression of the liver-specific gene Cyp7a1 was induced in developing EBs from culture day 15 onwards. These results indicate that mouse ES cells differentiate into hepatocytes derived from definitive endoderm as well as visceral endoderm in culture. No or weak effects of LIF on the genes expressed in both the yolk sac and liver, which included Afp, Fgb, Hnf4, Tdo2 and Ttr, were observed in the developing EBs (Fig. 4D). However, the induction of the Alb and Apcs genes, which are expressed in both

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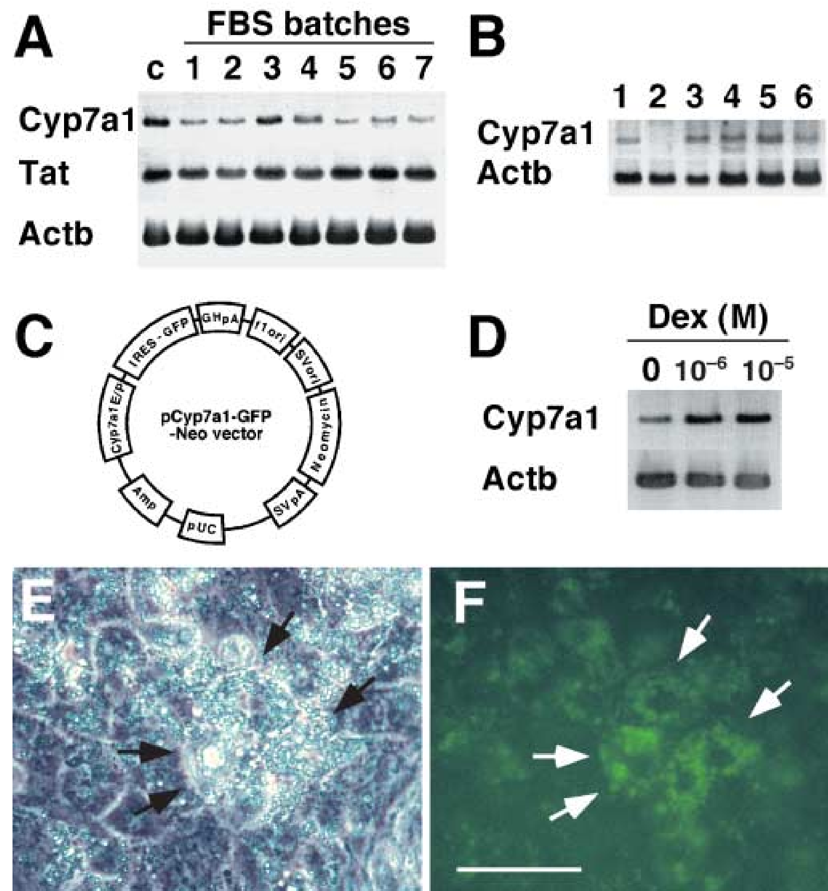

Figure 5 Efficiency of hepatic differentiation in developing EBs. (A) Hepatic differentiation of EBs cultured in seven different batches of FBS. Expression of Cyp7a1 and Tat was detected in all EBs cultured in the seven different batches of FBS as well as in the control FBS (c) used in the present study. (B) Frequency of the Cyp7a1 expression in developing EBs. Single EBs were cultured in individual wells for 21 days. RT-PCR analysis showed that 20 of the 34 EBs exhibited Cyp7a1 mRNA expression. Out of the 34 EBs, 5 EBs expressing Cyp7a1 (lanes 1, 3–6) and one EB expressing no Cyp7a1 (lane 2) are shown. (C) pCyp7a1-GFP-Neo vector construct. The plasmid carries the IRES-GFP gene under the control of the Cyp7a1 enhancer/promoter (Cyp7a1 E/P) and bovine growth hormone polyadenylation site (GH pA). The plasmid contains SV40 early promoter and origin (SV ori), the neomycin gene, and the SV40 polyadenylation signal (SV pA). (D) Effect of dexamethasone on the expression of Cyp7a1 mRNA in cultured EBs. Day 20 EBs were cultured in the presence of 10^{-6} and 10^{-5} M dexamethasone (Dex) for 24 h. Cyp7a1 mRNA expression was increased in the presence of dexamethasone. (E) Developing EBs carrying pCyp7a1-GFP-Neo vector on day 27. (F) The fluorescent image of the same field shown in (E). GFP positive epithelial-like cells (arrows) were observed in the multilayered region of the developing EBs. Scale bar, 100 μm.

the yolk sac and liver, was inhibited in the presence of LIF. LIF is presumed to permit the differentiation of visceral endoderm from primitive endoderm, and to inhibit the late differentiation or maturation of the visceral endoderm. Cyp1a2, Mat1a, Sth2 and Tat that are expressed higher in hepatocytes than in the yolk sac were down-regulated in the presence of LIF (Fig. 4D). In particular, the expression of the liver-specific gene Cyp7a1 was reduced in the presence of LIF (Fig. 4D). Our results demonstrate that hepatocytes from the definitive endoderm can be differentiated from mouse ES cells *in vitro*.

Efficiency of hepatic differentiation in developing EBs

To examine the effects of batches of FBS on hepatic differentiation in the developing EBs, we cultured EBs in seven different batches of FBS. Although the expression level of Cyp7a1 mRNA varied, the expression of Cyp7a1 and Tat was detected in EBs cultured in the seven different batches of FBS as well as in the control FBS used throughout the present study (Fig. 5A).

Next, we determined the frequency of Cyp7a1 expression in developing EBs. Single EBs formed by the

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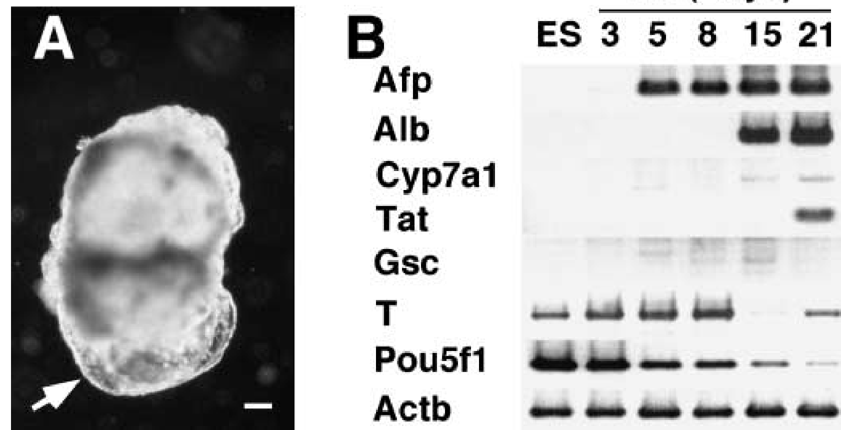

Figure 6 Expression of gastrulation-related genes and hepatic differentiation in developing EBs in suspension culture. EBs formed by the hanging drop culture were kept in suspension culture in the absence of LIF (days 3–21). (A) EBs in suspension culture on day 15. Arrow indicates yolk sac-like structure on developing EBs. Scale bar, 100 μm (B) RNAs were extracted from ES cells and developing EBs in suspension culture. RT-PCR was performed using primers for Afp, Alb, Cyp7a1, Tat, Gsc, T, Pou5f1 and Actb. Expression of Afp and Alb, which are expressed in both the yolk sac and liver, was up-regulated in cultured EBs. Actb was equally expressed in all samples.

hanging drop culture method were maintained in individual wells for 21 days. RT-PCR analysis showed that 20 of the 34 EBs (58.8%) exhibited Cyp7a1 mRNA expression (6 of the 34 EBs shown in Fig. 5B). In order to quantify the frequency of hepatic differentiation in the cultured EBs, we generated an ES cell line expressing humanized *Renilla reniformis* GFP under the control of the Cyp7a1 enhancer/promoter (Fig. 5C). Since the promoter activity of the Cyp7a1 gene is known to be less active, we added dexamethasone to the culture medium 24 h before the observations, because dexamethasone increases Cyp7a1 mRNA expression in rat primary hepatocytes (Hylemon *et al*. 1992). When day 20 EBs were cultured in the presence of 10^{-6} or 10^{-5} M dexamethasone for 24 h, the Cyp7a1 mRNA expression was increased (Fig. 5D). GFP-positive cells exhibiting an epithelial cell-like shape were observed in the multilayered region of the developing EBs on culture day 27 (Fig. 5E,F). We counted the number of GFP-positive cells under the fluorescent microscope, and then each EB was collected and the number of the nuclei in the EB was counted. As shown in Table 1, GFP-positive cells were observed in 14 out of 16 EBs (87.5%). Single EBs at 23 days in culture contained 1.5×10^4–1.5×10^5 nuclei, and the number of GFP-positive cells ranged between 1 and 92 in the 14 EBs (Table 1). Thus, the frequency of GFP-positive cells out of the total number of nuclei in each individual EB was calculated as 0.003% to 0.065%. The frequency of GFP-positive cells in the EBs showed a tendency to increase with the number of EB cells.

Table 1 Frequency of GFP-positive cells in individual EBs cultured for 23 days

EB	No. of nuclei in EB	No. of GFP-positive cells (%)*
1	1.5×10^5	92 (0.061)
2	1.4×10^5	91 (0.065)
3	1.2×10^5	13 (0.011)
4	7.4×10^4	18 (0.024)
5	6.8×10^4	15 (0.021)
6	5.5×10^4	9 (0.016)
7	4.9×10^4	16 (0.033)
8	4.1×10^4	2 (0.005)
9	4.0×10^4	2 (0.005)
10	3.8×10^4	22 (0.058)
11	3.8×10^4	3 (0.008)
12	3.5×10^4	1 (0.003)
13	3.3×10^4	0 (0)
14	2.8×10^4	3 (0.011)
15	2.7×10^4	3 (0.011)
16	1.5×10^4	0 (0)

*Percentage is calculated as the number of GFP-positive cells per the number of nuclei in a single EB.

Differentiation of EBs in suspension culture

When EBs were kept in suspension after the hanging drop culture, a yolk sac-like structure was observed in the EBs on day 15 (Fig. 6A). As in the case of the attached culture, Afp, Alb, and T were expressed at comparable levels in differentiating EBs (Fig. 6B). The expression of Pou5f1 decreased with the differentiation of the EBs (Fig. 6B). In contrast, the expression levels of Cyp7a1, Tat, and Gsc were lower in the developing EBs in suspension culture (Fig. 6B) than in attached culture (Fig. 4). Thus, the attached culture seems to be preferable for inducing hepatic differentiation in developing EBs.

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Discussion

Several groups, including us, have previously reported hepatic differentiation from mouse ES cells based on the induction of hepatocyte-related gene expression (Hamazaki *et al.* 2001; Chinzei *et al.* 2002; Miyashita *et al.* 2002; Yamada *et al.* 2002; Kuai *et al.* 2003). However, most of the hepatocyte-related genes such as Afp, Alb, transferrin, and apolipoproteins have been shown to be expressed in both hepatocytes and the visceral endoderm (Dziadek & Adamson 1978; Meehan *et al.* 1984; Sellem *et al.* 1984). Therefore, it remains unclear whether ES cells can differentiate into definitive endoderm *in vitro* (Abe *et al.* 1996). In the present study, we identified Cyp7a1 as a liver-specific gene, and examined its expression in developing EBs.

We showed that yolk sac as well as the liver expresses a variety of hepatocyte-related genes involved in protein transport (Alb, Apcs and Ttr), drug metabolism (Cyp1a2, Cyp2a12 and Sth2), urea synthesis (Cps1), amino acid metabolism (Mat1a, Tat and Tdo2), bile acid synthesis (Baat), transporter (Slco1b2), transcription (Foxa2, Hnf4 and Nr2f1), and blood coagulation (Fgb). Many transcription factors such as Foxa2, Gata4, Hnf1β, Hnf4 and Sox17 are expressed in both the definitive and visceral endoderm, and play pivotal roles in the differentiation of the visceral endoderm as well as in the differentiation of definitive endoderm and liver morphogenesis (Soudais *et al.* 1995; Duncan *et al.* 1997; Dufort *et al.* 1998; Coffinier *et al.* 1999; Kanai-Azuma *et al.* 2002). Thus, it is plausible that the gene expression profiles of the yolk sac are similar to those of the liver.

We showed that genes expressed in both the yolk sac and liver are induced in developing EBs. Furthermore, the expression of the liver-specific gene Cyp7a1 was induced in developing EBs, indicating that ES cells differentiate into hepatocytes as well as visceral endoderm *in vitro*. Similar gene expression patterns were also observed in the experiments using an R1 ES cell line (data not shown). We also tested the effects of different batches of FBS on hepatic differentiation in cultured EBs. Although the expression levels of Cyp7a1 varied in the EBs cultured in the different batches of FBS, the expression of Cyp7a1 was seen in the all EBs. Thus, our culture conditions may be independent of both ES cell lines and batches of FBS. In the present study, we identified Cyp7a1 as a gene that was expressed in hepatocytes, but not in the yolk sac. However, there is a possibility that the expression of Cyp7a1 may be permissively induced in the visceral endoderm differentiated in the cultured EBs. We showed that yolk sac tissues cultured in the EB differentiation medium did not express Cyp7a1 mRNA. In addition, the yolk sac-like structure formed in the cultured EBs did not express Cyp7a1, suggesting that the expression of Cyp7a1 is strictly suppressed in yolk sac tissues. Thus, Cyp7a1 is an excellent marker for the identification of hepatocytes in developing EBs.

CYP7A1 is the rate-limiting enzyme in the conversion of cholesterol to bile acids in the liver (Davis *et al.* 2002). We showed that Cyp7a1 mRNA is expressed in the foetal liver, and is restricted to perivenous hepatocytes in the adult liver as previously reported in rats (Massimi *et al.* 1998). The mRNA expression of Cyp7a1 is regulated by multiple factors involved in a circadian cycle, bile acid feedback inhibition, and stimulation by oxycholesterol, dexamethasone, and thyroid hormone (Hylemon *et al.* 1992; Davis *et al.* 2002; Noshiro *et al.* 2004). The promoter region located 0.4 kb upstream contains a glucocorticoid response element, binding sites for D-element-binding protein, DEC2, C/EBPα, Foxa2, and HNF-4, and bile acid response elements (Ramirez *et al.* 1994; Davis *et al.* 2002; Noshiro *et al.* 2004). These factors are known to be involved in the liver-specific expression of the Cyp7a1 gene. In addition, Ramirez *et al.* (1994) reported that a liver specific enhancer located 7 kb upstream is necessary for the expression of the rat Cyp7a1 gene. In the present study, we generated an ES cell line expressing GFP under the control of the Cyp7a1 enhancer (−7320 to −6086) and promoter (−376 to +27). These GFP-positive cells were observed in the multilayered region of developing EBs cultured for 27 days. The GFP-positive cells were clustered, and had an epithelial cell-like shape, suggesting that hepatocytes were differentiated in the EBs. The GFP-positive cells were observed in14 out of 16 EBs (87.5%). RT-PCR analysis showed that 20 of the 34 EBs (58.8%) expressed Cyp7a1 mRNA. Such a discrepancy may be caused by the sensitivity of the RT-PCR. Indeed, several EBs contained only 1–3 GFP-positive cells (see Table 1). We counted GFP-positive cells under the fluorescence microscope, and showed that the frequency of GFP-positive cells ranged from 0.003% to 0.065% in each individual EB. We assumed that the frequency calculated as above would be underestimated due to the following reasons:

- the activity of the Cyp7a1 enhancer/promoter is relatively low;
- Cyp7a1 is expressed in perivenous hepatocytes, but not in periportal hepatocytes;
- transgenes are silenced frequently in ES cells.

Thus, the frequency of hepatocytes differentiated in the EBs should be higher than that calculated in the present study. The Cyp7a1 enhancer/promoter would be useful for improving the culture conditions by

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monitoring hepatocyte differentiation from ES cells and hepatocyte-directed gene delivery.

Gastrulation-related genes including Gsc and Wnt3 are transiently expressed in developing EBs in a 5-day culture, suggesting that a gastrulation-like process takes place in cultured EBs *in vitro*. We found that LIF inhibits the differentiation of the primitive ectoderm and mesoderm from ES cells, and also the gastrulation-like process in cultured EBs. Under the influence of LIF, Cyp7a1 and Tat, which are expressed specifically and highly, respectively, in the liver were down-regulated. These results concur with the reports that LIF inhibits gastrulation and the formation of the primitive ectoderm in developing EBs (Shen & Leder 1992; Murray & Edgar 2001). Taken together, these results indicate that hepatocytes are likely to be differentiated from ES cells through a gastrulation-like process in EBs.

It is known that the onset of Alb expression in the yolk sac is later than that of Afp, Ttr, and Hnf4 *in vivo* (Dziadek & Adamson 1978; Sellem *et al.* 1984; Makover *et al.* 1989; Duncan *et al.* 1997). We showed that a yolk sac-like structure expressing Afp and Ttr was formed in the developing EBs in attached culture. However, the expression of Alb mRNA was low in the yolk sac-like structure. In addition, ALB antibody-positive cells were observed in a multilayered structure of EBs, but not in the yolk sac-like structure. It seems that the yolk sac formed in the developing EBs is not fully differentiated. When EBs were cultured in the presence of LIF, no yolk sac-like structure was formed in the EBs. Furthermore, the expression of Alb and Apcs, which are expressed in both the yolk sac and liver, was completely inhibited in the presence of LIF. LIF is presumed to inhibit the maturation of the visceral endoderm or the formation of yolk sac in developing EBs, although it permits the differentiation of visceral endoderm from primitive endoderm. Indeed, EBs cultured in the presence of LIF expressed Afp, Hnf4 and Ttr mRNAs. Thus, we assume that the induction of the Alb gene in the visceral endoderm might be suppressed, because the maturation of the visceral endoderm and formation of yolk sac were inhibited by LIF in developing EBs.

In the present study, we found that the expression of the liver-specific gene Cyp7a1 is induced more strongly in attached culture than in suspension culture. In both culture systems, the formation of a yolk sac-like structure and the expression of Afp and Alb were observed in developing EBs. However, the expression of Gsc and Cyp7a1 was quite low in the developing EBs in suspension culture, suggesting that the attached culture of EBs is more effective in inducing the gastrulation-like process and hepatic differentiation.

The visceral endoderm plays an important role in axis formation during early embryogenesis as well as in foetal development by the formation of a yolk sac, where the visceral endoderm secretes and absorbs metabolites (Jollie 1990; Beddington & Robertson 1999). The visceral yolk sac is composed of two layers of visceral endoderm and mesoderm, and the visceral endoderm is morphologically and physiologically similar to hepatocytes and intestinal epithelial cells (Jollie 1990). Indeed, yolk sac tissues express many hepatocyte-related genes and synthesize urea (data not shown), suggesting that the visceral endoderm has similar functions to hepatocytes. It is also known that visceral endodermal cells express the insulin and Pdx-1 genes (McGrath & Palis 1997). These results raise the possibility that the expression of hepatocyte- or pancreas-related genes in developing EBs denotes the differentiation of only visceral endoderm, but not of definitive endoderm. Thus, caution will be required to demonstrate the differentiation of definitive endodermal cells, including hepatocytes and pancreatic endocrine cells, from ES cells.

In the present study, we demonstrated that hepatocytes can be differentiated in cultured EBs. We previously reported that isolated cells from day-9 EBs can be integrated into the host parenchyma after the transplantation via the spleen (Chinzei *et al.* 2002). Thus, hepatocytes differentiated from mouse ES cells may be useful for cell replacement therapy. However, cultured EBs contain undifferentiated cells, and the transplantation of EBs frequently leads to teratoma formation in the recipient liver and spleen (Chinzei *et al.* 2002). Therefore, improving the purification process of hepatocytes is necessary prior to therapeutic applications.

Experimental procedures

Culture of ES cells

The mouse ES cell line BL6 was cultured in Knockout Dulbecco's modified Eagle's medium (Gibco BRL, Grand-Island, NY, USA) containing 20% foetal bovine serum (JRH Biosciences, Lenaxa, KS), 0.3 mM monothioglycerol (Sigma), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1000 U/mL recombinant mouse LIF (ESGRO, Chemicon, Temecula, CA, USA) on dishes coated with gelatin (Chinzei *et al.* 2002). For comparison with BL6, the ES cell line R1 (Hamazaki *et al.* 2001) was also employed, but the data do not appear in figures cited in this paper.

Differentiation of ES cells *in vitro*

To induce the formation of EBs, ES cells were suspended in a differentiation medium consisting of Iscove's modified Dulbecco's medium (Gibco BRL), 20% foetal bovine serum, 0.3 mM

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monothioglycerol, 100 U/mL penicillin, and 100 μg/mL streptomycin. ES cells were incubated by the hanging drop culture method (300 ES cells per 30 μL in each drop) for 2 days (Chinzei *et al.* 2002). The 36 EBs formed in the drops were cultured on a 60-mm dish coated with gelatin or cultured in suspension using an Ultra Low Attachment dish (Corning, Acton, MA) in the differentiation medium. In some experiments, EBs were formed and cultured in the presence of 1000 U/mL LIF. We also cultured EBs in the presence of seven different batches of FBS. Five different batches of FBS were obtained from JRH Biosciences (Lenaxa, KS), and others were purchased from Gibco BRL (Grand-Island, NY, USA) and Harlan Sera-Laboratory (Leicestershire, UK).

Immunocytochemistry

EBs were fixed with 80% acetone for 10 min and blocked with 2% rabbit sera. The cells were incubated with 500-fold diluted anti-rat albumin antibodies (ICN Biomedicals, Aurora, OH, USA) for 1 h, then with 200-fold diluted TRITC-labelled anti-goat antibodies for 30 min. After washing, cell nuclei were stained with a 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI)/anti-fade solution (Chemicon).

Tissue isolation

C57BL6 mice (Japan LSC, Shizuoka, Japan) were used in all experiments. Yolk sac and liver tissues were harvested from embryos at E10 to E18. Liver tissues were isolated from neonates at day 0–2 after the birth. Tissues of liver, pancreas, kidney, duodenum, small intestine, colon, stomach, oesophagus, lung, heart, cerebrum, skeletal muscle, testis, thymus, and spleen were isolated from 5-week-old male mice. Yolk sac tissues isolated from E13 embryos were cultured in the EB differentiation medium on 6-well plates as above.

RT-PCR

Total RNA was extracted from cultured cells and mouse tissues with a RNeasy total RNA system (Qiagen, Tokyo, Japan). cDNAs were synthesized from 1 μg of total RNA by using Superscript II reverse transcriptase and oligo (dT) primers (Gibco BRL) according to the manufacturer's instructions. PCR was performed by using FastStart *Taq* DNA polymerase (Roche Diagnostics, Penzberg, Germany) with the following conditions: 94 °C for 10 min followed by 30–38 cycles of denaturing at 94 °C for 30 s, annealing at the set temperature for each gene for 30 s, and extension at 72 °C for 50 s, followed by a final extension at 72 °C for 7 min. Unless otherwise noted, the PCR primers, annealing temperature, cycles, and size of each amplified gene are as follows: for Actb (Accession no.: X03672): 5′-AGA-CTT-CGA-GCA-GGA-GAT-G, 5′-ACT-CAT-CGT-ACT-CCT-GCT-TG; 57 °C; 30 cycles, 431 bp, for Afp (AK076197): 5′-CAC-TGC-TGC-AAC-TCT-TCG-TA, 5′-CTT-TGG-ACC-CTC-TTC-TGT-GA; 55 °C; 34 cycles, 300 bp, for Alb (AJ011413): 5′-GTC-TTA-GTG-AGG-TGG-AGC-AT, 5′-ACT-ACA-GCA-CTT-GGT-AAC-AT, 57 °C; 30 cycles, 569 bp, for Apcs (Y00426): 5′-AAA-GCA-TAG-GGA-GAC-ACC-A, 5′-GGC-TTT-CCA-TTG-ACC-CAA-A, 57 °C; 38 cycles, 471 bp, for Baat (U95215): 5′-CTG-GTG-GAT-TGA-TGG-AGT-T, 5′-CAT-GCA-CTT-TGC-TGT-TGA-G, 56 °C; 35 cycles, 501 bp, for Cps1 (Schofield *et al.* 1999): 5′-CAG-AGT-TCC-AGA-TGT-TGA-GAC, 5′-CTA-CCA-ATT-CGG-CTG-GAT-G; 56 °C; 35 cycles, 346 bp, for Cyp1a2 (X00479): 5′-GTC-ACC-TCA-GGG-AAT-GCA-GT, 5′-GGT-GAA-GGG-GAC-AAA-GGA-TGT; 63 °C; 33 cycles, 465 bp, for Cyp2a1 (BC018356): 5′-AGA-AAG-TGA-AGC-AGA-ACC-AG, 5′-CTT-GGG-AAC-TTG-AAA-CGG-A, 56 °C; 35 cycles, 646 bp, for Cyp7a1 (AK050260): 5′-AGG-ACT-TCA-CTC-TAC-ACC, 5′-GCA-GTC-GTT-ACA-TCA-TCC, 56 °C; 35 cycles, 453 bp, for Fgb (BC031715): 5′-GGC-CAG-CAA-ATA-CCA-AGT, 5′-CTA-TTG-CTG-TGG-GAA-GAA-GG, 57 °C; 30 cycles, 385 bp, for Fgf5 (AK028894): 5′-AAA-GTC-AAT-GGC-TCC-CAC-GAA, 5′-CTT-CAG-TCT-GTA-CTT-CAC-TGG; 54 °C; 35 cycles, 465 bp, for Foxa2 (X74937): 5′-ACT-GGA-GCA-GCT-ACT-ACG, 5′-CCC-ACA-TAG-GAT-GAC-ATG; 55 °C; 38 cycles, 169 bp, for Gsc (Y13149): 5′-TTC-CAG-GAG-ACG-AAG-TAC-C, 5′-CTG-GTC-TAC-ATT-GCC-ATC-AC, 54 °C; 35 cycles, 514 bp, for Hnf4 (D29015): 5′-ACA-CGT-CCC-CAT-CTG-AAG, 5′-CTT-CCT-TCT-TCA-TGC-CAG, 58 °C; 35 cycles, 270 bp, for Leap2 (AJ409055): 5′-CTT-TTG-CCA-AGA-TGC-TAC-AG, 5′-TAT-TCC-CCA-GAA-CAG-ATC-AC, 56 °C; 35 cycles, 293 bp, for Mat1a (BC011211): 5′-CAA-CAG-TCC-CCA-GAT-ATT-GCC, 5′-TCC-ACC-TTG-GTG-TAG-TCC-T; 58 °C; 35 cycles, 539 bp, for Nr2f1 (X74134): 5′-AGC-CAT-CGT-GCT-ATT-CAC-g, 5′-TTC-TCA-CCA-GAC-ACG-AGG-TC; 58 °C; 33 cycles, 570 bp, for Pou5f1 (M34381): 5′-GGC-GTT-CTC-TTT-GGA-AAG-GTG-TTC, 5′-CTC-GAA-CCA-CAT-CCT-TCT-CT, 55 °C; 30 cycles, 313 bp, for Slco1b2 (AB031959): 5′-GTG-AAT-GCC-CAA-GAG-ACA, 5′-CAG-AAG-GTA-CAC-AAG-AGT-ATC-C, 56 °C; 35 cycles, 486 bp, for Sth2 (L27121): 5′-ATC-CGA-AGT-GGA-TCC-AAA-C, 5′-GAA-GTT-GTC-CCA-TTC-TCT-CA, 57 °C; 38 cycles, 353 bp, for T (X51683): 5′-ATG-CCA-AAG-AAA-GAA-ACG-AC, 5′-AGA-GGC-TGT-AGA-ACA-TGA-TT; 54 °C; 35 cycles, 835 bp, for Tat (BC030728): 5′-ACC-TTC-AAT-CCC-ATC-CGA, 5′-TCC-CGA-CTG-GAT-AGG-TA; 57 °C; 35 cycles, 206 bp, for Tdo2 (BC018390): 5′-TGC-TGA-CCT-CAC-TTA-TGG-AC, 5′-CTC-CTA-ATC-GCT-CAC-CAT-CA, 58 °C; 33 cycles, 467 bp, for Ttr (D89076): 5′-CTC-ACC-ACA-GAT-GAG-AAG, 5′-GGC-TGA-GTC-TCT-CAA-TTC, 55 °C; 30 cycles, 224 bp, and for Wnt3 (M32502): 5′-ACA-CCT-GCA-AGT-AGT-GAG, 5′-CAA-AGG-TAA-GCC-TCG-AGT, 57 °C; 32 cycles, 306 bp. The primers were designed using different exons for each gene. The amplified products were separated by electrophoresis on a 1.2% agarose gel and stained with ethidium bromide. PCR products were subcloned into a pGEM-T vector (Promega, Tokyo, Japan) and specific amplification was confirmed by DNA sequencing.

In situ hybridization

Digoxigenin (DIG)-labelled cRNA probes were synthesized with a DIG RNA labelling kit (Roche Diagnostics) using the Cyp7a1

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and Alb cDNAs in the pGEM-T vector as templates. Male adult mouse livers were perfused with 4% paraformaldehyde (PFA) in PBS and excised. The liver was further fixed overnight in 4% paraformaldehyde and embedded in TissueTek OCT compound. Cryosections (5 μm) were rehydrated, digested with 1 μg/mL proteinase K in PBS at 37 °C for 7 min, and postfixed in 4% PFA in PBS for 20 min. Hybridization and wash procedures were described elsewhere (Asahina *et al.* 2002). Hybridized cRNA probes were detected using 5-bromo-4-chloride-3-indolyl-phosphate and nitroblue tetrazolium (Roche Diagnostics).

Transfection and establishment of transgenic cell lines

A plasmid vector containing hrGFP (Stratagene, La Jolla, CA, USA) under the mouse Cyp7a1 enhancer/promoter was constructed. A 1241 bp fragment of the mouse Cyp7a1 enhancer and a 418 bp fragment of the Cyp7a1 promoter were amplified by PCR using mouse genomic DNA as a template. Primer sequences are as follows: Cyp7a1 enhancer, 5′-GG-TAC-CGA-GGC-AGT-GCT-ACC-TC and 5′-GGTA-CCA-CGT-GAT-CTT-AGG-ATC-ATC (−7320 to −6086 from the transcription initiation site); Cyp7a1 promoter, 5′-GGTA-CCA-GGA-TGG-AAA-GCT-TCT-GCC-T and 5′-GGATCCACTAG-TCT-GTG-CTT-AGC-AAA-GCA-AGA-CGG (−376 to +27). The underlines indicate extrasequences to generate restriction enzyme recognition sites used for the vector construction. The EF-1a promoter in the pEF/myc/nuc vector (Invitrogen, Carlsbad, CA, USA) was replaced by the Cyp7a1 enhancer/promoter. Then, the IRES-GFP fragment was amplified by PCR using the pIRES-hrGFP-a1 vector (Stratagene) as a template, and inserted under the Cyp7a1 enhancer/promoter sequence. The pEF/myc/nuc vector contains a neomycin resistance gene driven by the SV40 early promoter and origin. The resulting vector pCyp7a1-GFP-Neo was used for electroporation of ES cells. The pCyp7a1-GFP-Neo vector (33 μg) was transfected into the ES cell line BL6 by electroporation using a Gene Pulser (Bio-Rad Laboratories, Richmond, CA, USA) at 960 μF and 250 V. Transfected clones were selected by growth in the presence of 150 μg/mL G418 (Gibco BRL). Following 9 days of selection, 24 colonies were collected. EBs formed from the independent 24 ES cell lines were individually cultured and monitored under the fluorescent microscope using an FITC filter set. Three ES cell lines that generated EBs containing GFP-positive cells were selected, and one clone was used in the present study.

Frequency of hepatic differentiation in cultured EBs

To estimate the frequency of Cyp7a1-positive EBs, single EBs obtained in the hanging drop culture were plated individually in 24-well plates. After 21 days in culture, expression of Actb and Cyp7a1 mRNAs was determined in each individual EB by RT-PCR. PCR for Actb and Cyp7a1 was performed at 28 and 40 cycles, respectively.

The frequency of Cyp7a1-positive EBs and the number of Cyp7a1-expressing cells in a single EB were estimated using an ES cell line transfected with the pCyp7a1-GFP-Neo vector. EBs formed from the ES cell line were cultured and GFP positive cells in the EBs were counted under the fluorescent microscope. Then, each EB was collected to prepare the number of the nuclei in the EB. To extract nuclei, the each individual EB was incubated with 1 mL of a crystal violet solution consisting of 1 mg/mL crystal violet (Sigma) and 21 mg/mL citric acid monohydrate at 37 °C for 24 h (van Wezel 1967). After the incubation, the solution was collected, and nuclei were counted with a counting chamber.

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