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Activation of *Cyp*1a1 and *Cyp*1a2 Genes in Adult Mouse Hepatocytes in Primary Culture

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Expression of *Cyp*1a1 and *Cyp*1a2 genes was investigated in adult C57BL/6NCrj mouse hepatocytes in primary culture for up to 5 days. When the cells were cultivated as monolayers on collagen-coated dishes, CYP1A1 mRNA species were prominently induced after treatment with 3-methylcholanthrene (MCA) throughout the observation period. Substantial induction of CYP1A2 mRNA by MCA was also observed at day 1 of cultivation, followed by a decrease to very low levels thereafter. In contrast, when cultivated on non-coated dishes, the hepatocytes formed multicellular aggregates (spheroids) and prominent induction of both mRNA species was found for up to 5 days. Constitutive expression of CYP1A2 mRNA in spheroid culture was maintained throughout the observation period, whereas that in monolayer culture decreased rapidly. The time-course of the induced CYP1A2 mRNA amounts after the treatment with MCA or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) followed the same pattern as that of CYP1A1 mRNA. Expressed amounts of CYP1A1 or CYP1A2 mRNA in spheroid culture were higher than or similar to the levels in the case of *in vivo* production, respectively. Induction of both mRNA species was also observed in hepatocytes from nonresponsive DBA/2NCrj mouse in spheroid culture, but the expressed amount after MCA treatment was far smaller than for C57BL/6NCrj cells, despite equivalent expression in the two strains after TCDD. Activities of aryl hydrocarbon hydroxylase (AHH) and acetanilide 4-hydroxylase (AAH) were elevated with either type of cultivation after treatment with MCA or TCDD. Ratios of AAH to AHH were not changed between the two cultures after 24 h treatment. However, the ratios in spheroid culture after 48 h treatment increased, whereas they did not change in monolayer culture. The present observations indicate that the spheroid culture is more suitable than the monolayer system for studying the mechanism of *Cyp*1a2 gene expression in adult mouse hepatocytes.

Key words: *Cyp*1a1 — *Cyp*1a2 — Mouse hepatocyte — Primary culture

Primary culture systems for adult hepatocytes are convenient for investigation of drug metabolism as well as proliferation or differentiation.[1] However, while the liver contains high levels of several kinds of metabolizing enzymes, serious disadvantages need to the overcome in studying the regulatory mechanisms of expression of P450 species in cultured hepatocytes. Rapid decline of total P450 content in culture systems has been reported by many investigators[2–19] and, despite attempts to inhibit the decline, such as addition of specific chemicals to the culture medium,[3, 9–11, 14–19] or cultivation of hepatocytes with special cell attachment factors,[13, 19] it has proved impossible to maintain *in vivo* content or inducibility of some species. A typical example is CYP1A2, which is constitutively expressed and is inducible by treatment with aromatic hydrocarbons *in vivo*. When hepatocytes are transferred to monolayer culture, its content decreases rapidly with little potential for induction except in limited cases.[18–23] Moreover, even in successful cases, the mRNA induction level decreases rapidly after the start of cultivation of hepatocytes. Maintenance of induced CYP1A2 protein for several days was reported in rat hepatocytes in the presence of a high concentration of dimethyl sulfoxide[18] and in mouse cells cultivated on Matrigel™ in the presence of 5-aminolevulinic acid.[19] However, in these reports, treatment with the inducer was started one or two days after the start of cultivation and no description was given concerning the inducibility during the later culture period. Since CYP1A2 is responsible for activating carcinogenic aromatic amines and heterocyclic amines,[23–26] its regulation is of considerable interest. A culture system in which it could be induced for at least a few days would therefore be of advantage.

Several attempts to maintain cellular differentiation characteristics of adult hepatocytes in primary culture have been reported.[27–31] In rat hepatocytes in primary culture, better maintenance of the differentiated functions is known to be achieved by monolayer cells in high cell density culture, in which cell growth is relatively suppressed.[32] However, full maintenance of *in vivo* functions is still difficult. For example, secretion of albumin,

Abbreviations used are: AAH, acetanilide 4-hydroxylase; AHH, aryl hydrocarbon hydroxylase; C57BL/6, C57BL/6-NCrj; cDNA, complementary DNA; DBA/2, DBA/2NCrj; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

one of the marker proteins of differentiated hepatocytes, gradually decreases in most cases.[33] However, Koide *et al.* recently reported that rat hepatocytes in multicellular spheroid culture continue to produce this protein at high levels for up to 14 days.[29]

The present paper deals with expression of *Cyp*1a2 and *Cyp*1a1 genes, in mouse hepatocytes during spheroid formation. The observations indicate that hepatocytes respond to 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin treatment by producing *Cyp*1a2 as well as *Cyp*1a1 gene transcripts at sufficiently high levels for up to 5 days. This is the first observation of *Cyp*1a2 gene transcript induction without decrease over a number of days in culture. We anticipate that this system will be convenient for investigation of expression mechanisms.

MATERIALS AND METHODS

Chemicals Materials for culturing hepatocytes were purchased from GIBCO Laboratories, Grand Island, NY, Collaborative Research Inc. Bedford, MA, and Kyokuto Seiyaku, Tokyo. Percoll and collagenase were products of Pharmacia, Uppsala, and Sigma Chemical Co., St. Louis, MO, respectively. Full length cDNAs of *Cyp*1a1 and *Cyp*1a2, cloned from mouse liver,[34,35] were generous gifts from Dr. Daniel W. Nebert, Univ. of Cincinnati, OH. 3-Hydroxybenzo[*a*]pyrene was supplied by the NCI Chemical Repository, Bethesda, MD. Chicken β-actin cDNA was obtained from Oncor, Inc., Gaithersburg, MD. Other routine chemicals were purchased from Seikagaku Kogyo, Dai-ichi Pure Chemicals and Wako Pure Chemicals, Tokyo.

Preparation of primary cultures of hepatocytes The livers of female C57BL/6 or DBA/2 mice (Charles River Japan, Inc.) weighing 25–30 g were subjected to collagenase perfusion and then Percoll isodensity centrifugation for isolation of viable hepatocytes using the method previously described.[36,37] The cells were dispersed in Waymouth MB752/1 medium containing bovine serum albumin (2 g/liter), insulin (0.5 mg/liter), transferrin (0.5 mg/liter), selenium (0.5 μg/liter) and dexamethasone (0.4 μg/liter), and seeded in dishes at a density of 2.5×10^6 cells/10 ml/100 mm dish or 5×10^6 cells/10 ml/100 mm dish, respectively, with or without 100 μg of collagen Type I coating. The cultures were maintained at 37°C in a CO_2-humidified incubator. Medium was renewed every day, but, since medium change itself provoked *Cyp*1a1 gene expression,[38] treatment with either 1.625 μM 3-methylcholanthrene or 3.1 pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was started 24 h after the last medium change. The inducers were dissolved in dimethyl sulfoxide, which itself had no influence on AHH activity at the routinely employed maximal concentration of 0.1%.

Hybridization of hepatocyte RNA with P450 probes Total RNA was prepared from the hepatocytes according to the guanidinium thiocyanate/phenol method[39] and used for hybridization as described by Maniatis *et al.*[40] Northern transfer experiments were performed after size-fractionation of the denatured RNA (20 μg) on formaldehyde containing 1.3% agarose gels. Hybridization was carried out at 42°C overnight in a mixture containing 50% formamide, 1×Denhardt, 5×SSC, 50 mM sodium phosphate, pH 6.4, salmon testis DNA at 0.25 mg/ml, and a ^{32}P-labeled cDNA probe. Washing was performed four times for 5 min with 2×SSC and 0.1% SDS at room temperature, and then twice for 15 min with 0.1×SSC and 0.1% SDS at 60°C. Exposure to Kodak X-ray film was carried out at −70°C with an intensifying screen (Du Pont).

Assay of enzymes The activity of AHH in hepatocytes was measured primarily as described previously.[35,41] The reaction mixture (1 ml) contained 50–100 μg protein of 8,000g supernatant of hepatocytes, 1.08 μmol of NADPH, 3 μmol of $MgCl_2$, 100 nmol of benzo[*a*]pyrene and 50 μmol of Tris-HCl, pH 7.5. The reaction was stopped after 30 min by addition of cold acetone and the metabolites were extracted with *n*-hexane, followed by extraction with 1 N NaOH. The emission at 522 nm with excitation at 396 nm was measured with 3-hydroxybenzo-[*a*]pyrene as a standard. Enzyme activity was expressed as pmol of 3-hydroxybenzo[*a*]pyrene produced per mg protein per min. Reaction mixture for assay of acetanilide hydroxylase activity was prepared by a modification of the method of Koop *et al.*[42] and contained 200–500 μg protein of 8,000g supernatant, 1 μmol of NADPH, 4 μmol of acetanilide and 50 μmol of Tris-HCl, pH 7.5, in a total volume of 1.0 ml. The reaction proceeded for 30 min at 37°C and then 3 ml of cold diethyl ether and 0.5 g of NaCl were added. Extraction with diethyl ether was repeated once more and the organic phases were combined, followed by evaporation under a stream of nitrogen gas. The residue was dissolved in a small volume of 50% methanol and subjected to high-performance liquid chromatography using a Zorbax ODS column (Du Pont; 4.6 mm×25 cm) with 50% methanol at a flow rate of 0.7 ml/min. Production of 4-acetamidophenol was quantitated by use of an internal standard.

RESULTS

Differences in *Cyp*1a1 and *Cyp*1a2 gene expression between monolayer and spheroid cultures When mouse hepatocytes were seeded on collagen-coated dishes, they spread within 6 h and formed monolayers within 24 h. In contrast, if dishes were not coated with any attachment factor, the hepatocytes did not spread in spite of anchoring to the dish, and subsequently aggregated. The aggre-

gates gradually grew by assembling together and detached to form floating spherical aggregates (spheroids) after 72 h. This process of forming spheroids was identical to that described earlier for rat hepatocytes,[29] except that rat hepatocytes required proteoglycans as cell attachment factors during spheroid formation, whereas those from the mouse did not. Furthermore, at day 5 of cultivation more than half of the aggregates of mouse hepatocytes were still anchored to the dish, while almost all rat hepatocytes had formed floating spheroids. Figure 1 shows the results of Northern-blot hybridizations of total RNAs from C57BL/6 mouse hepatocytes cultivated as monolayers or spheroids for up to 5 days. Treatment with 3-methylcholanthrene substantially induced both CYP1A1 and CYP1A2 mRNAs in either type culture at day 1. However, thereafter, in monolayer-cultured hepatocytes induction of CYP1A2 mRNA rapidly decreased. In contrast, induction levels of CYP1A1 mRNA were high even at day 5. In spheroid culture, although the induction pattern of the CYP1A1 mRNA was the same as that of monolayer-cultured cells, *Cyp*1a2 gene transcription after 3-methylcholanthrene treatment was still maintained at day 5. Identical induction patterns for both *Cyp*1a genes were observed after treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (data not shown). Constitutive expression of CYP1A2 mRNA in spheroid culture was maintained throughout the observation period, whereas that in monolayer culture was reduced to negligible levels. Expression of β-actin mRNA increased with increasing culture period and was higher in monolayer-cultured cells. It was not changed after 3-methylcholanthrene treatment.

Comparison of induction of *Cyp*1a genes between C57BL/6 and DBA/2 mouse hepatocytes We previously observed[37] induction of CYP1A1, but not CYP1A2, mRNA after treatment with aromatic hydrocarbons when hepatocytes from nonresponsive as well as responsive mouse strains were transferred to monolayer culture. Figure 2 demonstrates that the two *Cyp*1a genes in nonresponsive DBA/2 mouse hepatocytes in spheroid culture at day 3 were induced considerably after treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, but only slightly by 3-methylcholanthrene. This was in clear contrast to the similar amounts of CYP1A1 and CYP1A2 mRNAs expressed after treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3-methylcholanthrene in C57BL/6 mouse hepatocytes (Fig. 3). The same concentrations of the two inducers were employed with both strains. After treatment with 3-methylcholanthrene, amounts of CYP1A1 mRNA almost reached a plateau within 36 h and were maintained for up to 48 h in C57BL/6 mouse hepatocytes, while in DBA/2 the decrease started after 12 h. With 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, amounts of CYP1A1 mRNA maintained peak levels until 48 h in both strains. Alteration of CYP1A2 mRNA expression followed the same course as that of CYP1A1 mRNA.

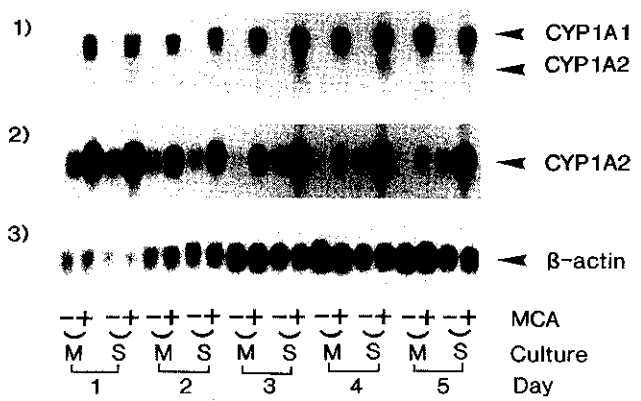

Fig. 1. Change of *Cyp*1a1 and *Cyp*1a2 gene expression after treatment with 3-methylcholanthrene. C57BL/6 mouse hepatocytes in either monolayer or spheroid culture were treated with 1.625 μM 3-methylcholanthrene (MCA) at the indicated culture times. For example, day 3 means treatment started 72 h after the beginning of cultivation. In each case, total RNA was prepared 10 h after the start of the treatment. Northern-blot hybridization of total RNA was performed using cDNA probes for mouse *Cyp*1a1 (1) and *Cyp*1a2 (2), and chicken β-actin (3). The same filter was used for all hybridizations. M, monolayer culture; S, spheroid culture.

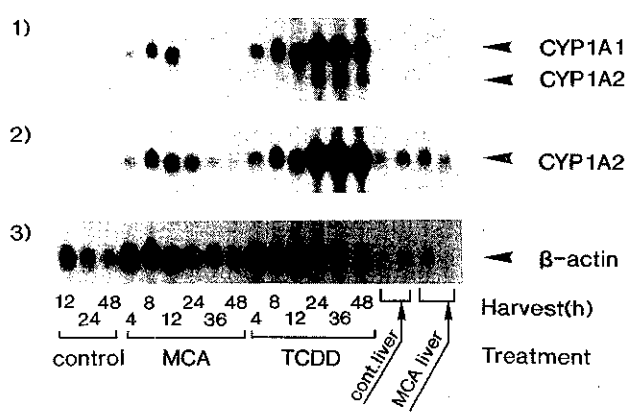

Fig. 2. Time-course of *Cyp*1a gene expression in DBA/2 mouse hepatocytes after treatment with 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. DBA/2 mouse hepatocytes in spheroid culture at day 3 were treated with either 3-methylcholanthrene (MCA) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Total RNA was prepared at the indicated harvest time points. Probes employed were the same as for Fig. 1. Liver RNAs were prepared 12 h after treatment with 3-methylcholanthrene at 30 mg/kg body weight.

Induced amounts of CYP1A2 mRNA were compatible with those observed *in vivo* after treatment of C57BL/6 mouse with 3-methylcholanthrene at 30 mg/kg body weight for 12 h, while those of CYP1A1 mRNA in culture were higher. With the same dosage of 3-methylcholanthrene, neither CYP1A1 nor CYP1A2 mRNA was induced in DBA/2 mouse liver.

Induction of enzyme activity Induced enzyme activities of AHH, and AAH after 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin treatment were measured in hepatocytes cultivated as either monolayers or spheroids (Table I). Treatment was started at the 3rd day of cultivation and enzyme activities were determined 24 h or 48 h later. Induced activities of both enzymes were lower after 24 h treatment than those after 48 h under both culture conditions. The ratios of AAH to AHH in monolayer culture were not changed between 24 h and 48 h treatment, and those at 24 h were almost the same between monolayer and spheroid culture. In contrast, they were significantly ($P<0.02$) elevated at 48 h in spheroid culture.

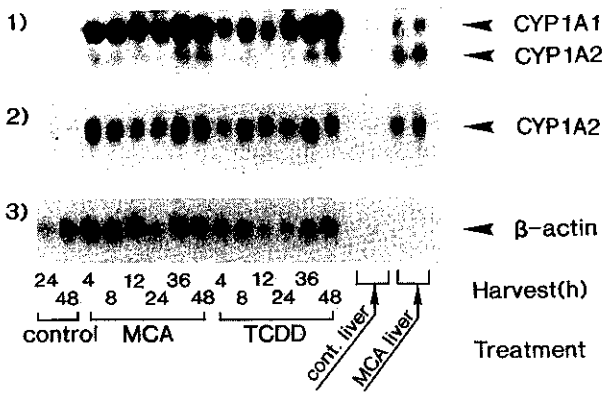

Fig. 3. Time-course of *Cyp*1a gene induction by 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in C57BL/6 mouse hepatocytes. C57BL/6 mouse hepatocytes in spheroid culture at day 3 were treated with either 3-methylcholanthrene (MCA) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Total RNA was prepared at the indicated times after the start of treatment. Probes were the same as for Fig. 1. Liver RNAs were prepared 12 h after treatment with 3-methylcholanthrene at 30 mg/kg body weight.

DISCUSSION

The present investigations revealed that inducibility of *Cyp*1a2 as well as *Cyp*1a1 gene expression after treatment with 3-methylcholanthrene was maintained at least throughout the initial 5 days of cultivation of hepatocytes as spheroid cultures, whereas in monolayer-cultured cells only *Cyp*1a1 gene expression was inducible at a comparable level to that in spheroid culture and *Cyp*1a2 gene induction rapidly decreased.

Although both *Cyp*1a1 and *Cyp*1a2 genes *in vivo* are inducible by the same kinds of aromatic hydrocarbons, it is considered that their regulation is independent. This conclusion is supported by an analysis of the nucleotide sequence of genomic *Cyp*1a2: xenobiotic responsive elements, assumed to be binding regions of a complex of the so-called *Ah* receptor with the inducer in the *Cyp*1a1 genome,[43] are not found in the proximal 5′-flanking region of *Cyp*1a2.[44] The regulation mechanisms of *Cyp*1a1 gene expression have been elucidated comparatively successfully and several regulatory factors have been

Table I. Induction of Aryl Hydrocarbon Hydroxylase (AHH) and Acetanilide-4-hydroxylase (AAH) in C57BL/6 Mouse Hepatocytes in Primary Culture

Treatment	For 24 h			For 48 h		
	AHH	AAH	AAH/AHH	AHH	AAH	AAH/AHH
Monolayer						
Control	2.2±0.5	n.d.	–	1.6±0.3	n.d.	–
MCA	182.9±15.5	104.3±45.2	0.56±0.22	230.9±10.0	172.9±27.1	0.75±0.15
TCDD	184.8±54.5	165.1±15.2	0.96±0.31	336.5±31.2	304.6±64.2	0.90±0.12
Spheroid						
Control	4.6±2.2	n.d.	–	3.1±0.3	n.d.	–
MCA	197.2±43.5	125.0±18.3	0.69±0.10	243.9±19.2	325.2±21.7	1.34±0.10*
TCDD	183.3±28.6	163.1±29.8	0.89±0.09	282.7±27.0	368.2±30.8	1.31±0.10*

The hepatocytes at day 3 of cultivation were treated with 3-methylcholanthrene (MCA) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and enzyme activities (pmol/mg protein/min) were determined 24 h or 48 h later. Each value represents the mean±SD from four experiments.
* Statistically significant difference ($P<0.02$) by Student's *t* analysis. n.d.: not detected.

suggested, including a cytosolic *Ah* receptor,[45] a short-lived suppressor protein,[46, 47] and cyclic AMP,[48] whereas knowledge of *Cyp*1a2 gene expression regulation mechanism is limited. One main reason is that a convenient cell culture system has not been available. Liver is the only organ which constitutively expresses CYP1A2 protein and is responsive to its induction, and expression of its mRNA in selected extrahepatic tissues is very small even in the presence of high concentrations of the potent inducer, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.[49]

Up till now, however, hepatocytes in primary culture have not been considered as a suitable tool for investigation of *Cyp*1a2 gene expression, because of the observed rapid decline in both constitutive expression and responsiveness to inducers.[4, 5] No established cell lines have yet been reported to express this gene. In 1988 two reports[20, 21] appeared concerning induction of CYP1A2 mRNA by 3-methylcholanthrene using rat hepatocytes in monolayer culture, and suggested that its expression is regulated primarily at the post-transcriptional level by mRNA accumulation. However, while culture conditions, including isolation procedure and medium ingredients, were improved, induction potential nevertheless rapidly decreased with increasing culture period, and their experiments were mostly performed using hepatocytes at the first day of cultivation. Sinclair *et al.*[19] observed induction and accumulation of CYP1A2 protein after administration of 3,4,5,3′,4′,5′-hexachlorobiphenyl in mouse hepatocytes cultivated on Matrigel™ in the presence of 5-aminolevulinic acid. They suggested that the accumulation might be due to stabilization of the induced CYP1A2 protein by binding to the inducer. Treatment with inducer was started from day 2 with daily medium change including the inducer, and it is uncertain from their paper whether the induction would be possible if exposure was started at a later culture period. Furthermore, a single treatment with 3.7 μM 3-methylcholanthrene at day 2 did not induce detectable levels of CYP1A2 protein in their experiments.

Multicellular spheroid culture of rat hepatocytes has been found to maintain several differentiation functions. For example, Koide *et al.* reported that cells in spheroids showed low growth activity even in the presence of epidermal growth factor and prominent albumin production.[29] Addition of proteoglycans is necessary for production of spheroids by rat hepatocytes.[28] It has been shown that transcriptional regulation of some liver-specific genes is significantly decreased when hepatocytes are placed in culture, resulting in a predominance of post-transcriptional regulation,[30] but this is reversed by addition of proteoglycans, glycosaminoglycans or other polyanions.[27] These observations suggest that improvements in culture conditions can restore *in vivo* expression patterns of P450 genes.

Little information is available on conditions for formation of spheroids by mouse hepatocytes in primary culture, but we found their cultivation on non-coated dishes relatively simple without any attachment factors, and the morphological time-course of their generation was identical to that of rat cells using proteoglycans. Within 72 h of culture the aggregates anchored to the dish surface, but subsequently half of them became detached, floating, by day 5. Induction of CYP1A1 and CYP1A2 mRNA in floating spheroid after this day was very low (unpublished observations). In the present experiments the fact that induction of CYP1A2 mRNA was maintained for at least the initial 5 days and then decreased suggests that a loss of induction potential occurs with floatation. Our observations do not yet allow any firm conclusions as to whether tight cell-cell interaction or communication in spheroids is necessary for maintenance of *in vivo* differentiated functions, but future analysis of the spheroid culture system should clarify this point.

Marker enzyme activities for CYP1A1 and CYP1A2 have been reported to be AHH and AAH, respectively.[24] In the present experiments the evidence of the alterations of relative ratios of these enzymes in spheroid culture 48 h after the inducer treatment, compared with the results in monolayer culture, suggests that more active CYP1A2 protein might be produced in spheroids. However, like many kinds of P450 species, substrate specificity is not absolutely exclusive and there is no information regarding the specificity of mouse CYP1A2 protein for AAH and AHH, so that it cannot be definitely concluded that active CYP1A2 enzyme protein is translated from the corresponding mRNA.

In conclusion, although earlier attempts to maintain CYP1A2 expression or induction beyond the first one or two days after transferring hepatocytes to primary culture were not successful, the present observations demonstrate that use of spheroid culture prolongs these functions, facilitating investigation of the regulatory mechanisms, especially at the transcriptional level.

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