J. Biochem. 120, 42-48 (1996)

Application of Primary Hepatocytes from p53-Knockout Mice for Studies of Expression of Cyp3a[1]

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Received for publication, November 29, 1995

CYP3A rapidly disappears in primary hepatocytes, although the primary cells are suitable for studies of the regulation of *CYP3A* genes. In the present study, we found that Cyp3a mRNA could be expressed in the primary hepatocytes from p53-knockout mice for at least 2 weeks when the cells were cultured in the presence of dexamethasone. Propoxycoumarin *O*-depropylase activity, which is known to be mainly catalyzed by CYP3A, was maintained at a level of 50% of the initial activity even after 5 days of culture, and the activity correlated with the expression level of Cyp3a mRNA in the primary hepatocytes from p53-knockout mice. The cells remained morphologically intact during 4 weeks. These results suggest that hepatocytes from p53-knockout mice are a useful tool for studies of the expression of Cyp3a.

Key words: Cyp3a, dexamethasone, hepatocytes, p53, primary culture.

We have already isolated three cDNA clones encoding distinct Cyp3a forms. Cyp3a-11 and Cyp3a-13 are major and minor forms in the mouse adult liver, respectively, and are induced by dexamethasone (*1, 2*). Cyp3a-16 is a fetal- and puberty-specific form and is expressed only in the liver (*3*). In order to investigate various properties, such as drug-metabolizing activity and gene regulation, of Cyp3a, we attempted to find convenient cell lines expressing Cyp3a.

Hepatocytes have been used as a convenient tool for pharmacological and toxicological studies (*4*), since a number of established hepatic cell lines exhibit only a limited spectrum of the normal hepatic functions (*5-7*). On the other hand, as a disadvantage in the use of hepatocytes, primary hepatocytes generally survive for only a short period of time and show a rapid decline of total P450 (*8-10*). Many attempts have been made to prolong the expression of P450s in primary hepatocytes: co-culture of the primary hepatocytes with a liver epithelial cell line (*4, 11*); culture on Matrigel (*12*), Vitrogen (*13*), collagen gel (*14*), or EHS gel (*15*); the addition of hormones (*9*), growth factors (*16, 17*), or specific chemicals (*18, 19*); spheroid culture (*20*); and the use of fetal hepatocytes (*21*). In the above-mentioned methods, several forms of P450s have been reported to be maintained in the primary hepatocytes as follows. 3-Methylcholanthrene highly induced CYP1A1 and CYP2A1 in rat primary hepatocytes on Vitrogen (*13*). CYP2Bs were highly induced by phenobarbital in primary hepatocytes, even after 40 days in culture on Vitrogen (*13*). Dexamethasone increased CYP3A mRNA dramatically from the basal levels in rat liver or zero time cultured cells on Matrigel (*12*). Moreover, the expression of Cyp1a and Cyp2b could be maintained for 3 days and 5 days, respectively, and inducers increased their expression markedly in the spheroid culture system (*20, 22*). However, long-term expression of Cyp3a has never been reported in primary hepatocytes from mice.

Recently, viral and cellular oncogenes or mutated tumor suppressor genes with immortalizing activity have been reported. These genes transformed primary cells, leading to tumorigenicity (*23, 24*). Hepatocyte cell lines have been established from transgenic mice carrying the temperature-sensitive SV40 large T-antigen gene. This cell line has been shown to possess inducibility of the *CYP1A1* gene by 3-methylcholanthrene (*25*). For rat and human hepatocytes transformed with SV40 large T-antigen, several differentiated features of normal hepatocytes, such as the expression of albumin, were retained (*26, 27*). Moreover, hepatocyte cell lines, which are nontumorigenic, were established from TGFα-transgenic mice (*28*). TGFα as well as HGF can enhance DNA synthesis in hepatocytes (*29, 30*).

p53, one of the tumor suppressor genes, behaves as a negative growth regulator. Transformed cells carrying a wild-type p53 gene were arrested in the G1 phase of the cell

[1] This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: P450, cytochrome P450; EGF, epidermal growth factor; EHS, Engelbreth-Holm-Swarm tumor; FCS, fetal calf serum; HGF, hepatocyte growth factor; L-15, Leibovitz's L-15 medium; SV40, simian virus 40; SDS, sodium dodecyl sulfate; SSC, sodium chloride-sodium citrate solution; TGFα, transforming growth factor α.

cycle (*23, 31, 32*). Recently, p53-knockout mice were established using the gene targeting method (*33, 34*). The fibroblasts prepared from p53-knockout mice acquired the capacity of immortality (*34, 35*), however, the properties of cultured hepatocytes are not known. Therefore, we focused on primary hepatocytes from p53-knockout mice.

Initially, we investigated DNA synthesis, expression of albumin mRNA and morphology of primary hepatocytes from p53-knockout mice, and whether or not the expression of Cyp3a could be maintained for a long period during culture of the cells.

MATERIALS AND METHODS

Materials—Williams' E and L-15 mediums were purchased from GIBCO BRL (Grand Island, NY). EGF and insulin were obtained from Takara Shuzo (Ohtsu). HGF was purchased from Toyobo (Osaka). Percoll and collagenase were products of Pharmacia (Uppsala, Sweden) and Yakult (Tokyo), respectively. Collagen-coated dishes and 96-well plates were obtained from Corning (Corning, NY). Amplified *Taq* polymerase was obtained from Roche Molecular Systems (Branchburg, NJ). Dexamethasone was purchased from Wako Pure Chemicals (Osaka). 7-Propoxycoumarin was a kind gift from Dr. Yamazoe of Tohoku University. [^{3}H]Thymidine and [α-^{32}P]dCTP were purchased from Amersham (UK).

Animals—p53-knockout mice were produced by Tsukada *et al.* using homologous recombination, and the judgment of wild (p53 +/+), heterogeneous (p53 +/−) or null (p53 −/−) mice was performed by the polymerase chain reaction method (*34*). Animals were maintained on a commercial chow and tap water *ad libitum*. Male mice at 8 weeks of age were used.

Hepatocyte Culture—Livers were perfused *in situ* with calcium-free Hanks' solution followed by the perfusion of Hanks' solution containing 0.5 mg/ml collagenase. The softened liver was minced and filtered through nylon mesh. The cell suspension was centrifuged at 50 × *g* for 2 min. The cells were resuspended in L-15 medium. Subsequently, the cell suspension was laid over isodensity Percoll followed by centrifugation at 50 × *g* for 15 min (*36*). The pellets were dispersed in Williams' E medium containing 10% FCS, insulin (0.4 μg/ml), EGF (20 ng/ml), and HGF (5 ng/ml). The cells were seeded at a density of 5×10^5 cells/ml in a 100 mm collagen-coated dish. The culture was maintained in a humidified incubator at 37°C under an atmosphere of 5% CO_2/95% air. The non-attached cells were discarded by aspiration 4 h after seeding, followed by a medium change. If necessary, dexamethasone dissolved in dimethyl sulfoxide (final concentration 0.5%) was added to the culture medium.

In Vitro Assay of Growth Factor-Induced Hepatocyte Proliferation—Isolated hepatocytes were seeded on collagen-coated 96-well plates at a cell concentration of 1×10^4 cells/well in 0.1 ml of Williams' E medium supplemented with 10% FCS. Four hours after seeding, the medium was removed by aspiration, followed by the addition of the above-mentioned Williams' E medium containing insulin, EGF or HGF, but not FCS. After 24 h of culture, 0.1 μCi of [^{3}H]thymidine was added to each well. Then the cells were incubated for another 24 h, and the incorporation of [^{3}H]-thymidine was measured.

Microscopic Observation of Primary Hepatocytes—Phase-contrast light micrographs were taken on a Olympus SC 35 camera (Tokyo) with an IMT-2 microscope (Olympus Optical Co., Tokyo).

Northern Blot Analysis—Total RNA was prepared from primary hepatocytes according to the acid-guanidinium phenol-chloroform method (*37*). Total RNA (10 μg) was electrophoresed in 0.8% agarose gel containing 18% formaldehyde and transferred to a nylon membrane (Nytran NY13, Schleicher & Schuell). The membrane was hybridized with the ^{32}P-labeled mouse *Cyp3a-11* (*1*), human albumin (provided by the Japanese Cancer Research Resources Bank), or mouse β-actin cDNAs (*38*) by using the DNA labeling system (Nippon Gene, Tokyo). Hybridization was carried out by the method of Sambrook *et al.* (*39*). The membrane was washed twice with 0.5 × SSC containing 0.2% SDS at 50°C for 30 min. The same membrane was used for experiments to determine the

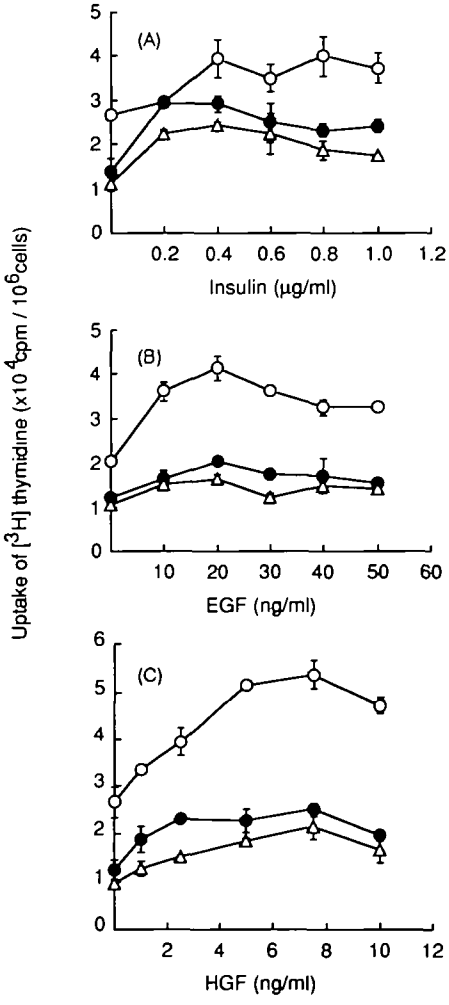

Fig. 1. **Effects of insulin, EGF, and HGF on the proliferation of primary hepatocytes from p53 (−/−), p53 (+/−), and p53 (+/+) mice.** Open circle, p53 (−/−) mice; closed circle, p53 (+/−) mice; open triangle, p53 (+/+) mice. All values represent mean ± SD (n = 3). [^{3}H]Thymidine incorporation into primary hepatocytes was measured with various concentrations of insulin (A), EGF (B), and HGF (C) in the culture medium. Three independent experiments gave similar results.

expression of albumin and β-actin mRNAs. Exposure to X-ray film (Fuji, Minamiashikaga) was carried out at −80°C with an intensifying screen for either 3 days or 2 weeks. RNA bands were quantified by scanning densitometry using Quick Scan R&D (Helena Laboratories, Beaumont, TX).

Enzyme Assay—7-Propoxycoumarin *O*-depropylase activity was assayed by the modified method of Aitio (*40*). Primary hepatocytes were seeded at 2×10^6 cells/60 mm dish. When 7-propoxycoumarin was added to the medium, the medium was also renewed. After 2 h, the reaction product in the medium was extracted with chloroform. The product in the organic phase was subsequently dissolved in 0.01 N NaOH containing 1 M NaCl. The concentration of 7-hydroxycoumarin in the aqueous alkaline phase was determined spectrofluorometrically, with excitation at 368 nm and emission at 456 nm. The number of living cells in each dish was also counted. After the measurement of the enzyme activity, cells were harvested with 0.05% trypsin/0.02% EDTA, and the number of non-staining cells was counted. The assays were carried out in duplicate. One unit of 7-propoxycoumarin *O*-depropylase activity was defined as the amount of enzyme catalyzing the formation of 1 pmol of 7-hydroxycoumarin/min/10^6 cells.

RESULTS

Effects of Insulin, EGF, and HGF on the Proliferation of Primary Hepatocytes from p53 (+/+), p53 (+/−), and p53 (−/−) Mice—To examine whether or not the proliferation of primary hepatocytes from p53 (−/−) mice was enhanced by growth factors, the primary cells were cultured in the presence and absence of insulin, EGF, or HGF, and the incorporation of [^{3}H]thymidine was measured (Fig.

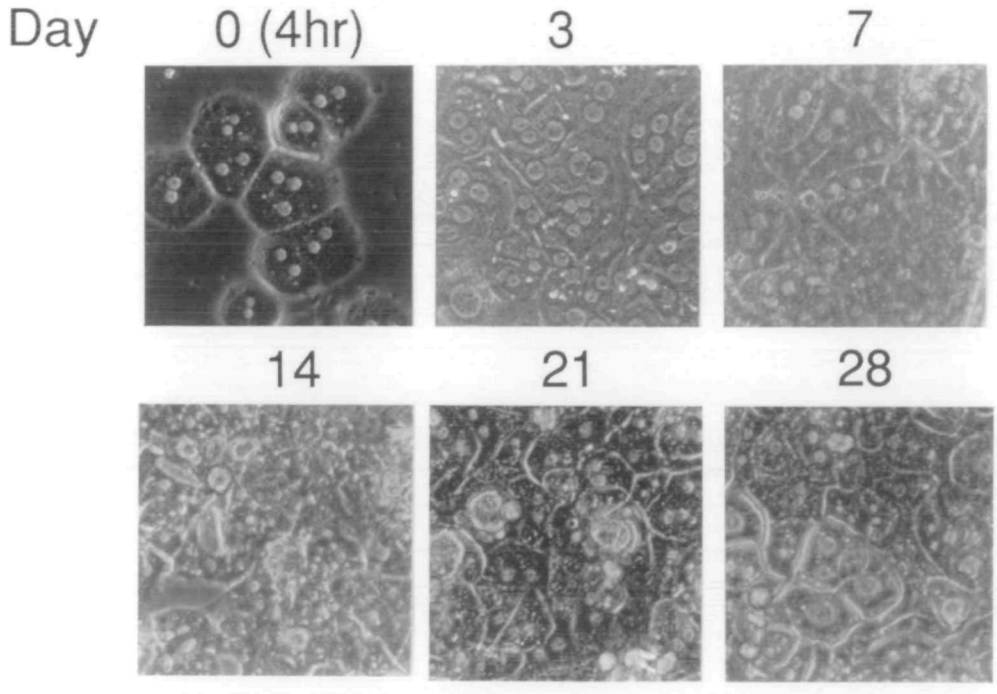

Fig. 2. **Inverted microscopic observation of primary hepatocytes from p53 (−/−) mice.** The medium containing dexamethasone (20 μM) was changed once a week. Magnification is ×300.

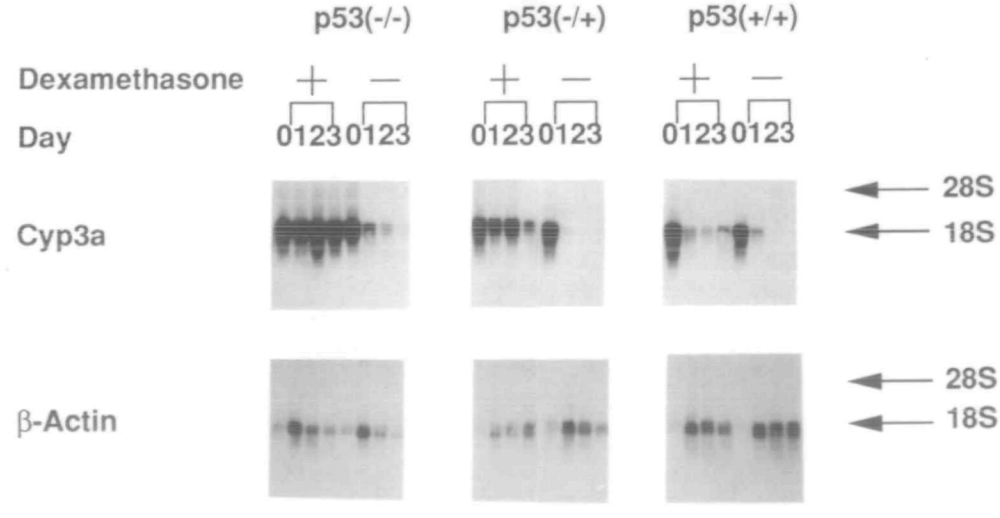

Fig. 3. **Northern blot analysis of Cyp3a mRNA in primary hepatocytes from p53 (−/−), p53 (+/−), and p53 (+/+) mice.** After 4 h of culture, the medium was changed to a fresh medium with (+) or without (−) dexamethasone (20 μM) and culture was continued for an additional 3 days. The sizes of ribosomal RNAs (28S and 18S) are indicated by arrows. X-ray film was exposed for 3 days.

1). The effect of each growth factor on DNA synthesis was examined. The primary hepatocytes from p53 (−/−) mice showed a higher capacity to incorporate added [^{3}H]thymidine as compared to the primary hepatocytes from p53 (+/+) mice in the presence of insulin (Fig. 1A), EGF (Fig. 1B), or HGF (Fig. 1C). These results correlated well with the observation on the proliferation of fibroblasts from p53-knockout mice (*34*). The extent of induction by these stimuli was similar (2-2.5 times) among primary hepatocytes from p53 (+/+), p53 (+/−), and p53 (−/−) mice. Since these growth factors augmented the proliferation of the primary hepatocytes, we added insulin (0.4 μg/ml), EGF (20 ng/ml), and HGF (5 ng/ml) to the culture medium in the following experiments.

Morphological Observation of the Primary Hepatocytes from p53 (−/−) Mice—The inverted microscopic observation of the primary hepatocytes from p53 (−/−) mice indicated that the morphology of day 3 was maintained at that of day 28 (Fig. 2). The cells from p53 (−/−) mice grown on the collagen-coated dishes were flat, polygonal cells with round nuclei and granular cytoplasm.

Expression of Cyp3a mRNA in the Primary Hepatocytes—Since the level of P450 including Cyp3a in the primary hepatocytes from p53 (+/+) mice rapidly decreases, we investigated the changes in the amount of Cyp3a mRNA in the primary hepatocytes from p53 (−/−) mice. In the presence of dexamethasone, the amount of Cyp3a mRNA in the primary hepatocytes from p53 (−/−) mice was maintained at a high level for 3 days, although the level in the primary hepatocytes from p53 (+/−) mice gradually declined and the level in the primary hepatocytes from p53 (+/+) mice decreased markedly after 1-day culture even in the presence of dexamethasone (Figs. 3 and 5B). The absence of dexamethasone in the culture medium caused the amounts of Cyp3a mRNA in the primary hepatocytes from all mice to fall rapidly to low levels. Cyp3a mRNA in the primary hepatocytes from p53 (+/−) and p53 (+/+) mice was not detectable after culture for 3 days. To determine further the period for which the expression of Cyp3a mRNA was maintained in the primary hepatocytes from p53 (−/−) mice in the presence of dexamethasone, we examined the expression of Cyp3a mRNA for up to 4 weeks. Figure 4 shows that the expression of Cyp3a and albumin mRNAs could be detected for 2 weeks.

Propoxycoumarin O-Depropylation in the Primary Hepatocytes—The activity of Cyp3a was measured using primary hepatocytes from p53 (−/−) and p53 (+/+) mice. 7-Propoxycoumarin *O*-depropylation is mainly catalyzed by the CYP3A family (*41*). The *O*-depropylase activity decreased markedly and was not detectable after 5 days of culture in the primary hepatocytes from p53 (+/+) mice, while this activity was maintained at a high level for 3 days and was detectable even after 21 days of culture in the primary hepatocytes from p53 (−/−) mice (Fig. 5A). The *O*-depropylase activity in 5-day-cultured cells from p53 (−/−) mice decreased to half of that in 4-h-cultured cells and the time courses of the activity and the expression of Cyp3a mRNA were biphasic. Although the activity of *O*-depropylase in the primary hepatocytes from p53 (−/−) mice was lower than that of the p53 (+/+) mice, the activity of *O*-depropylase in p53-deficient

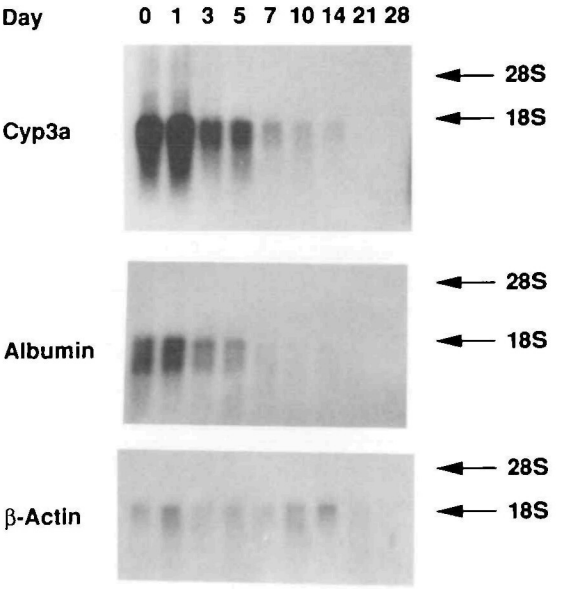

Fig. 4. **Time course of expression of Cyp3a and albumin mRNAs in primary hepatocytes from p53 (−/−) mice.** The medium containing dexamethasone (20 μM) was changed once a week. The sizes of ribosomal RNAs (28S and 18S) are indicated by arrows. X-ray film was exposed for 2 weeks.

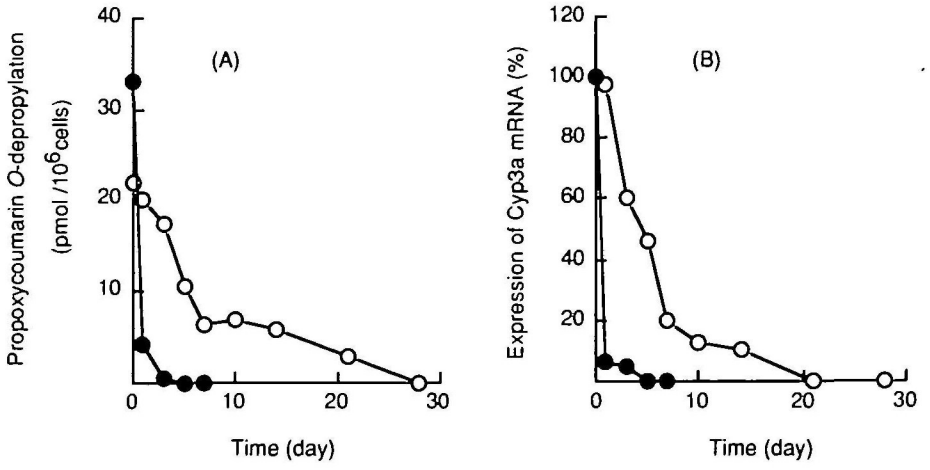

Fig. 5. **Correlation of 7-propoxycoumarin *O*-depropylase activity with the amount of Cyp3a mRNA in primary hepatocytes from p53 (−/−) and p53 (+/+) mice.** Open circle, p53 (−/−) mice; closed circle, p53 (+/+) mice. (A) 7-Propoxycoumarin *O*-depropylase activity. (B) The amount of Cyp3a mRNA. The level after 4 h was defined as 100%.

hepatocytes could be maintained longer than that in the hepatocytes from p53 (+/+) mice (Fig. 5A). There were no differences in the amount of Cyp3a mRNA between primary hepatocytes from p53 (−/−) mice and p53 (+/+) mice 4 h after the preparation. Furthermore, the level of this activity correlated well with that of Cyp3a mRNA in both hepatocytes (Fig. 5B).

DISCUSSION

Primary hepatocytes not only survive for only a short period of time, but also lose the ability to express P450 rapidly during culture (*8, 9, 12*). If primary hepatocytes can easily be cultured for a longer period of time without the loss of the liver-specific functions, this model should be widely applicable for biochemical as well as pharmacological and toxicological studies.

In this study, the primary hepatocytes from p53-knockout mice could be maintained for a sufficient period to do certain research. The DNA synthesis was enhanced by the simultaneous addition of insulin, EGF, HGF, and dexamethasone as reported by other laboratories (*9, 17, 19*). The addition of insulin (0.4 μg/ml), EGF (20 ng/ml) and HGF (5 ng/ml) and 10% FCS to the medium was optimal, because the growth factors have additive effects on the cell proliferation. The standard culture conditions containing the growth factors and 10% FCS might be expected to be suppressive to P450 induction in normal hepatocytes. On the other hand, a serum-free condition also resulted in the loss of Cyp3a mRNA after 1-day culture of primary hepatocytes from p53 (+/+) mice (data not shown). Although the concentration (20 μM) of dexamethasone seemed to be very high during the early days of primary culture, the incorporation of [^{3}H]thymidine was not inhibited by this concentration (data not shown). The expression of Cyp3a and albumin mRNAs was observed for only about 2 weeks in the presence of dexamethasone in the primary hepatocytes from p53 (−/−) mice (Fig. 4). In the absence of dexamethasone in the culture medium, the expression of Cyp3a mRNA was maintained for only 2 days in culture (Fig. 3), and dexamethasone was essential for the long-term expression of Cyp3a mRNA in primary hepatocytes from p53 (−/−) mice. Furthermore, S1 nuclease mapping analysis indicated that Cyp3a-11 mRNA was expressed for 2 weeks after culture (data not shown). Dexamethasone may act as an inducer of Cyp3a mRNA (*1*) and may also aid cell viability (*9, 42*), though this remains to be examined; we did not study the effect of other inducers of Cyp3a.

7-Propoxycoumarin *O*-depropylase activity in the hepatocytes from p53(−/−) mice was detectable for at least 3 weeks (Fig. 5A). CYP1A (*10, 43*) and CYP2A (*44*), as well as CYP3A (*41*), can catalyze the *O*-depropylation of 7-propoxycoumarin. However, the expression of Cyp1a-2, Cyp2a, Cyp2b, Cyp2c, Cyp2e, or Cyp4a was not detectable in primary hepatocytes from p53-knockout mice 1 week after the culture (data not shown), suggesting that the 7-propoxycoumarin *O*-depropylation in the primary hepatocytes from p53-knockout mice was mediated by Cyp3a. In addition, although the activity of *O*-depropylase was lower in the hepatocytes from p53-knockout mice compared to p53 (+/+) mice (0 day), the mRNA levels at this time were similar. This result may be due to the lack of specificity of the enzyme activity, because Cyp1a-2 and Cyp2a, as well as Cyp3a, were expressed at high levels in both primary hepatocytes (data not shown). The activities were detectable at 21 days, while the mRNA levels were no longer detectable at 21 days in the hepatocytes from p53-knockout mice (Figs. 5 and 4). p53 deficiency might cause stabilization of the Cyp3a protein in some way. The *O*-depropylase activity in the primary hepatocytes from p53 (−/−) mice 4 h after culture was only 1.2% of that in liver microsomes from p53 (+/+) mice (data not shown).

It has been reported that primary cells from many tissues of p53-knockout mice proliferate continuously (*34, 35*). However, no such effects of p53 deficiency on proliferative properties were observed in cardiac muscle cells and hepatocytes (*34*). We succeeded in culturing hepatocytes from p53-knockout mice for a longer time by the simultaneous addition of insulin, EGF, HGF, and dexamethasone to the culture medium. In the absence of dexamethasone in the culture medium, the hepatocytes from p53-knockout mice did not survive for a month (data not shown). HGF inhibited the growth of hepatocellular carcinoma cells such as Fao (*45*), whereas the DNA synthesis in the hepatocytes from p53 null type, as well as those of the wild type, was enhanced by about 2-fold by HGF (Fig. 1C). In addition, the frequency of cells carrying two nuclei (Fig. 2) and the expression of albumin (Fig. 4) suggest that p53-deficient hepatocytes reflect the properties of the cells from p53 (+/+) mice at early culture time. Although the hepatocytes remained morphologically normal, the cells did not proliferate further after 1 week of culture. Other factors may be necessary to enhance the proliferative potential of hepatocytes from p53-knockout mice.

In summary, the primary hepatocytes from p53-knockout mice can survive for at least a month. The Cyp3a mRNAs could be easily expressed for 2 weeks when the cells were cultured on collagen-coated dishes in the presence of the growth factors, FCS and dexamethasone.

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