Complete Rescue of Lethal Albino c14CoS Mice by Null Mutation of 4-Hydroxyphenylpyruvate Dioxygenase and Induction of Apoptosis of Hepatocytes in These Mice by in Vivo Retrieval of the Tyrosine Catabolic Pathway*

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Fumio Endo‡§, Shuji Kubo‡, Hisataka Awata‡, Koji Kiwaki‡, Hideki Katoh†, Yumi Kanegaei, Izumu Saito, Jun-ichi Miyazaki**, Tetsuro Yamamoto‡‡, Cornelis Jakobs§§, Shinzaburo Hattori¶¶, and Ichiro Matsuda‡

From the ‡Department of Pediatrics, the §§Division of Molecular Pathology, Graduate School of Medical Sciences, and the ¶¶College of Medical Sciences, Kumanomo University School of Medicine, Honjo 1-1-1, Kumanomo 860, Japan, the †Department of Genetics, Central Institute for Experimental Animals, 1430 Nogawa, Miyamae Kawasaki 216, Japan, the Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan, the **Institute of Development, Aging and Cancer, Tohoku University, Seiryo-machi 4-1, Aoba-ku, Sendai 980-77, Japan, and the §§Department of Pediatrics and Clinical Chemistry, Free University Hospital, de Boelelaan 1117, NL-1081 HV Amsterdam, The Netherlands

Hereditary tyrosinemia 1 (HT1) is characterized by progressive liver damage, from infancy, and by a high risk for hepatocellular carcinoma. HT1 is due to mutations in the fumarylacetoacetate hydrolase gene Fah, encoding the last enzyme in the tyrosine catabolic pathway. Lethal albino deletion c14CoS/mice and mice with target-disrupted Fah are models for HT1, but they die in the perinatal period, albeit with a different phenotype from that seen in HT1 in humans. We first asked whether homozygous null mutation of the 4-hydroxyphenylpyruvate dioxygenase geneHpdd could rescue the homozygous c14CoS/mice (c14CoS/c14CoS or Fah−/−). The double mutant Fah−/−Hpdd−/−mice appeared normal, at least until age 18 months, and there was no evidence of liver disease, findings that facilitated examination of the effect of Fah−/−on mature and unmodified hepatocytes in vivo. The hepatocytes of Fah−/−undergo rapid apoptosis, and acute death follows. Essentially the same phenomena were observed when Fah−/−Hpdd−/−mice were administered homogentisate intraperitoneally. These changes in liver pathology in Fah−/−Hpdd−/−mice after the administration of homogentisate are associated with massive urinary excretion of succinylacetone. These results suggest that accumulation of fumarylacetoacetate, maleylacetoacetate, or succinylacetone seems to trigger the endogenous process of apoptosis in hepatocytes that lack fumarylacetoacetate hydrolase activity. This apoptosis may be related to the development of hepatocellular carcinomas seen in HT1 patients and pharmaceutically treated fumarylacetoacetate hydrolase-deficient mice.

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‡ To whom correspondence should be addressed. Tel.: 81-96-373-5191; Fax: 81-96-366-3471; E-mail: endo@kaju.med.kumamoto-u.ac.jp.

3 The abbreviations used are: HT1, hereditary tyrosinemia 1; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; FAH, fumarylacetoacetate hydrolase; HPD, 4-hydroxyphenylpyruvate dioxygenase; PCR, polymerase chain reaction; pfu, plaque-forming units.
Hpd gene would rescue the homozygous c14CoS mice (c14CoS/c14CoS or Fah−/−) (5, 8, 9, 12). HPD catalyzes a complex reaction to form homogentisate from 4-hydroxyphenylpyruvate. The homozygous mutant Hpd allele from mouse strain III is expected to completely block tyrosine catabolism (13–15). As the formation of homogentisate exclusively depends on HPD activity, a complete block of tyrosine catabolism at this step would result in deprivation of homogentisate and its oxidative products. The outcome of the double mutants (Fah−/− Hpd−/−) itself is important for evaluating treatment strategies (4) and for understanding the mechanism of the related carcinogenesis (1, 16).

We report here that blocking of tyrosine catabolism concealed the liver damage in lethal albino deletion c14CoS mice. Retrieval of the tyrosine catabolic pathway in the double mutants by in vivo gene transfer or by injection of homogentisate resulted in apoptotic death of the hepatocytes. These observations provide new insights into the disease process of HT1. This model will aid in assessing and developing treatments for HT1 patients.

**EXPERIMENTAL PROCEDURES**

**Generation of Double Mutants**—Heterozygous c14CoS (Fah−/+)-mice (kindly provided by G. Schütz) were crossed with homozygous III mice (Hpd−/−), followed by the breeding of F1 × homozygous III mice. Mice with the genotype Fah−/− Hpd−/− were identified and used for the next breeding for generation of Fah−/− Hpd−/− mice. Homozygotes for the Fah− allele (Fah−/−) were identified by Southern blots using the RN.Fd probe (a gift from G. Schütz) as described (8, 9). Homozygotes for the Fah− allele (Fah−/−) were identified by the absence of exon 2 sequences and the presence of exon 8 sequences of the Fah gene after PCR amplification of regions containing each exon, respectively. The sequences of sense and antisense primers for PCR amplification of the regions of the mouse Fah gene were derived from the published cDNA sequence (12). Primer sets used for PCR amplifications were as follows (sense/antisense): a set for Fah gene exon 2, 5′-CACAAGCCTACCGAATCGG-3′/5′-CTATCGAAGACATGTTGATG; and a set for Fah gene exon 8, 5′-CAGGGAATCCGAAATGGC-5′/5′-CTGCTTGGGTTGAC-3′. The Hpd− allele was identified as described previously (15). DNA fragments were analyzed by electrophoresis on agarose gels and stained with ethidium bromide.

**Immunoblotting of FAH and HPD**—Liver samples were quick-frozen in dry ice and kept at −70 °C until use. The tissues were homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.4, and centrifuged at 11,000 g for 15 min at 4 °C. The supernatant (7.5 μg of protein) was subjected to SDS-10% polyacrylamide gel electrophoresis. Immunoblotting of HPD and FAH was carried out with antiserum directed against recombinant human HPD and FAH, respectively. Rabbits were immunized with glutathione S-transferase-human FAH and glutathione S-transferase-human HPD for production of antibodies, respectively.2 Preparations of human FAH cDNA (17) and HPD cDNA (18) was as described. Other conditions for SDS-polyacrylamide gel electrophoresis and immunoblotting were as described (18). Amounts of protein were determined by the Bio-Rad dye binding method. Hepatic HPD activity was measured as described previously (19).

**Recombinant Adenovirus**—A replication-defective recombinant adenovirus, human adenovirus type 5/13, carrying the E1A, E1B, and E3 regions and bearing a human HPD expression unit in the E1-deleted region, was prepared as described (20). Transcriptional orientation of the expression cassette is leftward, opposite the original orientation of the adenovirus, human adenovirus type 5 lacking the E1A, E1B, and E3 regions coding for the E1A, E1B, and E3 regions and bearing a recombinant adenovirus, snap-frozen and kept at −80 °C until use. A recombinant adenovirus, AdexCAGhOTC, which efficiently expresses human ornithine transcarbamylase in mouse liver when injected into the tail vein, was prepared as described previously (24). The effects of the intravenous administration of AdexCAGhOTC on the livers of C57BL/6 mice and ornithine transcarbamylase-deficient mice (sp+/−) were characterized (24).

**RESULTS**

**Phenotype of the Double Mutants (Fah−/− Hpd−/−)**—The mutant Hpd allele (Hpd−/) is from mouse strain III (Hpd−/) (13–15), a model for human hereditary tyrosinemia type 3 (27) that is characterized by elevation of blood tyrosine and the absence of visceral injuries (13, 27). There is a C to T transition at nucleotide +7 on exon 7 of the Hpd gene on chromosome 5 of the III mouse, the result being premature termination of translation (15). This stop codon mutation was associated with the skipping of exon 7 in its mRNA (15). The transcript with the termination codon has not been obtained by reverse transcription-amplification of RNA from the liver (15). Thus, the amount of the 14-kDa polypeptide that was synthesized from mRNA with the stop codon mutation appears to be negligible. On the other hand, the abnormally spliced transcript can be translated into a 38-kDa protein lacking the central part of the subunit of HPD. This 38-kDa protein seemed to be unstable. As a result, protein immunologically related to HPD was absent in III mouse liver.

Genotyping of the pups revealed that the double mutants (Fah−/− Hpd−/) survived and grew well (Fig. 1, A and B). Analysis of liver biopsy samples confirmed that FAH and HPD proteins were absent in these mice (Fig. 1C). The clinical phenotype of Fah−/− Hpd−/− mice was indistinguishable from those of Fah−/− Hpd−/− and Fah+/− Hpd−/− mice. The findings in liver sections from Fah−/− Hpd−/− mice (Fig. 2A) were normal and similar to those from Fah+/− Hpd−/− and III mice. Long-term investigations of Fah−/− Hpd−/− mice (12–18 months) revealed no evidence of hepatocellular carcinomas or preneoplastic lesions. Thus, the homozygous mutant Hpd allele from the III mouse not only rescued the lethal phenotype of Fah−/− mice, but also concealed the critical visceral phenotype of HT1.

Expression of HPD and Apoptosis of Hepatocytes in the Double Mutants—Phenotypically normal mice with inactive Fa provide a model in which the onset of visceral injuries of HT1 is controlled. At first, we attempted to retrovirally introduce the tyrosine catabolic pathway in these mice, as achieved by adenovirus-mediated in vivo expression of HPD in hepatocytes. Injection of the recombinant virus AdexCAGhHPD into the tail veins of the control mice (Fah−/− Hpd−/) resulted in expression of HPD in the liver (Fig. 1D) and was accompanied by a reduction in blood tyrosine levels. No animal injected with 5 × 106 pfu of AdexCAGhHPD was lost in the control experiments using C57BL/6, 2 F. Endo, S. Kubo, H. Awata, and I. Matsuda, unpublished data.
The death of the wild type as early as 6–12 h after administration (19). In the present study, livers were excised 6 h after the administration of AdexCAGHPD, and HPD activities in the liver were measured. These results indicated that the activities in treated Fah−/− Hpd−/− mice were similar to those in treated Fah+/+ Hpd−/− mice.

Fah−/− Hpd−/− mice treated with AdexCAGHPD were definitely ill from 12 h after the injection, and mobility was reduced and appetite lost. These animals died within 30 h after injection of the recombinant virus (Table I). In control experiments, we injected AdexCAGHTOC into the tail veins of Fah−/− Hpd−/−, III, or C57BL/6 mice; however, there were no significant changes in the clinical symptoms of these mice, and none were lost (Table I).

In liver sections obtained from the AdexCAGHPD-treated Fah−/− Hpd−/− mice, massive numbers of hepatocytes were nonviable. There was no infiltration of inflammatory cells, but there were small areas of bleeding (Fig. 2b). The damaged cells showed evidence of chromatic condensation. When the liver sections were investigated for 3′-OH DNA ends generated by DNA fragmentation (25), −15–25% of the hepatocytes in the observed regions were positive for the signals (Fig. 2c). Examination of nuclear DNA of the livers from the recombinant virus-treated Fah−/− Hpd−/− mice revealed fragmentation, with sizes corresponding to typical nucleosome units (Fig. 3). Thus, retrieval of HPD function in Fah−/− Hpd−/− mice resulted in apoptosis of hepatocytes. Treatment with AdexCAGhHTOC led to no significant changes in liver pathology in Fah−/− Hpd−/−, Fah+/+ Hpd−/−, or Fah+/+ Hpd+/+ mice.

Apoptosis of Hepatocytes by Homogentisate in the Double

FIG. 1. Generation of double mutants (Fah−/− Hpd−/−) and adenovirus-mediated expression of HPD in mice. A and B, rescue of a homozygous c3c5c5 mouse by a homozygous mutant Hpd allele (Hpd−/−) and genotype analysis. The deletion in c3c5c5 mice disrupted the Fah gene, resulting in the absence of exon 1 and 2 sequences (8). The results of PCR amplification of regions containing exon 2 (upper panel) and exon 8 (lower panel) of the Fah gene are shown in A. The results from PCR amplification and restriction enzyme digestion of a region containing exon 7 of the Hpd gene are shown in B. Also shown in B are an undigested fragment with normal sequences (386 base pairs (bp)) by the HindIII restriction enzyme and digested products due to the mutated sequence of the Hpd− allele by HindIII (272 and 114 base pairs) (15). The surviving Fah−/− Hpd−/− mice were able to generate offspring with the expected genotypes at the expected mendelian ratios when mated with Fah−/− Hpd−/−, Fah+/− Hpd−/−, and Fah+/+ Hpd−/− mice, respectively. C, immunoblot analysis of HPD and FAH in the liver. Lane 1, control human liver; lane 2, C57BL/6 mouse; lane 3, III mouse; lane 4, Fah−/− Hpd−/− mouse; lane 5, Fah−/− Hpd−/− mouse. FAH and HPD proteins were absent in the liver from the Fah−/− Hpd−/− mouse. D, adenovirus-mediated expression of human HPD in mouse liver. Lane 1, C57BL/6 mouse; lane 2, untreated Fah−/− Hpd−/− mouse; lane 3, adenovirus-treated Fah−/− Hpd−/− mouse; lane 4, untreated Fah−/− Hpd−/− mouse; lane 5, adenovirus-treated Fah−/− Hpd−/− mouse. FAH and HPD proteins were absent in the liver from the Fah−/− Hpd−/− mouse. The appearance of HPD protein in the livers of AdexCAGhHPD-treated Fah−/− Hpd−/− and Fah−/− Hpd+/− mice was noted 12 h after injection of 5 × 10^6 pfu of the recombinant virus. The dose was determined in preliminary experiments in which 5 × 10^5 pfu of the recombinant virus was effective in reducing blood tyrosine levels without apparent changes in liver histology (19). A significant reduction in blood tyrosine levels was evident as early as 3 h after injection of 5 × 10^6 pfu in Fah−/− Hpd−/− mice (from 20.7 ± 4.8 to 9.6 ± 2.1 mg/dl), and the levels reached those of controls (3.4 ± 1.1 mg/dl in treated mice versus 2.5 ± 1.6 in C57BL/6 mice) within 12 h.
The mice died within 16 h. (The mean dose given was 500 μg/kg. This amount of homogentisate corresponds to −20–25% of the daily intake of precursor amino acids, Phe + Tyr.) Liver sections from homogentisate-treated Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mice had massive numbers of dead hepatocytes with fragmentation of nuclei, as seen in the ultrastructural analysis (Fig. 2e). In addition, abnormalities in mitochondria were prominent. Approximately 20–30% of the hepatocytes gave positive signals by in situ detection of DNA fragmentation (data not shown). The double mutant mice treated with 5 mg of homogentisate did not die (Table I); however, ~2% of the hepatocytes were positive for in situ detection of fragmentation of DNA (data not shown). Nuclear DNA from the livers of homogentisate-treated Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mice showed typical fragmentation (Fig. 3). These apoptotic changes were nil in Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mice treated with

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**FIG. 2.** Histological appearance of lesions induced in Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mice by adenovirus-mediated expression of HPD or homogentisate injection. a, liver section from an untreated Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mouse (8 months old) showing a normal structure and no hepatocyte injury (hematoxylin eosin ×400). b, liver section from an Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mouse administered 5 × 10<sup>8</sup> pfu of AdexCAGhHPD (hematoxylin eosin ×400). Note the massive numbers of dead hepatocytes. c and d, fluorescence staining of DNA fragmentation in situ. The liver sections from Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mice administered 5 × 10<sup>6</sup> pfu of AdexCAGhHPD (magnification ×400) (e) or not (d) are shown after the staining. Many nuclei were positive for the signal when AdexCAGhHPD was administered (e). e and f, conventional electron microscopic views of hepatocytes from an Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mouse 12 h after injection of homogentisate (e) and from an untreated Fah<sup>−/−</sup> Hpd<sup>−/−</sup> mouse (f). Note the nuclear fragments with peripherally condensed chromatin (e). Mitochondria are swollen, and cytoplasmic vacuolations are evident (e) (original magnification ×2000). The bar indicates 1 μm.
Apoptosis of Hepatocytes in Mouse Model for HT1

AdexCAGhOTC. Liver sections from the homogentisate (10 mg)-treated animals with the genotypes Fah⁻/⁻ Hpd⁻/⁻, Fah⁻/⁻ Hpd⁻/⁺, and Fah⁻/⁺ Hpd⁻/⁻ showed no significant changes.

Blood Chemistry and Urinary Succinylacetone—To evaluate liver function in Fah⁻/⁻ Hpd⁻/⁻ mice before and after the administration of homogentisate or the recombinant adenovirus AdexCAGhHPD, serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, bilirubin, and blood urea nitrogen were measured before and 12 h after administration. In Fah⁻/⁻ Hpd⁻/⁻ mice on a normal diet, serum levels of aspartate aminotransferase and alanine aminotransferase activities were essentially the same as in III mice (Fig. 4, A and B), suggesting that the liver and kidney functions of the double mutants on the normal diet were normal. After the administration of homogentisate, the levels of transaminase activities were essentially the same as in Fah⁻/⁻ Hpd⁻/⁻ mice, suggesting either that small amounts

A small amount of succinylacetone was detected in the pooled urine of untreated Fah⁻/⁻ Hpd⁻/⁻ mice (Table III). The concentration of succinylacetone was markedly increased in urine from Fah⁻/⁻ Hpd⁻/⁻ mice given homogentisate, whereas it remained at a low level when homogentisate was administered to III mice (Fah⁻/⁻ Hpd⁻/⁻). Blood chemistry tests of III mice treated with homogentisate indicated no significant changes in aspartate aminotransferase and alanine aminotransferase, thereby suggesting that the slightly increased formation of succinylacetone in the liver of III mice after administration of homogentisate did not lead to hepatocyte injury. This observation was consistent with findings that liver sections obtained from homogentisate-treated III mice indicated no pathological changes. The urinary excretion of succinylacetone in untreated Fah⁻/⁻ Hpd⁻/⁻ mice was slightly higher than in III mice, suggesting either that small amounts
of homogentisate were formed in the livers of Fah−/− Hpd−/− mice or that homogentisate was included in the standard chow. The slight increase in succinylacetone seen in Fah−/− Hpd−/− mice did not correlate with increased serum levels of transaminases as none of the Fah−/− Hpd−/− mice fed the normal diet had significant changes in pathology, including carcinogenesis or premalignant lesions in the liver.

**DISCUSSION**

The c14CoS mice that carried a homozygous deletion on chromosome 7 were normalized when the tyrosine catabolism pathway was completely blocked at the step of oxidation of 4-hydroxyphenylpyruvate by mutant HPD. This suggested that the impairment of expression of hepatocyte-specific and developmentally regulated genes seen in c14CoS mice was due to oxidative product(s) of the homogentisate down the pathway of tyrosine catabolism or their derivatives. Because accumulation of homogentisate caused no abnormalities in the livers and kidneys of patients with alkaptonuria (28), who lacked homogentisate oxidase activity, oxidative product(s) of homogentic acid are the primary candidates causing various abnormalities seen in c14CoS mice. This is consistent with observations on c14CoS mice that express FAH following transgenic manipulations: the c14CoS mouse was rescued by transgenic expression of FAH (10). The FAH-deficient mice treated with NTBC, an inhibitor of HPD, survived but developed hepatocellular carcinomas (16). This may be due to possible differences in the extent of inhibition at the step of oxidation of 4-hydroxyphenylpyruvate. Our data suggest that liver carcinomas in the target-disrupted FAH-deficient mice could be caused by small amounts of the oxidative product(s) of homogentisate or their derivatives produced by an incomplete block of the oxidation of 4-hydroxyphenylpyruvate by NTBC.

The administration of homogentisate to Fah++/− Hpd−/−, Fah−/− Hpd++/−, and Fah−/− Hpd−/− mice did not lead to changes in liver pathology or in liver function tests. Severe liver damage and death of animals after the administration of homogentisate were seen only in mice with the genotype Fah−/− Hpd−/−. Similarly, the administration of recombinant adenovirus that expresses human HPD in the liver led to death of the mice with elevation of serum transaminases and apoptotic death of hepatocytes. Hepatic failure is highly suspected as the cause of death of these treated double mutant mice.

An extremely high concentration of succinylacetone, which seemed to be derived from fumarylacetocetate and maleylacetocetate, was found in urine from Fah−/− Hpd−/− mice after the administration of homogentisate, but not in urine from homogentisate-treated Fah−/−/− Hpd−/− mice. Taken together, these results suggest that metabolites derived from homogentisate, i.e. fumarylacetocetate (and maleylacetocetate) or its derivatives, cause acute apoptotic death of mature and unmodified hepatocytes in Fah−/− Hpd−/− mice. Fumarylacetocetate is strongly electrophilic (29); however, other substances have not been excluded as chemicals responsible for liver injury, including maleylacetocetate, succinylacetocetate, and succinylacetone. Accordingly, the development of hepatocellular carcinomas seen in pharmacologically treated FAH-deficient mice (16) may be triggered by events related to fumarylacetocetate and maleylacetocetate and their derivatives. Although our investigation suggests that metabolite(s) derived from homogentisate are required for liver damage, further investigations will be needed to identify the substance responsible for the liver injury and hepatocellular carcinomas in FAH-deficient mice and HT1 patients.

The morphological abnormalities seen in hepatocytes of lethal albinos deletion mice in the perinatal period (8, 9) are likely to represent the very early stage of cellular damages. In vivo inhibition of the tyrosine catabolic pathway by NTBC corrected the altered gene expression in the livers of mice with target-disrupted Fah and rescued the lethal phenotype (16). This altered gene expression seen in these mice (16) and in c14CoS mice in the perinatal period (6–11) is probably part of the cellular response to the apoptotic insults. Because mutant HPD apparently prevented both liver injury and carcinogenesis, the endogenous insults caused by the intracellular metabolite(s) may lead to apoptotic cell death or carcinogenesis in the livers of HT1 subjects. There are genetic diseases that predispose to cancer, such as defects in the repair pathway for DNA damage or increased sensitivity to chromosome breakage. An enhanced apoptosis has been demonstrated under experimental conditions in which DNA damage is increased or cell cycle regulation is impaired (30). These investigations support the notion that accumulation of DNA damage commits the cell to apoptosis or carcinogenesis (31). These DNA damages are caused either by endogenous insults produced by normal cellular metabolic events or by exogenous events (32). Our investigations, together with others on models and on HT1 patients, suggest that HT1 may be the first example of a genetic disease in which the accumulation of endogenous metabolite(s) due to a metabolic defect commits the cells to apoptosis or carcinogenesis.

Sudden apoptotic death of unmasked phenotype of HT1 in mature and unmodified hepatocytes had not been expected (1–12, 16), and these are implications for the pathogenesis and treatment of liver disease in HT1 patients. We suggest that mature and unmodified hepatocytes in those with the FAH defect cannot survive and that hepatocytes in the chronic form of HT1 have to be protected from a likely acute death. Both delayed development of the tyrosine catabolic pathway in the neonatal period and secondary inactivation of the pathway seem to contribute to survival of hepatocytes in HT1 patients. Indeed, HPD activities are reduced in the livers of HT1 patients (33), and this reduction is proposed to be part of the altered gene expression (34). If this inactivation is inadequate, acute death of hepatocytes is inevitable after the full expression of HPD, the result being the acute form of HT1. Under these circumstances, various cellular responses will occur as “adaptive dysregulation” or “adaptive mutation.” The pleomorphic appearance of the affected hepatocytes in the acute and severe forms of the disease (2) possibly reflects an unsuccessful process of survival of the fittest. These observations provide added support for the current prescription of NTBC for the treatment of HT1 patients (4).

Self-induced correction of mutations (revertant) on the Fah gene and proliferation of the corrected hepatocytes have been observed in the livers of chronic form HT1 patients (35), implying that hepatocytes corrected for FAH deficiency by gene transfer can expand in the HT1 liver. Following this observation, it was shown in mice carrying targeted-disrupted Fah that transplantation of hepatocytes that expressed FAH by retroviral mediated gene transfer under ex vivo conditions resulted in expansion of the cells in the liver (36). This provides an important model system in which one can investigate the fate of genetically corrected and transplanted hepatocytes in the liver where surrounding uncorrected cells were injured. The double mutant mice presented here will serve as another model for such experiments to investigate the fate of normalized hepatocytes in the damaged liver. In addition, both the double mutant mice and the target-disrupted FAH-deficient mice will be useful for the study of apoptosis of hepatocytes triggered by endogenous metabolite(s). Inhibition of the apoptosis of these hepatocytes with specific inhibitors will provide further understanding of hepatocyte injury and may provide alternative therapeutic interventions. Our preliminary exper-
ments suggest that homogentisate-induced apoptosis is completely inhibited by certain specific inhibitors of apoptosis. In addition, the double mutant will provide an opportunity to examine the process of development of hepatocellular carcinomas caused by endogenous metabolite(s) in HT1.

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