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# Fumarylacetoacetate hydrolase deficient pigs are a novel large animal model of metabolic liver disease☆

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**Abstract** Hereditary tyrosinemia type I (HT1) is caused by deficiency in fumarylacetoacetate hydrolase (FAH), an enzyme that catalyzes the last step of tyrosine metabolism. The most severe form of the disease presents acutely during infancy, and is characterized by severe liver involvement, most commonly resulting in death if untreated. Generation of $FAH^{+/-}$ pigs was previously accomplished by adeno-associated virus-mediated gene knockout in fibroblasts and somatic cell nuclear transfer. Subsequently, these animals were outbred and crossed to produce the first $FAH^{-/-}$ pigs.
FAH-deficiency produced a lethal defect in utero that was corrected by administration of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexanedione (NTBC) throughout pregnancy. Animals on NTBC were phenotypically normal at birth; however, the

*Abbreviations:* HT1, Hereditary tyrosinemia type I; FAH, Fumarylacetoacetate hydrolase; NTBC, 2-(2-Nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexanedione; FAA, Fumarylacetoacetate; AAV, Adeno-associated virus; SCNT, Somatic cell nuclear transfer; CFTR, Cystic fibrosis transmembrane conductance regulator.

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animals were euthanized approximately four weeks after withdrawal of NTBC due to clinical decline and physical examination findings of severe liver injury and encephalopathy consistent with acute liver failure. Biochemical and histological analyses, characterized by diffuse and severe hepatocellular damage, confirmed the diagnosis of severe liver injury. $FAH^{-/-}$ pigs provide the first genetically engineered large animal model of a metabolic liver disorder. Future applications of $FAH^{-/-}$ pigs include discovery research as a large animal model of HT1 and spontaneous acute liver failure, and preclinical testing of the efficacy of liver cell therapies, including transplantation of hepatocytes, liver stem cells, and pluripotent stem cell-derived hepatocytes.


## Introduction

Hereditary tyrosinemia type I (HT1; OMIM #276700) is an autosomal-recessive inborn error of metabolism caused by deficiency in fumarylacetoacetate hydrolase (FAH), an enzyme that catalyzes the last step of tyrosine metabolism (de Laet et al., 2013; Grompe, 2001; Sniderman King et al., 1993; Lindblad et al., 1977). The absence of FAH causes accumulation of the toxic metabolite fumarylacetoacetate (FAA) in hepatocytes and renal proximal tubules, the two major cell types that express FAH (Endo and Sun, 2002; Jorquera and Tanguay, 1997; Kubo et al., 1998). Clinically, individuals with HT1 commonly develop symptoms within the first few weeks of life; however, presentation is often variable, even within a family (Sniderman King et al., 1993). Acute onset of HT1 is characterized by severe liver involvement (Russo and O'Regan, 1990), most frequently leading to death, if untreated. The most common treatment for HT1 is a low-tyrosine diet combined with administration of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexanedione (NTBC) (Lindstedt et al., 1992), a potent inhibitor of 4-hydroxyphenylpyruvate dioxygenase (Fig. S1).

Two *Fah*-knockout mouse models have been previously described: the $c^{14CoS}$ albino mouse and the $Fah^{\Delta exon5}$ mouse (Gluecksohn-Waelsch, 1979; Russell et al., 1979; Grompe et al., 1993). *Fah*-knockout mice have proven a tremendous resource for translational research related to treatment of a metabolic liver disease by various cell and gene therapy approaches (Overturf et al., 1996; Paulk et al., 2010; Lisowski et al., 2012; Huang et al., 2011; Zhu et al., 2014). However, as has been demonstrated elegantly by the creation of cystic fibrosis transmembrane conductance regulator (CFTR) knockout pigs (Rogers et al., 2008), the pig is a more appropriate research model because of its similarity in size, anatomy, and biology to the human (Cooper et al., 2002). We have previously reported the generation and characterization of heterozygous $FAH^{+/-}$ pigs (Hickey et al., 2011) by using adeno-associated virus (AAV) and homologous recombination to target and disrupt the porcine *FAH* gene, located on chromosome 7 in the pig genome. An AAV vector was used to deliver a knockout construct targeted to exon 5 of *FAH* fetal pig fibroblasts with an average knockout targeting frequency of 5.4% achieved. Targeted $FAH^{+/-}$ fibroblasts were used as nuclear donors for somatic cell nuclear transfer (SCNT) to porcine oocytes, and multiple viable $FAH^{+/-}$ pigs were born. $FAH^{+/-}$ pigs were phenotypically normal, but had decreased *FAH* transcriptional and enzymatic activity compared to $FAH^{+/+}$ animals. Therefore, the goal of this study was to generate and characterize $FAH^{-/-}$ pigs in order to develop a more relevant preclinical model of an inborn error of metabolism than currently exists.

## Materials and methods

### Animals and animal care

$FAH^{-/-}$ pigs were produced in a 50% Large White 50% Landrace pig. All animals received humane care in compliance with the regulations of the Institutional Animal Care and Use Committee at Mayo Clinic. All animals were observed at least daily for clinical signs and symptoms consistent with HT1 and acute liver failure. All animals were weighed daily until reaching a weight of 20 kg; animals were subsequently weighed twice weekly. NTBC (Yecuris, Portland, OR) was administered to the animals orally mixed within a portion of daily chow rations. Pregnant sows were given 50–100 mg of NTBC per day for the duration of gestation. Weaned piglets were administered 1 mg/kg NTBC per day until day 30. The animals were observed until they consumed the entire portion of medicated food.

### PCR genotyping

Pig tissue, from ear or tail, was added to 100 μL of lysis buffer (stock lysis solution = 440 μL 0.01% SDS; 40 μL 10 mg/mL proteinase K; 20 μL 0.5 M EDTA). Following a 90-min incubation at 50 °C and 30-min incubation at 95 °C, 0.5 μL of the lysed tissue were used for each 25 μL PCR reaction using BIOLASE DNA Polymerase (Bioline, Taunton, MA) with the following three primers: WT-F: TTTCCTCCGCAGGTGACTAC; MUT-F: GGGAGGATTGGGAAGACAAT; R-GACAACATGCTGCTGG ACAC. PCR conditions were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; 72 °C for 5 min. PCR generated products of either 168 bp (wild-type allele) or 232 bp (mutant allele), which were electrophoresed on a 2.5% TAE agarose gel and visualized with ethidium bromide staining.

### FAH protein assays

For western blot analysis, liver samples were homogenized in cell lysis buffer (Cell Signaling, Danvers, MA) and isolated total protein separated by SDS-PAGE, followed by immunoblotting onto a polyvinylidene fluoride membrane (TransBlot Turbo, BioRad, Hercules, CA). The primary antibodies against FAH (Wang et al., 2002) and beta-Actin (#4970; Cell Signaling, Danvers, MA) were detected with a secondary HRP conjugated anti-rabbit antibody (Cell Signaling, Danvers, MA), and imaged