Murine Models of the Human Porphyrias: Contributions Toward Understanding Disease Pathogenesis and the Development of New Therapies

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Abstract

Mouse models of the human porphyrias have proven useful for investigations of disease pathogenesis and to facilitate the development of new therapeutic approaches. To date, mouse models have been generated for the major porphyrias, with the exception of X-linked protoporphyria (XLP) and the ultra rare 5-aminolevulinic acid dehydratase deficiency porphyria (ADP). Mouse models have been generated for all three autosomal dominant acute hepatic porphyrias, acute intermittent porphyria (AIP), hereditary coproporphyria (HCP), and variegate porphyria (VP). The AIP mice, in particular, provide a useful investigative model as they have been shown to have acute biochemical attacks when induced with the prototypic porphyrinogenic drug, phenobarbital. In addition to providing important insights into the disease pathogenesis of the neurological impairment in AIP, these mice have been valuable for preclinical evaluation of liver-targeted gene therapy and RNAi-mediated approaches. Mice with severe HMBS deficiency, which clinically and biochemically mimic the early-onset homozygous dominant AIP (HD-AIP) patients, have been generated and were used to elucidate the striking phenotypic differences between AIP and HD-AIP. Mice modeling the hepatocutaneous porphyria, porphyria cutanea tarda (PCT), made possible the identification of the iron-dependent inhibitory mechanism of uroporphyrinogen III decarboxylase (UROD) that leads to symptomatic PCT. Mouse models for the two autosomal recessive erythropoietic porphyrias, congenital erythropoietic porphyria (CEP) and erythropoeitic protoporphyria (EPP), recapitulate many of the clinical and biochemical features of the severe human diseases and have been particularly useful for evaluation of bone marrow transplantation and hematopoietic stem cell (HSC)-based gene therapy approaches. The EPP mice have also provided valuable insights into the underlying pathogenesis of EPP-induced liver damage and anemia.

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Keywords

mouse models; porphyrias; acute hepatic pophyrias; erythropoietic porphyrias; inborn errors of heme biosynthesis

1. Introduction

The inherited porphyrias are metabolic disorders resulting from the defective activity of specific enzymes in the heme biosynthetic pathway [Fig. 1]. Of the eight major hereditary porphyrias, seven are autosomal dominant or recessive disorders due to loss-of-function (LOF) mutations that lead to enzyme deficiencies, while X-linked protoporphyria (XLP) is caused by gain-of-function (GOF) mutations in the erythroid-specific 5-aminolevulinic acid synthase 2 (*ALAS2*) gene [Table 1] [1, 2]. Thus far, a human disorder associated with deficiency of the housekeeping 5-aminolevulinc acid synthase (ALAS1) has not been reported.

Porphyrias are classified as hepatic or erythropoietic, depending on whether the excess production of porphyin precursors and/or porphyrins occur primarily in the liver or in the erythron [1, 2]. The hepatic porphyrias include three autosomal dominant porphyrias, acute intermittent porphyria (AIP), hereditary coproporphyria (HCP), and variegate porphyria (VP), and the ultra rare autosomal recessive 5-aminolevulinic acid dehydratase deficient porphyria (ADP). These diseases are characterized by acute neurovisceral attacks that are precipitated by factors that induce heme biosynthesis, including cytochrome P450 (CYP450)-inducing drugs, hormonal changes, and fasting [2, 3]. Porphyria cutanea tarda (PCT) is considered to be a hepatocutaneous porphyria, as it presents with cutaneous lesions but the primary site of porphyrin accumulation is the liver. Erythropoietic porphyrias include congenital erythropoietic porphyria (CEP), erythropoietic protoporphyria (EPP), and XLP, and present with cutaneous photosensitivity that leads to skin fragility and blisters or pain and swelling [1, 2].

To date, mouse models have been generated for all of the major hereditary porphyrias, with the exception of XLP and the ultra rare ADP [Table 1]. Most were generated via gene targeting, while a mouse model for EPP and HCP were identified in N-ethyl-N-nitrosourea (ENU) mutagenesis screens [4, 5]. As heme is essential to life, homozygous knockout mice for any of the heme biosynthetic genes are embryonic lethal [4, 6–8]. Spontaneous mouse models have not been identified for any of the porphyrias thus far. However, larger animals with various porphyrias have been identified, including cats with AIP and CEP [9, 10] and EPP in cows [11]. While some of the porphyrias can be chemically induced in mice [e.g. Griseofulvan induces pathological changes similar to those in EPP-associated liver damage [12, 13]], this review focuses on genetically modified mouse models.

Here, each of the porphyric mouse models are briefly described, highlighting the similarities in clinical and biochemical phenotypes with their respective human counterparts. The major contributions of these porphyric mouse models towards furthering our understanding of disease pathogenesis and for preclinical evaluation of novel therapeutic approaches are highlighted.

2. Methods

A pubmed search was performed using the following terms: acute intermittent porphyria mouse model, variegate porphyria mouse model, hereditary coproporphyria mouse model, aminolevulinic acid dehydratase deficiency porphyria mouse model, acute porphyria mouse model, hepatic porphyria mouse model, porphyria cutanea tarda mouse model, congenital erythropoeitic porphyria mouse model, erythropoeitic protoporphyria mouse model, and X-linked protoporphyria mouse model. Publications describing the generation and characterization of the mouse models, as well as those that showcase the usefulness of the porphyric mice for understanding disease pathogenesis and/or for preclinical evaluation of novel therapeutic approaches were selected, and the findings were summarized.

3. Acute Hepatic Porphyrias (AHPs)

The AHPs include three autosomal dominant porphyrias: AIP, VP, and HCP, which are due to half-normal activities of hydroxymethylbilane synthase (HMBS), protoporphyrinogen oxidase (PPOX), and coproporphyrinogen III oxidase (CPOX), respectively, and the ultra rare autosomal recessive ADP, caused by loss-of-function aminolevulinic acid dehydratase (*ALAD*) mutations (Table 1). All four AHPs present with acute neurovisceral attacks, which typically start with crippling abdominal pain and may progress to include tachycardia, hypertension, motor weakness, and seizures [1, 2]. The attacks are triggered by various porphyrinogenic factors, including fasting, CYP450-inducing drugs, and hormonal fluctuations, all of which induce the hepatic expression of *ALAS1*, the first and rate-limiting enzyme of the heme biosynthetic pathway [2, 3]. When hepatic *ALAS1* is induced, the respective enzyme deficiency becomes rate-limiting, leading to insufficient heme production and depletion of the 'free' heme pool. This leads to derepression and further induction of hepatic *ALAS1*, resulting in the marked elevation of the putative neurotoxic porphyrin precursors, 5-aminolevulinic acid (ALA) and porphobilinogen (PBG) [1, 2].

To date, mouse models have been developed for the three autosomal dominant acute porphyrias, AIP, HCP, and VP. The AIP mouse model has been particularly useful for studies of disease pathogenesis and for evaluating novel therapeutic approaches.

3.1. ATP Mouse Model: T1/T2 mice

Homozygous *HMBS* knockout mice were embryonic lethal, while heterozygotes with half-normal activity were viable but did not accumulate urinary ALA when administered the prototypic porphyrinogenic drug, phenobarbital (PB) [7]. Therefore, compound heterozygous mice with ~30% of normal HMBS activity were generated and designated T1/T2 mice [7]. The T1 hypomorphic allele was generated by insertion of the Neomycin gene under the transcriptional control of the phosphoglycerate kinase promoter in antisense orientation in exon 1, leading to disruption of the house-keeping *Hmbs* isoform. The T2 allele had a competitive splice acceptor site in intron 1. Mice homozygous for the T1 allele were viable and had ~55% of normal HMBS activity. T2 homozygotes were embryonic lethal, while T2 heterozygotes had ~57% of normal HMBS activity [7].

The T1/T2 mice have baseline hepatic *Alas1* mRNA expression and urinary and plasma ALA and PBG concentrations that are slightly increased (2 to 6-fold) compared to normal. When administered PB, hepatic *Alas1* mRNA expression is markedly increased, leading to massive accumulation of plasma and urinary ALA and PBG, thereby biochemically mimicking a porphyric acute attack [7]. These mice do not develop overt neurologic manifestations of an acute attack. Rather, they develop chronic and progressive motor axonal degeneration, similar to changes seen in patients with severe attacks, by 6 months of age [7, 14]. This is accompanied by neuromotor impairment, including decreased rotarod and muscular performances. Of note, the axonal motor degeneration occurred regardless of whether the mice were induced with PB or not [14].

3.1.1. Investigations of Disease Pathogenesis in T1/T2 mice—The pathogenesis of the acute neurovisceral attacks has been long debated, particularly whether the accumulated porphyrin precursors, ALA and/or PBG, are neurotoxic, or if heme deficiency in nervous tissues leads to neurologic dysfunction [15]. In particular, ALA is thought to be neurotoxic, as it is elevated in all four acute hepatic porphyrias (whereas PBG is not elevated in ADP) and it is a structural analogue of the inhibitory neurotransmitter, γ-aminobutyric acid (GABA). ALA has also been shown to exhibit pro-oxidative properties *in vitro* [16–19]. The fact that liver transplantation completely stopped the recurrent acute attacks in patients with AIP clearly demonstrates that the liver is the main site of pathology [20]. Conversely, when livers of AIP patients with recurrent attacks were 'domino' transplanted into non-porphyric patients, these patients developed acute porphyric attacks [21]. While these studies and others [22–24] strongly support that ALA and/or PBG are neurotoxic and are the main mediators of the attacks, studies in the T1/T2 mice suggest that other mechanisms may also contribute.

As noted above, the T1/T2 mice develop axonal degeneration without exposure to porphyrinogenic factors, hence with only slight elevations of plasma and urinary ALA and PBG [14]. Thus, it has been proposed that neuronal heme deficiency may be involved in the development of the porphyric neuropathy [14]. Related to this, it was shown that the T1/T2 mice have lower heme saturation and enzymatic activity of Cyp1a1 in the brain following treatment with β-naphthoflavone, a Cyp1a1-inducer, compared to normal mice [25].

More recent studies have demonstrated mitochondrial energetic defects in the T1/T2 mice, suggesting that this, too, may play a role in disease pathogenesis. In the livers of PB-induced T1/T2 mice, the activities of the TCA cycle and downstream respiratory chain complexes were markedly decreased, presumably due to a large amount of succinyl-CoA being shuttled away from the TCA cycle to be used as substrate for the massively induced ALAS1 enzyme [26]. Enzymatic activities of the respiratory chain complexes were also decreased in skeletal muscles and brains of the PB-induced T1/T2 mice, leading to decreased ATP production in muscle [27]. Mitochondrial impairments in the brain and muscle were postulated to be due to the neurotoxic effects of ALA overproduced by the liver, although in the brain, the activity of heme-dependent respiratory enzymes were decreased, suggesting that heme deficiency may also contribute [27].

Additionally, these mice have been useful for the identification of potential biomarkers for AIP [28], for the assessment of porphyrinogenicity of certain drugs [29], and to investigate alterations in glucose metabolism induced by fasting in AIP livers [30].

3.1.2.   Preclinical Studies in T1/T2 mice—The most effective standard treatment for the acute attacks is intravenous administration of hemin, which provides exogenous heme for the negative feedback inhibition of hepatic ALAS1 [31]. Although patients generally respond well, its effect is slow, and frequent administration is associated with side effects such as phlebitis and iron overload [32]. While liver transplantation is curative, it is a high-risk procedure associated with limited organ availability. Therefore, alternative therapeutic approaches for the AHPs that are more effective, faster-acting, and safe are desirable.

Due to the fact that the T1/T2 mice can be induced into a biochemical acute attack with PB, these mice have been very useful for preclinical evaluation of various therapeutic strategies, including hepatocyte transplantation. Yin et al. showed that engraftment of < 3% of normal hepatocytes into the T1/T2 livers reduced the PB-induced elevations of plasma ALA and PBG by ~50% [33]. These disproportional findings suggest that the porphyrin precursors readily cross cellular membranes and are efficiently metabolized by the transplanted cells.

The effectiveness of liver-targeted gene therapy strategies for AIP, including adeno-viral [34, 35] and adeno associated viral (AAV)-mediated approaches, has been evaluated in the T1/T2 mice. In particular, Yasuda and Unzu demonstrated that gene transfer of the human *HMBS* cDNA via AAV2/8- and AAV2/5-vectors, respectively, provided complete and sustained protection against the induced biochemical acute attacks and improved neuromotor function in the T1/T2 mice [36, 37]. Notably, transduction of only 5-10% of hepatocytes was sufficient to completely prevent the PB-induced acute attacks [36]. AAV2/5-mediated gene therapy was further evaluated in Phase I Clinical Trials and proved to be safe and well-tolerated in human AIP patients, but no appreciable decreases in urinary ALA and PBG were achieved, presumably due to inadequate HMBS expression in the liver [38]. Thus, recent efforts have been directed towards optimizing the therapeutic vector to achieve high transgene expression, either by 1) incorporating human *ALAS* drug responsive enhancing sequences (ADRES) into the promoter or 2) modifying the *HMBS* cDNA to include the p.I291M and p.N340S variants, which synergistically increase kinetic stability of the HMBS enzyme [39, 40]. The ADRES, previously identified in the distal 5'-flanking region of the human *ALAS1* gene, have been shown to activate transcriptional activity of *ALAS1* in response to porphyrinogenic drugs [41]. Administration of an AAV2/8 vector harboring two tandem ADRES immediately upstream of the promoter (AAV2/8-ADRES-HMBS) led to markedly increased hepatic HMBS activities in the T1/T2 mice, particularly upon exposure to factors that precipitate attacks, including porphyrinogenic drugs, fasting, and estrogen [40]. Importantly, compared to a 'control' AAV vector lacking the ADRES elements (AAV2/8-HMBS), the vector dose of AAV2/8-ADRES-HMBS could be reduced by 10-fold to achieve the same therapeutic effect. Treatment with an AAV2/8 vector that included the p.I291M and p.N340S variants in its *HMBS* transgene (AAV-HMBS-I291M/N304S) also resulted in markedly elevated hepatic HMBS activities in the T1/T2 mice, representing a 3-fold decrease in effective vector dose relative to the AAV2/8-HMBS 'control' vector [39].

Recently, the T1/T2 mice have been used for preclinical studies of a lipid nanoparticle (LNP)-encapsulated short interfering RNA (siRNA) specifically targeting hepatic *Alas1* (Alas1-siRNA). Intravenous administration of the Alas1-siRNA effectively prevented and treated induced biochemical attacks in these mice and did not perturb the activities of representative hemoproteins [24]. Importantly, when administered during an induced attack, the Alas1-siRNA reduced the markedly elevated plasma ALA and PBG levels more rapidly and effectively than did a single intravenous hemin infusion. Subsequent efforts developed a N-acetylgalactosamine (GalNAc)-conjugated Alas1-siRNA (GalNAc-Alas1) that can be administered subcutaneously and demonstrated its efficacy in the T1/T2 mice [42]. Recent Phase I/II Clinical trials for a subcutaneously administered GalNAc-ALAS1 have indicated this approach to be safe and effective in human AIP patients (R.J.D., unpublished data).

3.2. VP Mouse Model: *PPOX*R59W$^{+/-}$ Mice

The prevalence of VP is highest in South Africa due to an ancestral founder mutation, c. 175C>T (p.R59W), that was brought to the Cape by a Dutch couple in 1680. A mouse model of VP carrying the p.R59W mutation was generated via gene targeting by Medlock et al [43]. Heterozygous mice (R.59W$^{+/-}$) had approximately half-normal PPOX activity and accumulated porphyrins in a pattern similar to human VP patients. However, administration of phenobarbital (PB) did not result in clinical manifestations of an acute attack or elevation of urinary ALA or PBG (M.Y., personal communication).

3.3. HCP Mouse Model: *Cpox*$^{+/W373X}$ Mice

Recently, a mouse line carrying the c.1118G>A (p.W373X) mutation in its *Cpox* gene was identified in a genome-wide ENU study aimed to identify novel regulators of erythropoiesis [4]. Heterozygous mice (Cpox$^{+/W373X}$) had half-normal hepatic *Cpox* mRNA and presented with a mild microcytic anemia. Homozygous mice were not viable and died in uetro by embryonic day 9.5.

Female Cpox$^{+/W373X}$ mice had slightly elevated (3 to 4-fold) urinary and fecal porphyrins, with an increased coproporphyrin III:I ratio in faeces, and normal baseline urinary PBG. Male heterozygotes, on the contrary, had normal porphyrin profiles and were not further characterized. Porphyrinogenic stimuli, including fasting (15 hr/day, two consecutive days) and PB administration (75, 80, 85, and 90 mg/kg/day; 4 consecutive days), did not induce apparent acute neurovisceral symptoms or significantly increase urinary PBG in Cpox $^{+/W373X}$ females [4].

3.4. Homozygous Dominant AHPs

Biallelic loss-of-function mutations in *HMBS, CPOX,* and *PPOX* genes lead to rare conditions known as homozygous dominant (HD)-AIP, HD-HCP, and HD-VP, respectively [22, 44–51]. Unlike the autosomal dominant forms, these diseases present in infancy with severe and progressive neurological impairment and/or marked cutaneous manifestations with growth retardation and short stature. Of interest, with the exception of one HD-HCP patient [46], acute neurovisceral attacks have not been reported in patients with the HD-AHPs.

HD-AIP patients have less than 4% residual HMBS activity and constitutively elevated urinary ALA and PBG. In addition to gross psychomotor retardation, patients have early-onset ataxia, nystagmus, and dystonia [22]. Patients typically have short stature and die in childhood. Interestingly, of the five HD-AIP patients reported to date, four had c.500G>A (p.R167Q), c.499C>T (p.R167W), or c.518G>A (p.R173Q) mutations: one was homozygous for p.R167W [22], two siblings were heterozygous for p.R167Q and p.R167W [49], while one was heterozygous for p.R167W and p.R173Q [44]. In all cases, their parents were asymptomatic AIP heterozygotes.

A mouse model for HD-AIP has been generated and characterized in an effort to understand the striking differences in clinical presentation between human AIP and HD-AIP. Mouse models for HD-HCP and HD-VP have not been generated to date.

3.4.1. HD-AIP Mouse Model: R167Q$^{+/+}$ Mice and Investigations of Disease Pathogenesis—*Hmbs* knock-in mice carrying the 'common' human HD-AIP mutations, p.R167Q and p.R173W, were generated by gene targeting. Mice homozygous for p.R173W were embryonic lethal, while p.R167Q homozygotes (R167Q$^{+/+}$) survive to adulthood and have a normal lifespan [52]. R167Q$^{+/+}$ mice are severely runted and display profound neurological abnormalities similar to the human HD-AIP patients, including early-onset ataxia, delayed neuromotor development, and impaired rotarod performance. These mice also recapitulate the biochemical features of human HD-AIP, having only ~5% of normal HMBS activity and constitutively elevated plasma and urinary ALA and PBG in the absence of porphyrinogenic factors [52]. Central nervous system (CNS) histology was grossly normal, but CNS myelination was delayed and overall myelin volume was decreased. When compared with the T1/T2 mice, a striking biochemical difference was that the R167Q$^{+/+}$ mice had much higher elevations of ALA and PBG in their CNS tissues. The finding that the T1/T2 mice accumulated high levels of porphyrin precursors in their livers and plasma following PB induction, but not their CNS tissues, suggested that ALA and PBG do not readily cross the blood-brain-barrier [52]. Taken together, these studies suggest that the severe HD-AIP neurological phenotype results from decreased myelination and the accumulation of locally produced neurotoxic porphyrin precursors within the CNS. Importantly, these findings suggest that liver-directed therapies for heterozygous AIP, including hemin, RNAi-mediated silencing of hepatic *ALAS1*, and liver transplantation, are likely not effective for HD-AIP, as they do not address these CNS issues.

4. Porphyria Cutanea Tarda (PCT)

PCT, the most common human porphyria, is characterized by the development of blisters on sun-exposed skin. It is unique among the inherited porphyrias in that it occurs both in a Type 1 sporadic form, in the absence of an *UROD* mutation, and in a Type 2 familial form, in which an *UROD* mutation is inherited in an autosomal dominant pattern (Table 1). Clinically, the two forms are indistinguishable, although age of disease onset is significantly lower in Type 2 familial patients [53]. The disease becomes active when patients are exposed to predisposing factors that lead to hepatic iron overload, including hemochromatosis, excessive alcohol consumption, estrogens, and viral infections such as hepatitis C and HIV [1–3]. While the half-normal activity of UROD predisposes to PCT, additional susceptibility

factors are required to activate disease in Type 2 familial patients, like the Type 1 sporadic patients. Patients with active disease display marked porphyrinuria, predominantly consisting of uroporphyrin and 7-carboxylate porphyrin. Mouse models for PCT have played a particularly important role in defining the underlying pathogenic mechanism of this hepatocutenous porphyria.

4.1. Mouse Models for Type 1 Sporadic and Type 2 Familial PCT and Investigations of Disease Pathogenesis

To model Type 2 autosomal dominant familial PCT, *Urod* knockout mice were generated by gene targeting. Much like human *UROD* heterozygotes, mice heterozygous for the *Urod*-null allele (Urod$^{+/-}$) did not display porphyrinuria unless predisposing factors were present [54]. Administration of a single intraperitoneal dose of iron, combined with ALA to bypass the rate-limiting step of heme biosynthesis (i.e. ALAS1), led to marked porphyrinuria by 3 weeks. Subsequently, these mice were bred against mice null for the hemochromatosis *(Hfe)* allele to produce mice heterozygous for the Urod-null allele and homozygous null for the hemochromatosis *(Hfe)* allele (Urod$^{+/-}$/Hfe$^{-/-}$). Urod$^{+/-}$/Hfe$^{-/-}$ mice accumulated high levels of hepatic iron and developed porphyuria at 14 weeks of age without ALA supplementation or other exogenous precipitating factors, establishing that iron alone is sufficient to cause the porphyric phenotype in mice [54]. Assessment of hepatic UROD enzymatic activity showed that, contrary to the Urod$^{+/-}$/Hfe$^{+/+}$ mice with intact Hfe which had about half-normal UROD activity, the Urod$^{+/-}$/Hfe$^{-/-}$ mice had only ~14% of WT. Notably, the marked decrease in UROD activity occurred without changes to UROD protein levels, indicating that the catalytic activity of UROD was abolished. These findings confirmed previous reports that an iron-dependent inhibitor of UROD is formed in symptomatic Type 2 familial PCT [55–58].

In a follow up study, Phillips *et al.* demonstrated that a similar iron-dependent mechanism of hepatic UROD inhibition is observed in a mouse model for Type 1 sporadic PCT, generated by administrating iron, ALA, and polychlorinated biphenyls (PCB) to C57BL/6 wildtype mice [59]. Analyses of liver cytosolic extracts isolated from the sporadic PCT and familial PCT (Urod$^{+/-}$/Hfe$^{-/-}$) mice identified a competitive inhibitor of recombinant human UROD that proved to be uroporphomethane, an iron-oxidized product of uroporphyrinogen [59]. Thus, these studies established that Type 2 familial PCT and Type 1 sporadic PCT share a common pathogenic mechanism, namely, the iron-dependent formation of an UROD inhibitor in the liver that leads to transient decrease of hepatic UROD activity and disease activation.

5. Erythropoietic Porphyrias

The erythropoietic porphyrias include two autosomal recessive disorders, CEP and EPP, which are caused by loss-of-function mutations in the uroporphyrinogen III synthase (*UROS*) and ferrochelatase (*FECH*) genes, respectively. XLP is an X-linked disease caused by gain-of-function mutations in the *ALAS2* gene (Table 1). These disorders are characterized by cutaneous photosensitivity that results from the massive accumulation of

photoreactive porphyrins within the bone marrow and circulating erythrocytes, which eventually are deposited in the skin.

5.1. CEP

Major clinical features of human CEP include marked cutaneous photosensitivity with blistering, hemolytic anemia, and erythrodontia, which is reddish-brownish discoloration of teeth [1, 2]. Clinical severity can vary, from non-immune hydrops fetalis *in utero* to late-onset disease with only mild cutaneous photosensitivity in adulthood. Genotype/phenotype correlations predict disease severity, as the activity of the encoded UROS enzyme deficiency generally correlates with clinical severity. The most common presentation is severe, transfusion-dependent CEP due to homozygosity for the c.217T>C (p.C73R) mutation [60]. Biochemically, CEP patients have markedly elevated erythrocyte and urinary uroporphyrin I (Uro I), and coproporphyrin I (Copro I) isomers, which are phototoxic, non-physiologic substrates that are not utilized to synthesize heme.

5.1.1. CEP Mouse Models: UROS[P248Q/P248Q] and UROS[C73R/C73R] Mice—

Previously, it was shown that *Uros* knockout mice were homozygous lethal [6]. Therefore, Ged *et al.* generated a homozygous knock-in mouse model carrying the human severe *UROS* mutation, c.743C>A (p.P248Q; designated UROS[P248Q/P248Q]) [61]. Bishop et al. generated mice with the most common and severe human genotype, p.C73R/p.C73R (UROS[C73R/C73R]), and mice with later-onset genotypes, p.V99L/p.V99L (UROS[V99L/V99L]) and p.C73R/p.V99L (UROS[C73R/V99L]) [62]. Much like their human counterparts, the UROS[P248Q/P248Q] and UROS[C73R/C73R] mice had severe disease, with 1% of normal UROS activity in their erythrocytes and urinary Uro I concentrations that were increased >990-fold compared to that in normal mice. Both mouse models developed a profound hemolytic anemia and had an abundance of fluorocytes in their bone marrow and peripheral blood [61, 62]. Splenomegaly and erythrodontia were also apparent. A moderate skin photosensitivity was documented in the UROS[P248Q/P248Q] mice [61].

In contrast, the UROS[C73R/V99L] and UROS[V99L/V99L] mice had ~11 and 19% of normal erythrocyte UROS activity, respectively [62]. The UROS[C73R/V99L] mice had a very mild anemia, while adult UROS[V99L/V99L] mice were hematologically normal. Importantly, these findings suggested that only ~10% of wildtype UROS activity may be necessary to correct the pathology in CEP patients.

5.1.2. Preclinical Studies in UROS[P248Q/P248Q] Mice—For non-transfusion CEP

patients, efforts to keep the hematocrit above 35 with fresh erythrocyte transfusions has proven to be effective [63]. To date, the only curative treatment for transfusion-dependent CEP patients is bone marrow transplantation (BMT), which not only is a high-risk procedure, but also requires a suitable donor. Therefore, efforts have been directed to develop novel therapeutic approaches for this disease. Both the UROS[P248Q/P248Q] and UROS[C73R/C73R] are excellent models to evaluate new therapies for CEP, as they recapitulate most of the clinical and biochemical features of the severe human disease.

Robert-Richard *et al.* demonstrated that *ex vivo* lentiviral-mediated gene transfer of the human *UROS* cDNA into hematopoietic stem cells (HSC), followed by re-injection into the

UROS$^{P248Q/P248Q}$ mice, achieved complete and long-term enzymatic, metabolic, and phenotypic correction of disease [64]. Of note, transduction of only ~40% of HSC was sufficient to achieve this effect, due to the survival advantage of the corrected cells over non-transduced cells. That the corrected cells have a survival advantage over non-transduced cells is consistent with the finding that UROS-deficient erythrocytes have a shorter half-life and increased osmotic fragility compared to UROS-sufficient erythrocytes [65].

Blouin *et al.* showed in the UROS$^{P248Q/P248Q}$ mice that administration of Bortezomib, a clinically approved proteasome inhibitor, resulted in decreased UroI levels in circulating erythrocytes and urine and reversion of skin photosensitivity, although it did not correct the anemia [66]. The biochemical and phenotypic correction was shown to occur through rescue of the P248 mutant protein from premature proteosomal degradation. These studies suggested the therapeutic potential of proteasome inhibitors and/or pharmacological chaperones to treat CEP patients with certain *UROS* mutations, such as p.P248Q and the common p.C73R, which retain intrinsic catalytic activity but are mis-folded and targeted to the proteasome degradation pathway. In fact, Urquiza *et al.* recently demonstrated that ~75% of *UROS* mutations reported to date lead to decreased stability of the enzyme and premature proteosomal degradation [67], and therefore, are candidates for such therapeutic approaches. As long-term treatment with proteasome inhibitors such as Bortezomib may be difficult, due to high rates of acquired drug resistance and cytotoxicity such as peripheral neuropathy [68, 69], a pharmacological chaperone with better safety profile would be highly advantageous.

To this end, Urquiza and colleagues screened a library of compounds and identified ciclopirox (CPX), a FDA-approved synthetic antimicrobial, as a pharmacological chaperone that stabilizes and restores the activity of the UROS enzyme, both *in vitro* and *ex vivo* [67]. Notably, they demonstrated that CPX binds UROS at an allosteric site that is distant from the active site, and therefore, does not interfere with the enzyme's catalytic function. Daily oral administration of sub-toxic doses CPX to the UROS$^{P248Q/P248Q}$ mice via their diet resulted in significant reduction of erythrocyte, hepatic, and urinary Uro I and Copro I porphyrin concentrations, as well as decreased splenomegaly, suggesting improved hemolysis. Erythrocyte PPIX levels were increased, supporting restoration of heme biosynthesis in the treated mice [67]. The effectiveness and safety of this therapeutic approach in human CEP patients has yet to be determined.

5.2. EPP

EPP, the most common childhood porphyria, is primarily characterized by extremely painful photosensitivity that is accompanied by marked elevation of free protoporphyrin IX (PPIX) in plasma and erythrocytes [1, 2]. A mild microcytic anemia occurs in 20 to 60% of patients [70], but the etiology of the anemia is unclear. 10-20% of patients experience cholelithiasis, while ~5% of patients develop liver failure due to hepatobiliary involvement, requiring liver transplantation [71]. Since the accumulated PPIX released from erythroid cells is lipophilic, it accumulates in the liver and is secreted into the bile. The hepatic accumulation of PPIX leads to cholestasis and hepatobiliary fibrosis, but the precise underlying pathogenic mechanism leading to liver failure in EPP is not fully understood. Studies in an EPP mouse model have suggested possible mechanisms that lead to severe hepatobiliary disease.

5.2.1   EPP Mouse Model: _Fech^{m1Pas}_ Mice—The first genetically modified mouse model for EPP, described by Tutois _et al.,_ was identified in an ENU mutagenesis study and designated _Fech^{m1Pas}_ mice [5]. Boulechfar _et al._ later determined the molecular lesion to be a c.293T>A substitution (p.M98K) of the _Fech_ gene [72]. Heterozygous mice were clinically and biochemically normal, while homozygous mice (_Fech$^{m1Pas/m1Pas}$_) displayed biochemical and clinical features mimicking a severe form of human EPP. They had ~3 to 6% of FECH activity and ~25-fold increase in erythrocyte protoporphyrin IX (PPIX) levels compared to normal [5]. Clinical findings included mild to moderate photosensitivity under normal husbandry conditions and marked normocytic hemolytic anemia by 3 to 6 months of age.

These mice also displayed severe hepatobiliary involvement, with apparent jaundice and enlarged livers and spleens from the early days of life onwards [5]. PPIX was markedly accumulated in the liver, associated with elevated plasma transaminases and hyperlipidemia [73]. Histopathologic examination of the livers showed characteristic PPIX deposits predominantly in bile canaliculi, but also in hepatocytes, and lobular and portal macrophages, and in the lumen of small bile ducts, similar to findings in human EPP patients with severe hepatic disease. Bile duct proliferation and fibrosis with portoportal bridging, sclerosing cholangitis, and hepatolithiasis were also noted [74, 75].

5.2.2.   Investigations of Disease Pathogenesis Using _Fech$^{m1Pas/m1Pas}$_ mice—Previously, it was suggested that EPP-induced liver injury results from the cytotoxic effects of PPIX and obstruction of bile canaliculi by PPIX [76, 77]. In support of this, studies in the _Fech$^{m1Pas/m1Pas}$_ mice showed that the extent of hepatocyte damage correlated with the amount of intralobular PPIX deposition and with the proportion of portal tracts containing small bile ducts with PPIX deposition [74]. However, more recent studies found that bile flow was increased in the _Fech$^{m1Pas/m1Pas}$_ mice compared to normal [78]. Additionally, bile composition was significantly altered in these mice, with marked increases in cytotoxic bile acids and decreased lipid content [75, 78]. As PPIX was shown to bind strongly to phospholipids (PL) _in vitro,_ it was proposed that the large efflux of PPIX into the canaliculi leads to PL depletion and the formation of lipid-free cytotoxic bile, which then causes damage to bile duct epithelium, and, consequentially, biliary fibrosis and sclerosing cholangitis [75].

Whereas the originally described _Fech$^{m1Pas/m1Pas}$_ mice were on a BALB/cByJCrl genetic background, these mice have since been backcrossed onto C57BL/6JCrl and SJL/JOrlCrl backgrounds [79]. Compared to the BALB/cBYlCrl _Fech$^{m1Pas/m1Pas}$_ mice, the SJL/JorlCrl _Fech$^{m1Pas/m1Pas}$_ mice had mild anemia and hepatobiliary disease, while C57BL/6JCr1 _Fech$^{m1Pas/m1Pas}$_ mice had a moderate anemia and severe hepatocyte damage associated with markedly elevated PPIX levels in the cytosol of hepatocytes and Kupffer cells [79]. Notably, the C57BL/6J _Fech$^{m1Pas/m1Pas}$_ mice had little PPIX in the bile canaliculi and were protected from the severe bile duct epithelial cell damage, presumably due to reduced efflux of PPIX and reduced synthesis and export of cytotoxic bile acids [75]. The marked differences in the severity of anemia and hepatobiliary disease of the _Fech$^{m1Pas/m1Pas}$_ mice on these genetic backgrounds suggest that modifier genes are likely involved in the variable expression of anemia and liver disease in EPP.

Studies by Han et al. proposed that heme-regulated elF2α kinase (HRI) may be an important modifier gene that contributes to the clinical severity of EPP and other hematologic disorders [80]. HRI is a heme-regulated protein kinase that phosphorylates the α-subunit of the eukaryotic translational initiation factor 2 (eIF2α). Phosphorylation of eIF2α leads to global cessation of protein synthesis. In heme-deficient states, hepatic HRI expression is activated, thus promoting eIF2α phosphorylation and cessation of protein synthesis to protect against excess globin production. Compared to BALB/cBYlCrl $Fech^{m1Pas/m1Pas}$ mice with intact HRI ($Fech^{m1Pas/m1Pas}$/HRI$^{+/+}$). BALB/cBY1Cr1 $Fech^{m1Pas/m1Pas}$ mice homozygous null for HRI ($Fech^{m1Pas/m1Pas}$/HRI$^{−/−}$) had 30-fold higher PPIX, a more severe anemia, and markedly more severe hepatocellular toxicity and cutaneous photosensitivity [80].

To date, the pathogenesis of the microcytic hypochromic anemia in EPP is not fully understood. While EPP patients often have laboratory tests indicative of iron-deficiency (e.g. microcytic hypochromic anemia with low serum iron, decreased ferritin and transferrin saturation), there are conflicting reports on the therapeutic benefit of iron supplementation in these patients. Some studies report improvement of cutaneous photosensitivity and increased hemoglobin levels following iron supplementation, suggesting that the exogenous iron was efficiently incorporated into the excess PPIX to form heme, and consequentially, PPIX levels were decreased [81]. However, the majority of studies show that iron supplementation exacerbates photosensitivity and/or PPIX accumulation [82–84], consistent with the fact that iron-sufficient conditions induce ALAS2 translation via the iron response element (IRE) and promote erythroid heme biosynthesis, thereby resulting in increased PPIX production.

While initial description of the BALB/cByJCrl $Fech^{m1Pas/m1Pas}$ mice included a normocytic hemolytic anemia [5], more recent studies have demonstrated these mice to have a microcytic hypochromic anemia with absence of sideroblasts, little or no hemolysis, and no bone marrow or spleen erythroid hyperplasia [79, 85]. Lyoumi et al. demonstrated that the microcytic anemia in the BALB/cByJCrl $Fech^{m1Pas/m1Pas}$ mice is not associated with iron deficiency, as serum ferritin levels, hepcidin mRNA levels, and total body iron amounts were normal [85]. However, iron distribution was altered in the $Fech^{m1Pas/m1Pas}$ mice, with reduced iron amounts in liver, kidney, and heart, and increased splenic iron compared to normal. Of particular interest, serum transferrin levels were elevated by 2-fold in the $Fech^{m1Pas/m1Pas}$ mice, while serum iron levels were normal, resulting in low transferrin saturation levels that mimicked an iron-deficient state [85]. Taken together, these studies support that iron supplementation is not beneficial for the microcytic anemia associated with FECH deficiency.

5.2.3.   Preclinical Studies in $Fech^{m1Pas/m1Pas}$ Mice—Recent clinical studies have demonstrated that an α-melanocyte stimulating hormone analogue effectively protects against the photosensitivity in EPP patients by darkening their skin [86]. However, the only curative therapy to date is bone marrow transplantation (BMT). The EPP mice have been used extensively for preclinical studies of BMT and HSC-mediated gene therapy approaches.

Transplantation of normal bone marrow cells into 3-4 week $Fech^{m1Pas/m1Pas}$ mice mice dramatically decreased erythrocyte PPIX accumulation but only partially corrected the liver disease [87]. Notably, BMT into neonates led to complete correction of erythrocyte PPIX levels and skin photosensitivity and almost entirely prevented liver damage [88].

When FECH-deficient HSCs were transduced with retroviral vectors expressing the human FECH enzyme and re-implanted into adult $Fech^{m1Pas/m1Pas}$ mice, complete metabolic correction and reversal of skin photosensitivity were achieved only if the transduced cells were pre-selected prior to implantation [89, 90]. *Ex vivo* gene transfer using lenti-viral vectors precluded the need for pre-selection, but high transduction rates (> 50%) associated with myloablative pre-conditioning were necessary, as there wasn't a natural selective advantage of corrected cells over FECH-deficient cells [91]. Therefore, Richard *et al.* developed a bicistronic SIN-lentiviral vector co-expressing the human *FECH* cDNA and methylguanine DNA methyltransferase (MGMT) G156A mutant, which provides resistance against alkylating agents [temozolomide or bis-chloroethylnitrosourea (BCNU)] in transduced cells, thereby permitting drug-mediated *in vivo* selection of corrected cells [92]. Administration of this vector, followed by moderate doses of BCNU into adult $Fech^{m1Pas/m1Pas}$ mice led to progressive expansion of transduced cells (up to 95%) and the complete correction of PPIX levels and photosensitivity. Further, the investigators developed a bi-promoter lentiviral vector in which the expression of FECH is driven by the erythroid-specific chimeric HS40 enhancer/ankyrin promoter, while the expression of the MGMT G156A mutant is driven by the ubiquitous human PGK promoter. The erythroid-restricted expression of FECH was sufficient to achieve full correction of PPIX and photosensitivity [92]. Of note, HSC gene therapy in adult $Fech^{m1Pas/m1Pas}$ mice did not correct the liver disease, indicating that early intervention may be necessary to fully prevent this condition. Thus far, there have not been clinical trials for gene therapy approaches in human EPP patients.

5.2.3.   EPP Mouse Model: *Fech* Exon 10 Deletion Mice—A mouse with deletion of *Fech* exon 10, a mutation identified in human EPP, was generated by Magness *et al* [8]. Homozygous mice were embryonic lethal, while heterozygous mice had ~37% of normal FECH activity in their livers, presumably due to a dominant-negative effect of the mutant allele on the wildtype allele. These mice were clinically mild compared to the $Fech^{m1Pas/m1Pas}$ mice, with erythrocyte PPIX levels only ~9-fold higher than normal controls and no liver disease. A mild photosensitivity, manifested as edema and necrosis on depilated skin, was observed only after 48 to 72 hours of light exposure.

5.2.4.   EPP Mouse Model: Partially Humanized Emi/mPas1 Mice—Over 90% of patients with EPP have a pathogenic mutation on one *FECH* allele and the c.315-48C (IVS3-48T>C) 'low expression' allele on the other. This change leads to the increased use of an alternative 3'-splice site that is 63 nt upstream of the normal one, leading to an aberrant transcript with 63 additional nucleotides. Two in-frame stop codons in this additional sequence target this aberrant transcript for nonsense-mediated decay. As a result, the 'low expression allele' has −30% of normal FECH activity. Individuals heterozygous for a pathogenic *FECH* mutation without the 'low expression' allele are asymptomatic, and

therefore, correction of the low expression allele alone is therapeutic. To facilitate the development of novel therapeutic approaches for EPP, Barman-Aksozen *et al.* generated mice that harbor the human *FECH* intron 3 with the IVS3-48T>C polymorphism (designated Emi mice) [93]. Homozygous Emi mice were embryonic lethal, therefore, Emi mice were bred against the *Fech*^{m1Pas/m1Pas} mice to generate Emi/mPas1 mice. Emi/mPas1 mice were even more severely affected than the *Fech*^{m1Pas/m1Pas} mice, with 6 to 8–folder higher erythrocyte PPIX levels, increased prenatal mortality, more pronounced hepatosplenomegaly, and overall higher bilirubin levels. Within minutes of exposure of shaved skin to 400-420 nm light (1.69-2.3 J/cm^2), behavior indicative of pain was observed and erythema became evident by 2-3 hours post-exposure.

RT-PCR analyses revealed that the partially humanized *Emi* allele led to unexpected splicing events, with the majority of transcripts completely lacking exon 3. Only a small number of transcripts used the aberrant splice site that is 63 nucleotides upstream of the normal one, as described in humans carrying the 'low expression' allele [93]. Presumably, the exon 3-lacking transcripts were stable and resulted in non-functional protein, leading to lethality of homozygous Emi mice and a very severe EPP phenotype in the Emi/m1Pas mice.

6. Discussion

To date, mouse models have been generated for all eight major human porphyrias, with the exception of XLP and the ultra-rare ADP. These mouse models of the human porphyrias have clearly provided relevant and important information on the pathophysiology of their human counterparts as well as the opportunity to provide proof-of-concept and preclinical data for various therapeutic strategies. For the acute porphyrias, the 'inducible' T1/T2 AIP mice have been particularly useful for preclinical evaluation of liver-directed gene therapy and RNAi-mediated approaches that specifically knock down hepatic *ALAS1* mRNA expression [24, 35–37, 89, 90]. A subcutaneously administered RNAi therapeutic is currently in Phase III Clinical Trials and is emerging as a safe and effective alternative treatment for human AHP patients. If important modifying genes that predispose or protect against the acute attacks are discovered, they could be evaluated in these mice. While human AIP, VP, and HCP patients have half-normal activities of their respective enzymes, HMBS, PPOX, and CPOX, it is notable that mice with 50% reduction of these enzymes do not display PB-induced acute biochemical attacks [4, 7, 43]. For AIP, decreasing the HMBS activity to ~30% of normal, as was the case in the T1/T2 mice, resulted in an 'inducible' phenotype [7, 14]. Therefore, it is likely that further reduction of CPOX and PPOX activities are necessary to produce HCP and VP mice, respectively, that display acute biochemical attacks. The recently generated HD-AIP mice, which capture many of the clinical and biochemical features of the human disease, have provided important insights as to why autosomal dominant AIP and HD-AIP have vastly different manifestations. To date, there is no effective treatment for HD-AIP. These mice facilitate studies aimed to develop new therapeutic approaches for HD-AIP.

Mouse models for PCT have played a particularly important role in delineating the pathogenic mechanism of this hepatocutaneous porphyria. In addition to facilitating the understanding of the role of iron overload in the etiology of PCT, they made possible the

identification of the specific inhibitor of UROD that forms in the liver during active disease [54, 59]. In the future, these mice may serve in preclinical studies to evaluate newer approaches to efficiently treat PCT.

Mouse models for the erythropoietic porphyrias, particularly the UROS$^{P248Q/P248Q}$ mice for CEP and the *Fech$^{m1Pas/m1Pas}$* for EPP, clinically and biochemically mimic the severe forms of their respective human diseases and have been valuable for preclinical evaluation of BMT and/or HSC gene therapy approaches. The *Fech$^{m1Pas/m1Pas}$* mice have also provided important insights into the underlying pathogenic mechanisms of the liver damage and hematologic abnormalities in EPP. The fact that the *Fech$^{m1Pas/m1Pas}$* mice have vastly different clinical severity depending on their genetic background provides a unique opportunity to identify potential modifying genes for EPP. Efforts to generate a partially humanized EPP mouse model carrying the common 'low expression' allele were hampered by unexpected missplicing events that led to skipping of exon 3 in the majority of *Fech* transcripts, rather than use of the −63 nt aberrant splice site that is normally used in human patients with this allele. While this limits the use of this mouse model for evaluating therapies aimed to correct the aberrant splicing caused by the 'low expression' allele, these mice may be useful for assessing the impact of various therapies on the marked cutaneous photosensitivity in EPP, given their pronounced photosensitive phenotype.

In summary, mouse models of the human porphyrias have contributed significantly to furthering our understanding of disease pathogenesis and have facilitated the development of new therapeutic approaches for these disorders. Recent advances in gene editing technology, in particular the clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 system, has made generation of mouse models much more rapid and affordable, while offering the capacity to alter multiple genes simultaneously. Furthermore, this system is applicable to various animal species, including non-human primates, which are not readily amenable to conventional genetic manipulation techniques. Thus, going forward, additional porphyric animal models in mice and other species, perhaps with more complex genetic modifications, are likely to be generated using these newer technologies.

Acknowledgements

Conflicts of Interest

MY and RJD are past recipients of research grants from Alnylam Pharmaceuticals and Recordati Rare Diseases. They are co-inventors of a patent licensed to Alnylam Pharmaceuticals for RNAi therapy of the AHPs. RJD is a consultant for Alnylam Pharmaceuticals, Mitsubishi Tanabe Pharma Development America and Recordati Rare Disease.

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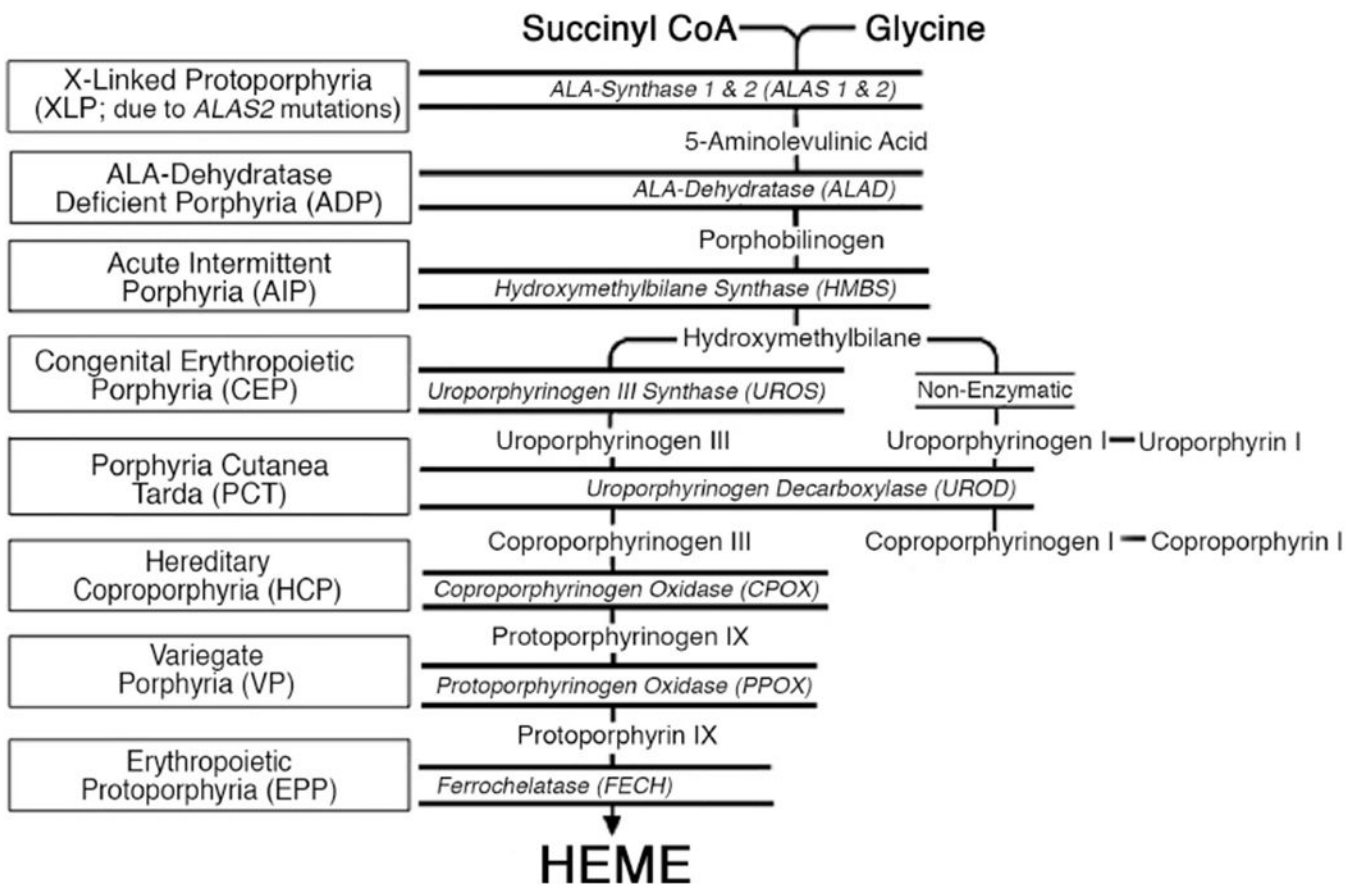
Figure 1.
Schematic of the human heme biosynthetic pathway and the porphyrias resulting from the indicated enzymatic defect. Heme is synthesized from succinyl CoA and glycine through eight enzymatic steps. The heme biosynthetic enzymes are italicized, and the resulting porphyrias are shown in boxes. Note that there are two isozymes for the first enzyme, 5-aminolevulinic acid synthase (ALAS): a housekeeping enzyme, ALAS1, encoded by a gene that is under negative feedback regulation of the end product heme, and an erythroid-specific enzyme, ALAS2, that is regulated by iron response proteins and erythroid transcription factors.

Table 1.

Classification of the porphyrias and available porphyria mouse models

Classification	Gene	LOF/	Inheritance[**]	Mouse Model
Acute Hepatic Porphyrias (AHPs)				
Acute Intermittent Porphyria	Hydroxymethylbilane Synthase (*HMBS*)	LOF	AD	T1/T2 R167Q$^{+/+}$[*][***]
Hereditary Coproporphyria	Coproporphyrinogen Oxidase (*CPOX*)	LOF	AD	Cpox$^{+/W373X}$
Variegate Porphyria	Protoporphyrinogen Oxidase (*PPOX*)	LOF	AD	PPOX$^{R59W+/-}$
ALA-Dehydratase Deficiency Porphyria	ALA-Dehydratase (*ALAD*)	LOF	AR	N/A
Hepatocutaneous Porphyria				
Porphyria Cutanea Tarda Type 1	-	-	Sporadic	Iron/ALA/PCB
Porphyria Cutanea Tarda Type 2	Uroporphyrinogen Decarboxylase (*UROD*)	LOF	AD	Urod$^{+/-}$/Hfe$^{+/-}$
Erythropoietic Cutaneous Porphyrias				
Congenital Erythropoietic Porphyria	Uroporphyrinogen Synthase (*UROS*)	LOF	AR	Uros$^{P248Q/P248Q}$ Uros$^{C73R/C73R}$
Erythropoietic Protoporphyria	Ferrochelatase (*FECH*)	LOF	AR	Fech$^{m1Pas/m1Pas}$ Fech Exon10 Emi/mPas1
X-linked Protoporphyria	ALA-Synthase 2 (*ALAS2*)	GOF	X-L	N/A

N/A, not available; ALA, aminolevulinic acid; PCB, polychlorinated biphenyls

[*] LOF = Loss-of-Function; GOF = Gain-of-Function

[**] AD = Autosomal Dominant; AR = Autosomal Recessive; X-L = X-Linked

[***] R167Q$^{+/+}$ mice model homozygous dominant AIP