The contribution of mouse models in the rare disease alkaptonuria

Short title: Alkaptonuria mouse models

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Abstract

Alkaptonuria is an ultra-rare autosomal recessive disorder of tyrosine metabolism, whereby the homogentisate 1,2-dioxygenase (HGD) enzyme is deficient, causing an elevation of its substrate homogentisic acid (HGA). Overtime, elevated HGA causes connective tissue ochronosis, leading to a severe and early onset osteoarthropathy. The use of HGD deficient mouse models in this metabolic bone disease have provided the opportunity to investigate AKU pathophysiology and potential treatments. An ENU mutagenesis AKU mouse model (BALB/c *Hgd*$^{-/-}$) provided the means to explore the onset of pigmentation in cartilage and treatment of AKU with nitisinone, an inhibitor of the upstream enzyme forming HGA. This work provided evidence that nitisinone could not only lower circulating HGA, but could also prevent ochronosis and halt disease progression, leading to its off-label use at the National Alkaptonuria Centre (Liverpool, UK) and its subsequent testing in human clinical trials (DevelopAKUre). Recently, a new targeted AKU mouse model (*Hgd tm1a -/-*, C57BL/6) has been established, offering a *LacZ* reporter gene for localising gene expression and LoxP and FRT sites that enabled generation of an inducible and liver-specific HGD knockout model (*Hgd tm1d MxCre +/-*). This conditional model determined the importance of the liver as a target organ for future gene/enzyme replacement therapies in AKU. The contribution of AKU mouse models has clearly accelerated the treatment and knowledge of this rare disease, and will continue to be used.

Keywords: mouse models; alkaptonuria; ochronosis; pathophysiology; therapy; nitisinone; conditional knockout

1. Alkaptonuria

Alkaptonuria (AKU; OMIM #203500) is an iconic ultra-rare disease, being the first disease found to conform to autosomal recessive Mendelian inheritance, described by Sir Archibald Garrod in 1902 [1]. It wasn't until 1958 that La Du determined the cause of this metabolic bone disease to be a defect in the homogentisate 1,2-dioxygenase (HGD; EC 1.13.11.5) enzyme [2]. HGD converts homogentisic acid (HGA) into maleylacetoacetic acid in the tyrosine metabolism pathway, therefore HGD deficiency leads to HGA accumulation in the blood and tissues, despite efficient urinary excretion. This excess HGA eventually leads to the formation and deposition of a dark pigment into connective tissues [3,4] such as the joints, the cardiovascular system [5], eyes, and skin [6]. This pigment deposition, termed ochronosis, causes affected tissue to become stiff and brittle [7], particularly affecting articular cartilage, leading to a severe and early osteoarthropathy, in addition to other musculoskeletal disorders such as tendon rupture and heart valve stenosis.

The primary metabolic consequences of AKU are relatively straightforward to study; metabolites such as HGA and tyrosine from urine [8,9] and blood [10] samples can be measured and monitored. More recently, technology and software has led to metabolomic analysis in AKU, enabling the wider consequences of this metabolic disease to be studied, which can be performed on blood/urine samples that are relatively easy to obtain [11]. Studying the ochronotic process in human joints however has been much more limited. Currently, only joints from patients undergoing joint replacement [7] or autopsy [6] are available to study as it is highly unethical and impractical to investigate younger, less diseased joints. Therefore, the only insight into human joint pathology in AKU is at the end stage of the disease.

The development of HGD deficient AKU mouse models has allowed examination of joints from the very onset of pigmentation. In addition, AKU mouse models can be used to study metabolites relevant to AKU, to further characterise disease pathophysiology and to investigate new and experimental treatments.

2. Animal models of disease

Model organisms such as fruit flies, worms, zebra fish and rodents have been used for many years to study elements of human disease. Many genes are conserved between species, in addition to many basic processes such as metabolism, cell division and growth. Due to easy and cost effective maintenance and breeding, similarities to humans, in terms of phylogenetics and physiology, and ability to manipulate the genome, the house mouse (*Mus musculus*) is the most common laboratory

model of human disease. The use of DNA manipulation technologies on the mouse genome to create transgenic, knock-in and knockout mouse models accelerated the use of mouse models in research, with thousands of strains now available.

2.1 Early animal work in AKU

Many reports have documented spontaneous AKU in various animals such as the rabbit [12], chimpanzee [13], orangutan [14] and macaque [15], where urine was found to darken after standing, leading to the identification of AKU. However, no evidence of osteoarthropathy or pigmentation was found. In addition, these findings were all isolated case reports. Early animal work involved attempts to induce ochronosis by injection of HGA into rabbits, with intra-articular injection shown to produce joint ochronosis resembling that of human lesions [16]. Intravenous and intraperitoneal injection failed to produce ochronosis in this rabbit model. A dietary model was also reported, with rats fed a diet excess in tyrosine (8%) for a minimum of 9 months, which appeared to induce ochronosis [17].

These early reports of AKU occurred before the advent of genome modifying technology; the first transgenic mouse model was developed in 1976 and the first knockout mouse in 1987 [18]. After the injection of HGA into rabbits to induce ochronosis in 1961, which did not attempt to create a genetic model of AKU, it was not until the 1990s that a murine model for AKU was discovered.

2.2 Identification of human and murine *HGD*

Preceding the first AKU mouse model was a series of studies that determined the location of human and murine *HGD*, and characterised a range of human *HGD* mutations. In 1993, the human *HGD* gene was mapped to chromosome 3q2 via a homozygosity study [19]. Following this, a homologous murine mutation was then discovered in 1994 on chromosome 16 [20]. Inter-species linkage studies then confirmed this human chromosome 3q2 location [21]. Human *HGD* was then further mapped to chromosome 3q13.33 and shown to comprise of 14 exons encoding a 445-mer protein [22]. By 2011, 115 known mutations had been identified in the human *HGD* gene, with exons 6, 8, 10 and 13 showing the highest prevalence of mutations [23]. These mutations range from missense mutations (66.4%), small deletions (12.2%) and insertions (12.2%), causing frameshift and splice mutations, and nonsense mutations (6%). To date, 203 different human *HGD* variants have been identified [24], which can all be found in the *HGD* mutation database; http://hgddatabase.cvtisr.sk/ [25].

2.3 Mutagenesis mouse model of AKU

The first AKU mouse model was generated by ethylnitrosourea (ENU) mutagenesis, whereby random mutations are induced into the genome [26]. The AKU phenotype was identified by Montagutelli et al in 1994 at the Pasteur Institute (Paris) due to darkened cage bedding, caused by elevated HGA in the urine turning a dark brown/black colour [20]. Subsequent mice were confirmed to have AKU by urine spots on sodium hydroxide-soaked filter paper, which causes AKU urine to turn brown in 30–60 seconds (alkaline conditions speed up the colour change; urine eventually turns dark after standing for several days). An example of dark stained cage bedding from AKU mice is shown in Figure 1. This *Hgd* mutation was then backcrossed onto the BALB/cByJ and C57BL/6J murine backgrounds. Urine analysis then confirmed that HGA was elevated to >0.55mol/mol creatinine in homozygous mice and was non-detectable in wildtype and heterozygotes.

In 1997, the murine *Hgd* gene was cloned and the ENU-induced mutation was identified in 1998 to be a splice mutation that resulted in a severely truncated HGD protein with <6% activity compared with wildtypes [27,28]. Despite the extreme level of pigmentation seen in AKU patient cartilage, no connective tissue pigmentation in these homozygous AKU mice was found, despite elevated urinary HGA [20,29]. Ochronosis however was later detected using Schmorl's staining in a crossbred murine model of AKU, $Hgd^{-/-}Fah^{-/-}$, derived from the original ENU mutagenesis mouse from the Pasteur Institute, showing microscopic pigmentation synonymous to human ochronosis [30]. Pigmentation was shown to initiate in the pericellular matrix of chondrocytes found in calcified articular cartilage [30]. Although pigmentation was identified, the complex $Hgd^{-/-}Fah^{-/-}$ model was not deemed a suitable model for AKU due to the confounding hereditary tyrosinaemia type I (HT-1; OMIM #276700) $Fah^{-/-}$ (fumarylacetoacetic acid hydrolase; EC 3.7.1.2) mutation that exhibits severe hepatic and renal pathology. Furthermore, the model requires nitisinone treatment (see section 2.3.1) from birth to survive by blocking the tyrosine pathway upstream of the fatal $Fah^{-/-}$ mutation, with subsequent removal in adulthood to cause spontaneous loss of heterozygosity from $Hgd^{+/-}Fah^{-/-}$ to $Hgd^{-/-}Fah^{-/-}$ in a subset of mice to generate the AKU genotype.

In 2014, a consistent murine model of AKU in the BALB/c background, $Hgd^{-/-}$, was described with no confounding HT-1 pathology [31]. This AKU model demonstrated relatively stable elevated plasma HGA levels, the first reported in an AKU animal model, and extensive chondrocyte pigmentation via a modified Schmorl's stain. It was shown here that initial chondrocyte pigmentation is pericellular, progressing to the intracellular compartment. The ability to score the number of pigmented chondrocytes in the tibio-femoral joint showed a linear increase in pigmentation with age. This AKU

mouse model has provided the means to study early ochronosis in a predictable and systematic manner.

Establishment of this BALB/c *Hgd*$^{-/-}$ AKU model has led to work looking at various aspects of the disease, such as preventing HGA formation and therefore disease progression with nitisinone (discussed in section 2.3.1)[31,32], metabolomic evaluation of the wider effects of nitisinone [11,33], investigation of the ultrastructure of murine AKU calcified cartilage by electron microscopy [34], expression of enzymes related to tyrosine metabolism (Wilson et al, manuscript in preparation) and the effectiveness of tyrosine dietary restriction on nitisinone-induced tyrosinaemia [35]. This mouse model would be of valuable use to other areas of AKU research, providing an *in vivo* model in which to investigate phenomena such as inflammation, amyloidosis and oxidative stress, which have so far been investigated only in alkaptonuric cells and tissues [36,37].

2.3.1 Nitisinone-treatment in the BALB/c *Hgd*$^{-/-}$ AKU mouse model

Until recently, only palliative treatment such as analgesics and arthroplasty [38] were available for AKU, with low-protein diets, vitamin C and physiotherapy proven clinically ineffective [3,39,40]. A drug called nitisinone (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; NTBC), which blocks the enzyme 4-hydroxyphenylpyruvic acid dioxygenase (HPPD; EC 1.13.11.27) forming HGA, was shown to completely prevent chondrocyte pigmentation in the BALB/c *Hgd*$^{-/-}$ mouse model [31]. This mouse study followed a human trial of nitisinone that saw a reduction in HGA, but was deemed inconclusive and ineffective due to no clinical joint improvement, assessed by hip rotation, which is now deemed an inappropriate primary outcome [41]. Further work in this mouse model showed that nitisinone can arrest, but not reverse, ochronosis progression, and also demonstrated a clear-dose response between nitisinone and plasma HGA [32].

The investigation of nitisinone in this BALB/c *Hgd*$^{-/-}$ model was highly influential, providing evidence of HGA reduction and disease prevention. This led to the off-label use of nitisinone at the National Alkaptonuria Centre (NAC, Liverpool, UK; http://www.akusociety.org/aku-national-aku-centre.html)[42,43] and a series of human clinical trials (DevelopAKure; http://www.developakure.eu/) investigating the use of nitisinone in AKU [44,45], where its use has proven safe and effective.

Although the use of nitisinone in these clinical trials has so far been very successful, nitisinone can only treat, not cure the disease. The next big step in single-gene diseases like AKU would be to treat the underlying genetic cause with gene/enzyme replacement therapy.

2.3.2 Limitations of the ENU mutagenesis AKU mouse model

It is clear that the ENU mutagenesis *Hgd*$^{-/-}$ AKU mouse model has greatly contributed to our knowledge of AKU, in terms of both disease pathophysiology and treatment and will continue to be used for AKU research. ENU mutagenesis however is not a targeted approach to create a model of genetic disease due to the high frequency of DNA mutations that ENU causes [26,46]. Other unknown and uncharacterised mutations could potentially exist in this mutagenesis AKU model that could affect the phenotype, as this ENU model has never been fully sequenced. Newer, more complex mouse models offering additional tools are now available, whereby a specific gene is targeted.

2.4 Targeted and conditional mouse model of AKU

A new targeted mouse model of AKU (*Hgd tm1a -/-*) in the C57BL/6 background has been generated using a mutant knockout-first *Hgd* allele [47] obtained from the UC Davis Knockout Mouse Project (KOMP) Repository (https://www.komp.org/), eliminating the uncertainty of other potentially confounding mutations arising from a mutagenesis approach [48]. This mouse, which has a gene trap cassette inserted into the fifth *Hgd* intron (see figure 2), exhibits the same AKU phenotype observed in the BALB/c *Hgd*$^{-/-}$ mouse, of elevated HGA and joint pigmentation.

The new targeted *Hgd tm1a -/-* AKU model provides confidence that no confounding mutations affecting the AKU phenotype, with regards to HGA and pigmentation, are present in BALB/c *Hgd*$^{-/-}$ (others mutations affecting disease aspects not yet investigated could exist). The mutagenesis model however has no ability to be manipulated further nor does it possess other tools. The targeted *Hgd tm1a* model on the other hand has a *LacZ* reporter gene (figure 2) within the gene trap cassette situated in the *Hgd* locus, which enabled precise localisation of HGD expression to hepatocytes and proximal convoluted cells of the kidney cortex and also determined when embryonic expression initiated [48]. Furthermore, this knockout-first *Hgd tm1a* allele contains FRT and LoxP sequences (figure 2) which enabled a double transgenic conditional *Hgd* knockout mouse to be generated using Flp/Cre recombination [47,49]. The use of Mx-1 Cre recombinase (*MxCre*) allowed inducible and liver-specific knockout of *Hgd* mRNA by removal of the LoxP flanked (floxed) sixth *Hgd* exon in the *Hgd tm1d MxCre +/-* conditional mouse model [48]. This model was used to identify the liver as the target for future AKU enzyme/gene therapy, concluding that the minimum *Hgd* mRNA level required to rescue AKU lies between approximately 25-40%.

The presence of LoxP sites within the conditional *Hgd tm1d* conditional knockout mouse, to date used only to remove HGD from the liver, has the potential to be crossed with other Cre mouse lines that can change how, when and where *Hgd* is knocked out. For example, the *Slc22a6*-CreER^{T2} knock-in line, a tamoxifen-inducible Cre system specific to proximal convoluted tubules, would provide kidney specific HGD knockout [50].

2.5 Limitations of AKU mouse models

Both the mutagenesis and targeted AKU mouse models reflect the metabolic nature of AKU with elevated urinary and plasma HGA that appear stable over the lifetime of the mice. The degree of pigmentation however appears to reflect early stage joint disease even when aged up to 52-80 weeks which is comparable with 40-60 years in humans [51], with no pigmentation observed in the superficial hyaline cartilage (see figure 3), despite humans exhibiting a severe blanket pigmentation present in all layers of articular cartilage [7,31,48]. Furthermore, AKU patients develop an early-onset and severe osteoarthritis, which is not seen in the AKU mouse models, with Preston et al reporting that the rate of development of osteoarthritis in the BALB/c *Hgd*$^{-/-}$ model was not greater than the spontaneous osteoarthritis rate reported for the background strain [31]. The absence of osteoarthritis in these mice is likely due to the ochronotic phenotype being mild and early-stage.

In addition to the absence of macroscopic and microscopic hyaline cartilage pigmentation, there has been no pigmentation observed in other connective tissues, such as tendons, ligaments, and the cardiovascular system in mice. Endogenous vitamin C production in mice [52] has been suggested as a protective mechanism against the formation of ochronotic pigmentation due to its anti-oxidant activity. In the 1990s, the absence of pigmentation in the first ENU BALB/c *Hgd*$^{-/-}$ mice aged up to 14 months was attributed to vitamin C production. Differences in urinary excretion of HGA in mice and humans may also have explained the lack of joint pigmentation. However, with subsequent detection of ochronotic pigment and plasma HGA levels that were 3 to 4-fold greater than human levels [31], differences in ochronosis severity cannot be attributed to increased urinary excretion nor vitamin C production. Furthermore, treatment with vitamin C in human patients has not proven to be clinically effective [38]. Lack of severe pigmentation in mice could be attributable to their smaller size and quadrupedal gait, and therefore reduced mechanical loading of the joints compared to humans. Furthermore, if ochronotic pigment deposition is a time-dependant process, the short mouse lifespan may prevent the accumulation of ochronotic pigment to the severe level observed in humans. As discussed in section 2.2, several different *HGD* mutations exist in human AKU patients, which can have different impacts on the residual activity of the enzyme [24,25,53]. The BALB/c *Hgd*$^{-/-}$ model, was shown to have <6% HGD activity caused by a splice mutation that causes skipping of

either one or two exons resulting in a truncated protein [20]. Residual activity in the *Hgd tm1a -/-* model has not been reported, however no mRNA is detected after the gene trap which causes early termination of transcription [48]. A recent study reporting the first human genotype-phenotype data however suggests that different residual HGD activity due to the nature of the different mutations is likely not responsible for the observed differences in ochronosis between mice and humans, as no differences in serum or 24-hour urine HGA, eye pigmentation and hip bone density were found between patients with 1% or 31-34% residual HGD activity [24].

2.6 Future of AKU mouse models

Both the mutagenesis BALB/c *Hgd*$^{-/-}$ and targeted *Hgd tm1a -/-* model show the same AKU phenotype of elevated plasma and urine HGA and joint pigmentation, therefore either model is suitable for future AKU research. Although these AKU mouse models may not exhibit a severe joint pathology like the human condition, they provide insight into early joint disease that currently cannot be investigated in human patients. Biochemically however, these models represent the AKU phenotype very well, showing the same metabolic features of elevated HGA and response to nitisinone. AKU mouse models provide controlled conditions in which to test potential therapies where the effectiveness can be investigated to a much greater extent than is possible in humans, in addition to the opportunity to conduct research into the slow, progressive pathophysiology of AKU, which is not achievable in human patients where disease manifestation takes decades to present. The requirement for these AKU mouse models will exist whilst new therapies and potential treatments are being established, such as enzyme replacement and gene therapy, the latter being the ultimate cure for AKU and other single gene Mendelian diseases. Studying a rare disease such as AKU can also provide valuable lessons for other more common diseases such as osteoarthritis, as AKU exhibits a severe and early osteoarthropathy, with AKU mice providing the means to study early-stage disease. Additionally, attempts to treat the metabolic defect in AKU, via enzyme replacement or gene therapy, will undoubtedly uncover fundamental information applicable to other genetic disorders of the liver and kidney.

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• Special interest

•• Outstanding interest

Figures + Legends

Figure 1. BALB/c *Hgd*$^{-/-}$ AKU mice. Mice with deficient homogentisate 1,2-dioxygenase (HGD) activity (shown on the right) are unable to metabolise homogentisic acid (HGA), leading to its excretion into the urine, causing dark urine stained cage bedding after several days of exposure to the air. A wildtype mouse is shown on the left. Image used with the permission of Dr Jean Louis Guenet.

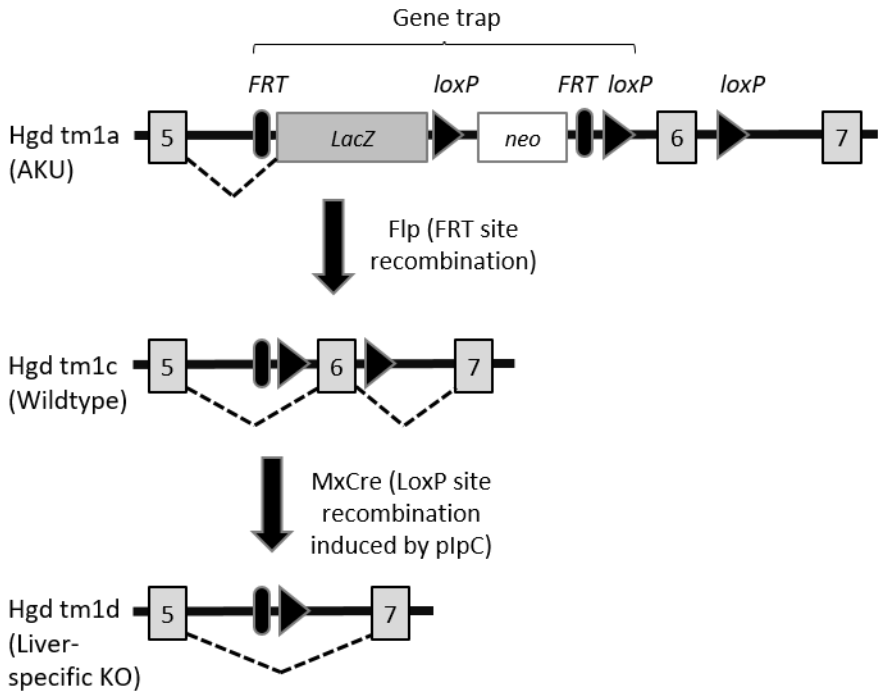

Figure 2. Schematic of the modified *Hgd* allele in the targeted mouse model of AKU. *Hgd tm1a* homozygous mice exhibit an AKU phenotype due to the presence of a gene trap cassette between exons 5 and 6 in the *Hgd* gene. To obtain *Hgd tm1c*, FRT site recombination via Flp removed the gene trap, leaving exon 6 in the *Hgd* gene flanked by LoxP sites. *Hgd tm1c* exhibits a wildtype phenotype. Introduction of *MxCre* into *Hgd tm1d* mice enables inducible (by injection of pIpC) and liver-specific HGD knockout (KO). pIpC = polyinosinic:polycytidylic acid.

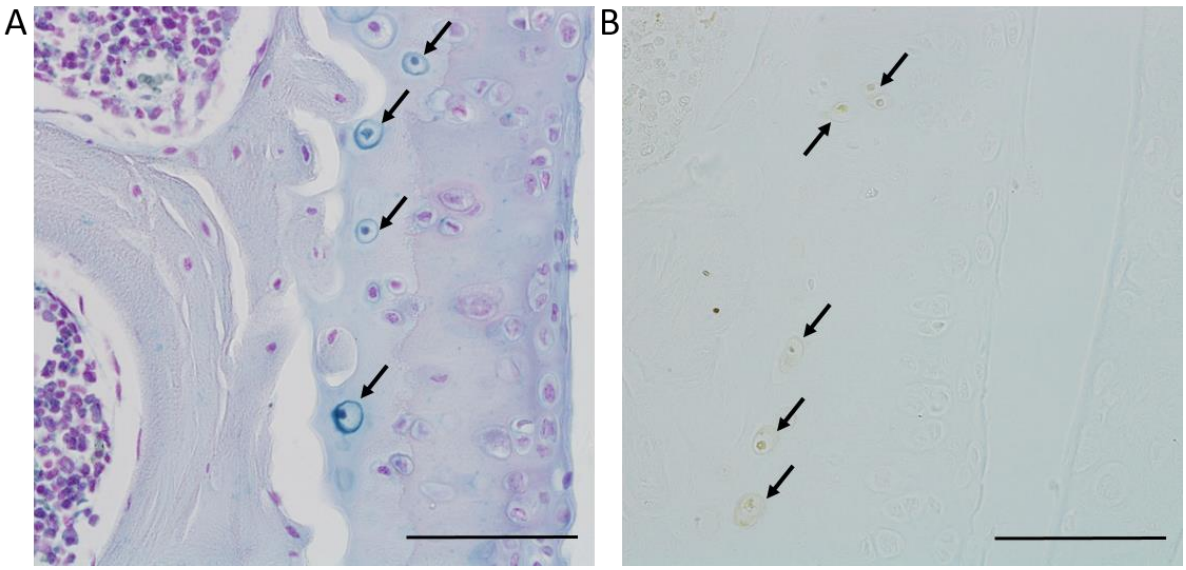

Figure 3. Knee joint pigmentation in AKU mice. (A) Schmorl's staining of ochronotic pigment associated with chondrocytes found within the calcified articular cartilage of the tibia, in the tibio-femoral joint of *Hgd tm1a -/-* mice. No pigmentation is observed in the superficial hyaline cartilage. (B) shows the presence of native ochronotic pigment with no staining. Scale bar: 50µM.