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Recombinant AAV-directed gene therapy for type I glycogen storage diseases

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Abstract

Introduction—Glycogen storage disease (GSD) type Ia and Ib are disorders of impaired glucose homeostasis affecting the liver and kidney. GSD-Ib also affects neutrophils. Current dietary therapies cannot prevent long-term complications. In animal studies, recombinant adeno-associated virus (rAAV) vector-mediated gene therapy can correct or minimize multiple aspects of the disorders, offering hope for human gene therapy.

Areas covered—A summary of recent progress in rAAV-mediated gene therapy for GSD-I; strategies to improve rAAV-mediated gene delivery, transduction efficiency and immune avoidance; and vector refinements that improve expression.

Expert opinion—rAAV-mediated gene delivery to the liver can restore glucose homeostasis in preclinical models of GSD-I, but some long-term complications of the liver and kidney remain. Gene therapy for GSD-Ib is less advanced than for GSD-Ia and only transient correction of myeloid dysfunction has been achieved. A question remains whether a single rAAV vector can meet the expression efficiency and tropism required to treat all aspects of GSD-I, or if a multi-prong approach is needed. An understanding of the strengths and weaknesses of rAAV vectors in the context of strategies to achieve efficient transduction of the liver, kidney, and hematopoietic stem cells is required for treating GSD-I.

Keywords

AAV; endoplasmic reticulum; gene therapy; glucose-6-phosphatase; glucose-6-phosphate transporter; glucose homeostasis; glycogen storage disease; hepatic gene delivery

1. Introduction

Glycogen storage disease type I (GSD-I), also known as von Gierke disease, consists of two autosomal recessive disorders, GSD-Ia (MIM232200), deficient in glucose-6-phosphatase- α (G6Pase- α or G6PC) and GSD-Ib (MIM232220), deficient in the glucose-6-phosphate transporter (G6PT or SLC37A4) [1,2]. The incidence of GSD-I is approximately 1 in 100,000 with GSD-Ia accounting for 80% of the cases [1,2]. Human *G6PC*, encoding G6Pase- α , is a single copy gene on chromosome 17q21 [3] that encodes a 357 amino-acid hydrophobic protein [4] with 9 helices that anchor it in the endoplasmic reticulum (ER) membrane [5] (Figure 1). G6Pase- α catalyzes the hydrolysis of glucose-6-phosphate (G6P)

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to glucose and phosphate. Human *G6PT* is a single copy gene on chromosome 11q23 [6] that encodes a 427 amino-acid hydrophobic protein [7] with 10 helices that anchor it in the ER membrane [8] (Figure 1). Based on mutational and active site labeling studies, the amino acids comprising the active site of G6Pase- α include Arg-83, His-119, and His-176 [9] which lie on the luminal side of the ER, inaccessible to the cytoplasm (Figure 1). Therefore, G6Pase- α activity is dependent upon transport of cytoplasmic G6P across the ER membrane by G6PT [1,2]. G6PT belongs to the organophosphate:phosphate antiporter family of the major facilitator super-family and was recently shown to transport G6P into the ER lumen while moving phosphate out [10]. The two members of the G6Pase- α /G6PT complex are coupled functionally. A detrimental mutation in either protein prevents the other functioning efficiently and leads to the same metabolic phenotype [1,2]. The primary function of the G6Pase- α /G6PT complex is to maintain interprandial blood glucose homeostasis (Figure 2). It does so by regulating the terminal step of gluconeogenesis and glycogenolysis in the liver and kidney, catalyzing the hydrolysis of G6P to glucose [1,2]. The hallmark of GSD-I is hypoglycemia following a short fast. Associated with this is an elevation of G6P in the cytoplasm which stimulates alternative metabolic pathways leading to the synthesis and excessive accumulation of glycogen in the liver and kidney, which promotes progressive hepatomegaly and nephromegaly (Figure 2). The hepatomegaly is further exacerbated by an increase in neutral lipid accumulation in the liver. Other major metabolic consequences of elevated cytoplasmic G6P are hypercholesterolemia, hypertriglyceridemia, hyperuricemia, and lactic acidemia that also characterize the clinical pathophysiology of GSD-I. Long-term complications include growth retardation, osteoporosis, gout, pulmonary hypertension, renal disease and hepatocellular adenomas (HCAs) which may undergo malignant transformation [1,2]. G6Pase- α is expressed primarily in the gluconeogenic organs of the liver, kidney and intestine [11], while G6PT is expressed ubiquitously [12], suggesting that G6PT has a wider range of biological roles. This led to the expectation that the additional clinical manifestations of neutropenia and neutrophil dysfunction (Figure 2), unique to GSD-Ib, are a direct, rather than indirect, consequence of G6PT mutation. This has now been confirmed [1,2]. Recent studies reveal that inactivation of G6PT in neutrophils leads to enhanced apoptosis that explains, at least in part, neutropenia and neutrophil dysfunction seen in GSD-Ib [13].

Both GSD-Ia and GSD-Ib are childhood lethal if untreated. The metabolic abnormalities of both diseases can be partially controlled with dietary therapy augmented by drug therapy. Patients 3 years or older are prescribed uncooked cornstarch, a slow release carbohydrate, to prolong the length of euglycemia between meals [14]. Younger patients typically receive nocturnal nasogastric infusion of glucose [15] because their pancreatic amylase activity, which is required to hydrolyze raw starch, is insufficiently developed [14]. Despite the benefits of dietary therapy, efficacy is frequently limited by poor compliance. For GSD-Ib patients manifesting neutropenia and/or neutrophil dysfunction, which predispose them to frequent infections and inflammatory bowel disease, granulocyte colony-stimulating factor (G-CSF) therapy is commonly added [16,17]. While these combined therapies are sufficiently successful to attain near normal growth and pubertal development for patients, the underlying pathological processes remain uncorrected and patients continue to suffer from hyperlipidemia, hyperuricemia, hypercalciuria, hypocitraturia, and lactic acidemia [18,19]. As a result, long-term complications, including renal disease and HCAs still persist in GSD-I patients and become increasingly more significant with age. Moreover, while G-CSF therapy shows no significant short-term toxicity, all patients receiving extended therapy do develop splenomegaly [17]. A major complication for patients with severe chronic neutropenia is myelodysplasia/acute myeloid leukemia [20,21]. Together, the various downsides of the combined dietary and drug therapy make somatic gene therapy an attractive alternative for targeting the hydrophobic G6Pase- α and G6PT proteins to the ER

membrane. Vectors based on recombinant adeno-associated virus (rAAV) are being exploited for this therapy.

2. rAAV vectors

2.1 Evolution of rAAV vectors

AAV is a member of the *Parvoviridae* family which require a helper virus to facilitate productive infection and replication in vertebrate and insect cells [22,23]. The genome of AAV is a single-stranded DNA of approximately 4.7 kb that consists of two open reading frames (*rep* and *cap*) flanked by 145 b inverted terminal repeats (ITRs). The four nonstructural Rep proteins, which are under transcriptional control of two promoters (p5 and p19) are involved in DNA replication, genome packaging, site-specific integration, and regulation of gene expression. A single p40 promoter directs the production of the three structural capsid proteins, VP1, VP2 and VP3 from a single transcript with 3 alternative splicing patterns. These proteins assemble into the virion shells and are involved in cell binding, internalization and trafficking within the targeted cells. The ITRs are the only cis-acting elements required for genome replication and packaging. The rAAV vectors are constructed by replacing the *rep* and *cap* genes with the promoter and gene of interest, leaving the ITRs as the only viral DNA sequences in the vectors.

All AAV serotypes are capable of infecting multiple tissues, although the exact tissue tropism is determined by the different capsid proteins. For gene therapy, the advantages of rAAV vectors are the lack of pathogenicity, wide range of infectivity and ability to establish long-term transgene expression [22–26]. In the last decade, 11 AAV serotypes and over 120 capsid variants have been categorized into six different phylogenetic clades [27–35] and the crystal structures of several AAV serotypes have been solved [36–40]. Much of the current rAAV vector development focuses on improving vector transduction efficiency and tropism through modification of both vector genomes and capsid proteins [reviewed in 41–43].

2.2 Self-complementary rAAV vectors

A major rate-limiting step in the life cycle of AAV is the need to convert the single-stranded (ss) DNA genome into double-stranded (ds) DNA prior to gene expression [44,45]. Since both the plus and minus strand ssDNA genomes are packaged with equal efficiency in the viral life cycle, this can be achieved *in vivo* by two mechanisms: base-pairing individual, but complementary, recombinant vector genomes, which reduces the effective vector dose; and DNA replication. The self-complementary AAV (scAAV) vectors, sometimes called dsAAV vectors, were developed to circumvent these mechanisms by packaging the gene of interest as a single inverted repeat that can spontaneously reanneal into a dsDNA genome [46–48]. These vectors arose from the observation that when an AAV genome is half wild type size, some replication intermediates fail to resolve at each ITR. As a result, packaging produces a mixture of virions which contain either one or two monomeric genomes per virion, or a single copy of a dimeric inverted repeat genome. With an scAAV, the packaged virions also consist of a similar mixture of monomeric and dimeric virions. The proportion of dimeric to monomeric genomes varies widely between preparations, from ~5% to >50% dsDNA genomes [48] but can be improved to >90% dimeric by mutating one of the AAV ITR [49,50]. The packaging capacity of ssAAV is reported to be 4.1–5.2 kb [48] and the primary compromise in using an scAAV is the 50% reduction in recombinant gene capacity to ~2.2 kb of unique transgene sequences.

Several studies suggested that packaging constraints for AAV are not as strict as previously reported [51,52]. Grieger and Samulski [51] reported that AAV serotype 1–5 can package up to 6 kb DNA and Allocca *et al.* [52] reported that rAAV2/5 can not only package up to 8.9 kb DNA but also mediate efficient transgene expression both *in vitro* and *in vivo*. In

contrast, three recent studies [53–55] reported that the AAV packaged genomes never exceed 5.2 kb but these normal-sized vectors could produce large functional transgene products following transduction. Wu *et al.* [53] proposed that these conflicting observations can be explained if the host cell reconstructs larger AAV genomes through mechanisms of annealing and homologous recombination between overlapping deletion variants. Clearly more needs to be understood before any gene therapy strategy might consider using larger genomes.

The scAAV vectors do display enhanced transduction efficiency in comparison with ssAAV vectors [48]. In primate studies, a factor IX transgene packaged in scAAV vectors serotype 7 and 8, was expressed in the liver 10- to 100-fold higher than ssAAV genomes packaged in the same serotype [56,57]. In another primate study, the macaque β -subunit of choriogonadotropin packaged in either scAAV2/7 or scAAV2/8 was expressed with a 2-log higher efficiency in the liver, compared to ssAAV genomes of the same serotype [58].

2.3 Strategies to improve transduction efficiency and evade immune responses

Many factors influence AAV transduction efficiency, and strategies to improve expression usually focus on the selection of the appropriate AAV serotype, the modification of capsid proteins, methods to minimize activation of the host immune system, and the selection of the most appropriate transgene promoter.

Different AAV serotypes exhibit different tropisms, due to the distribution of capsid-specific receptors on target cells [reviewed in 59]. Since this natural tropism can limit their utility for certain tissues, especially upon systemic application, rAAV vectors are often pseudotyped by cross-packaging an AAV genome of one serotype into the capsid of another serotype, yielding an infectious vector with the tropism of the new capsid [60,61]. This improves transduction of selected tissues *in vivo* while circumventing problems of preexisting immunity. The two organs specifically affected in GSD-I are the liver and kidney [1,2]. Both rAAV2/1 [62] and rAAV2/8 [63–66] have been used as gene delivery vehicles for GSD-I and both transduced the liver efficiently. However, only rAAV2/1 transduced the kidney [62]. Zincarelli *et al.* [67] compared the tropism of AAV serotypes 1–9, introduced via tail vein injection in mice, by following the distribution and kinetics of a recombinant luciferase gene. They found that overall rAAV2/9 exhibited the best viral genome distribution and highest transgene expression. For liver expression, rAAV2/1, rAAV2/7, rAAV2/8, and rAAV2/9 were best, but for kidney all AAV serotypes expressed low levels of luciferase [67]. Schievenbusch *et al.* [68] compared the efficacy of rAAV2, rAAV2/8, or rAAV2/9 carrying hepatocyte growth factor driven by a kidney tubular epithelial-restricted Ksp-cadherin-promoter to attenuate the course of chronic renal disease in a mouse model of renal tubulointerstitial fibrosis. They found that rAAV2/9 was the most effective for combined kidney- and liver-directed gene transfer.

To achieve higher transduction efficiency at lower doses, AAV capsid mutants have been developed to improve receptor binding and virus uptake. Tyrosine-modified rAAV vectors, in which tyrosine residues on the surface of AAV capsid proteins are converted to phenylalanine residues by site-directed mutagenesis, have markedly improved transduction efficiency [69–71]. The mechanism is attributed to the absence of tyrosine-phosphorylation sites on the capsid proteins, which results in reduced ubiquitination and proteasome-mediated degradation of the vector. The AAV capsid shell can be customized further using chimeric/mosaic capsids, site-directed or insertional mutagenesis, peptide display libraries, and chemical conjugation to create epitopes that mediate binding to receptors on the cell types of interest [41–43].

Minimizing the induction of host immune responses is critical in viral gene therapy. In general, AAV is a poor activator of both innate and adaptive immunity compared to other commonly used viral vectors, such as adenovirus (Ad) [72]. However, the encoded transgene product and AAV capsid proteins, presented during transduction, are targets for host immune responses. It is also important to recognize that studies of immune activation in mouse and canine models are not always predictive of the outcome in primates [73–77]. The activation threshold to AAV is relatively high in small animals compared to primates. Healthy human subjects carry AAV capsid-specific CD8⁺ T cell populations that expand upon rAAV-mediated gene transfer. Gao *et al.* [76] compared immune responses to transgene expression in the liver of mice and nonhuman primates and showed that primates elicited much stronger cytotoxic T cell responses to the transgene that correlated with the loss of transgene expression. This finding has been extended by Hurbert *et al.* [77] who examined the relationship between pre-existing antiviral titers, vector dose, vector copy number in the liver, and vector-mediated gene expression. In mice and monkeys with equivalent pre-exposure anti-AAV2/8 titers, a high-dose administration of the rAAV2/8 vector led to comparable genome copy numbers in the liver. However transgene expression was 1.5-logs lower in monkeys than mice, suggesting vector fate, or expression efficiency, differed between mice and monkeys.

Other experimental factors also impact the immunogenicity of rAAV-mediated gene delivery, including the route of administration, dose range, host species, and the nature of the transgene promoter. For instance, Wang *et al.* [78] compared the stability of rAAV2-mediated human factor IX gene expression following hepatic injection versus intramuscular injection. Hepatic injection elicited weak immune responses and high gene doses were well tolerated with sustained factor IX expression. In contrast, intramuscular injection elicited strong local immune responses that correlated with transient expression of the transgene. While different vectors and genes may lead to different outcomes, route of injection is clearly critical. Strategies to overcome humoral immunity to the AAV capsids have been reviewed by Mays and Wilson [72] and include capsid protein engineering, the use of alternative serotypes, and immunosuppression therapy during primary virus exposure.

The choice of transgene promoter not only impacts targeting efficiency and tissue-specific expression, but also the level of immune response or tolerance to the therapy. Ziegler *et al.* [79] compared the efficacy of gene therapy for murine Fabry disease by intravenous administration of rAAV2 expressing human α -galactosidase A under the control of either a ubiquitous CMV promoter, or a liver-restricted enhancer/promoter. The liver-specific promoter resulted in significantly higher levels of enzyme expression, compared to the CMV promoter, and little to no anti- α -galactosidase antibody formation. In contrast, ubiquitous expression from the CMV promoter elicited transgene-specific antibodies, that correlated with a reduction in transgene expression over time. Using a murine model of GSD-II, Franco *et al.* [80] compared a liver-specific promoter to a hybrid CMV enhancer/chicken β -actin promoter (CBA) on the expression of α -glucosidase using an rAAV2/8 vector. The liver-specific promoter directed ~60 fold higher hepatic transgene expression than the hybrid CBA promoter at 12 weeks post-infusion. Moreover, sustained α -glucosidase production was only observed with the liver-restricted promoter and correlated with the absence of anti- α -glucosidase antibody. The hybrid CBA promoter elicited CD4⁺/CD8⁺ lymphocytes and a humoral response [80]. Clearly the use of tissue-restricted promoter offers additional mechanisms to minimize immunity and enhance gene therapy efficacy.

2.4 Integration of rAAV vectors

The rAAV vectors rarely integrate into the host genome, remaining predominantly as circular episomal elements [81]. Approximately 0.1% of vector genomes that enter cells do integrate into host chromosomal DNA [82], mainly via homologous recombination with the

host genome or insertion at sites of host genome damage, although hotspots are reported at CpG islands and ribosomal DNA repeats [83,84].

AAV has not yet been identified as an etiologic agent of human disease but the risk of insertional mutagenesis should not be overlooked. Donsante *et al.* [85] showed that neonatal mice treated with an rAAV vector expressing the human β -glucuronidase gene driven by a β -actin promoter/CMV enhancer exhibited an increased incidence of hepatocellular carcinoma. Integrated AAV vector proviruses were observed in four different tumors from four different mice, and all mapped to a 6-kb region of chromosome 12. Whether this is a directed integration or coincidence, and whether the AAV vector and/or transgene is tumorigenic directly or indirectly, remain to be understood. Since HCAs are a severe long-term complication of GSD-I that develop in 70–80% patients over the age 30 and in 10% of patients undergo malignant transformation [1,2], it is critical that these issues are investigated further before contemplating an rAAV-mediated gene therapy in human GSD-I patients.

3. Animal models for GSD-Ia and GSD-Ib

For GSD-Ia there are two animal models, a transgenic mouse and a naturally occurring GSD-Ia dog. The GSD-Ia (G6pc^{-/-}) mice, generated by gene targeting [86], manifest all the symptoms of human GSD-Ia, namely hypoglycemia, growth retardation, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and mild lactic acidemia [86,87]. If left untreated, the mice rarely survive weaning and live no more than 3 months, mimicking the juvenile lethality seen with untreated human patients. The GSD-Ia dog was generated by crossbreeding a carrier Maltese, heterozygous for the p.M121I mutation in G6Pase- α , with a beagle [88]. The canine model which is outbred and immune competent similarly manifests all the typical symptoms of the human disorder, but exhibits a marked lactic acidosis more typical of human GSD-Ia patients, than the milder lactic acidemia seen in the G6pc^{-/-} mice. The average survival of GSD-Ia dogs historically, even with dietary therapy, is 5 to 8 weeks [88,89]. Therefore, the GSD-Ia dog is a valuable transitional model in the preclinical progression of any gene therapy methodologies pioneered in the smaller, faster breeding, more readily manipulated, GSD-Ia mice.

For studying GSD-Ib there is only a transgenic mouse model. The GSD-Ib (G6pt^{-/-}) mice, generated by gene targeting [90], manifest all the metabolic and myeloid defects of human GSD Ib, namely hypoglycemia, growth retardation, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, mild lactic acidemia, neutropenia, neutrophil dysfunction, and elevated serum G-CSF levels.

4. Gene therapy for GSD-Ia

4.1 Gene therapy in murine GSD-Ia

While the focus of this review is rAAV vector-mediated gene transfer for GSD-Ia, it is worth mentioning an early feasibility study using an Ad vector. Zingone *et al.* [91] showed that a first generation Ad vector expressing murine G6Pase- α under the control of a constitutive RSV promoter provided a short term metabolic correction in G6pc^{-/-} mice, followed by a rapid loss of the G6Pase- α transgene expression. To improve the efficacy, Koeberl *et al.* [92] used a helper-dependent Ad vector, which resulted in a sustained restoration of hepatic G6Pase- α activity to 20–40% of wild type enzyme activity at age 7 months. The infused mice exhibited normalized fasting hypoglycemia and reduced hepatic glycogen storage but this strategy failed to achieve uniform long term survival of G6pc^{-/-} mice and a dose-limiting hepatotoxicity was observed. Consequently, the focus shifted to rAAV vectors.

The G6Pase- α proteins are highly conserved, sharing 87–91% amino acid sequence identity across the mouse, dog and human. In the murine model of GSD-Ia, several promoters and gene constructs have been investigated in combination with different AAV serotypes. The gene constructs include murine, canine, and human cDNAs encoding G6Pase- α directed by promoters consisting of : (i) the hybrid chicken β -actin promoter/CMV enhancer (CBA); (ii) the canine *G6PC* gene promoter/enhancer (cGPE) [63]; (iii) a minimal human *G6PC* promoter/enhancer (hmGPE) [64]; and (iv) an extended human *G6PC* promoter/enhancer (hGPE) [65]. Both ssAAV and scAAV vectors have been investigated. The human, canine and murine G6Pase- α proteins have been shown to be functionally equivalent. Each is enzymatically active when expressed in the mouse, and each has the ability to couple functionally with murine G6PT to maintain interprandial glucose homeostasis and alleviate the symptoms of the disease.

In the first murine study [93], neonatal G6pc^{-/-} mice were infused with ssAAV2-CBA, an AAV2 vector carrying murine G6Pase- α directed by CBA. Immediately following ssAAV2-CBA infusion, glucose therapy was terminated. This approach failed to normalize hypoglycemia or sustain the life of G6pc^{-/-} mice presumably because slow uncoating of the AAV2 serotype delayed G6Pase- α expression [94]. To address this, Sun *et al.* [93] used a two-step strategy. In the first step, neonates were co-infused with Ad-G6Pase- α [91], to provide an immediate short term expression of G6Pase- α , and ssAAV2-CBA, to establish a longer term expression. In the second step, mice received an additional infusion of just ssAAV2-CBA, at age 2 weeks. This strategy corrected blood metabolic abnormalities for the full 12 months of the study. The mice grew normally, with only mild glycogen accumulation in the liver and kidney, and there were no premature deaths. Notably, the transgene was delivered to both the liver and kidney, resulting in the sustained expression of a functional G6Pase- α /G6PT complex in both organs. While a promising outcome, an Ad free strategy [95,96] is preferable.

Since those studies, novel AAV serotypes with improved potency, and broadened tropism, have become available [31–35]. Ghosh *et al.* [62] infused G6pc^{-/-} mice with two serial doses of either ssAAV2/1-CBA or ssAAV2/8-CBA, one at birth (2×10^{14} viral particles (vp)/kg) and the second at age 1 week (5×10^{13} vp/kg). Following the first vector infusion, dietary glucose therapy was terminated. Both ssAAV2/1-CBA and ssAAV2/8-CBA delivered the transgene to the liver and markedly improved their survival. However, only ssAAV2/1-CBA resulted in a sustained expression of the transgene in both the liver and kidney, and was studied further. The ssAAV2/1-CBA-infused animals grew normally throughout the 57-week study. They exhibited normal blood glucose levels, although fasting blood glucose levels were not examined. All of the infused animals exhibited normal metabolic profiles and, notably, an absence of anti-G6Pase- α antibody. G6Pase- α activity, measured between age 24 and 57 weeks, was restored to 11% of wild type activity in the liver and 7% in the kidney. While there was no histological abnormality in the livers of infused animals, they did exhibit mild hepatomegaly. Pathology analysis also showed that during most of the study, the mice exhibited normal kidney histology. However at age 57 weeks, increased glycogen accumulation was apparent along with some histological evidence of kidney disease that included glomerulosclerosis and tubular dilatation [62]. Despite this, the serum levels of creatinine, an index of altered renal function, remained within the normal range. Since G6Pase- α is primarily expressed in the proximal tubular epithelial cells in the kidney cortex [11], these results suggest that 7% of wild-type renal G6Pase- α activity, when uniformly expressed throughout the kidney tissues, cannot prevent longer term kidney complications. Clearly correction of the initial metabolic symptoms of GSD-Ia can be attained, but liver correction appears to be easier than kidney correction. This may explain why the current dietary therapies for human GSD-Ia patients, targeting the

liver's control of symptomatic hypoglycemia, result in good glucose homeostasis, but fail to prevent longer term kidney complications [18,19].

A more appropriate level of gene expression was obtained using the natural promoter/enhancer of the *G6PC* gene. Koeberl *et al.* [63] infused 2-week-old *G6pc*^{-/-} mice with 2×10^{14} vp/kg of ssAAV2/8-cGPE, an rAAV2/8 vector carrying canine G6Pase- α under the control of the canine *G6PC* promoter/enhancer at nucleotides -1372 to -11 relative to the translation initiation codon. During the 28-week study, the infused animals grew normally, exhibited normal serum triglyceride and cholesterol profiles, but only partially normalized blood glucose levels. Hepatic G6Pase- α activity was 48% of wild type activity at age 4 weeks and declined to 20% of wild type activity at age 28 weeks but this was insufficient, with the mice exhibiting subnormal fed and fasting blood glucose levels. These findings contrast to those of Ghosh *et al.* [62] who found that restoration of just 11% of wild type hepatic G6Pase- α activity could normalize blood glucose levels in the treated mice. The reason for this discrepancy is unknown, but differences in infusion strategies, AAV serotypes, and other experimental parameters could all contribute. Histological analysis of the liver similarly revealed only partial histo-morphological correction and hepatic glycogen content had risen well above normal levels at age 28 weeks. Consistent with Ghosh *et al.* [62], rAAV2/8-mediated gene transfer resulted in little or no renal G6Pase- α expression.

The most promising results come from GSD-Ia studies using rAAV vectors containing the human *G6PC* promoter/enhancer [64,65]. Koeberl *et al.* [64] infused *G6pc*^{-/-} mice with scAAV2/8-hmGPE, an scAAV2/8 vector expressing human G6Pase- α under the control of the human *G6PC* promoter/enhancer extending from the translation initiation codon to nucleotide -382. Two-week-old *G6pc*^{-/-} mice were infused with one of four different doses: 1×10^{13} , 1×10^{12} , 3×10^{11} , and 1×10^{11} vp/kg. All 4 doses increased the survival and corrected metabolic abnormalities. The best outcome was observed at the highest dose, which restored hepatic G6Pase- α activity to wild type levels when estimated by quantitative enzyme phosphohydrolase assays [64]. However, histochemical detection of G6Pase activity on the same liver tissues contradicted this finding, showing only a generalized low-level expression throughout the hepatocytes, with foci containing significantly higher levels of enzymatic activity, compared to wild type mouse livers. The reason for the discrepancy between the phosphohydrolase assay and histochemical staining is unclear. However, at age 26 weeks, levels of blood glucose and fasting blood glucose were lower in the treated mice than wild type littermates, and there was an elevation in hepatic glycogen content accompanied by mild hepatomegaly [64].

While ssAAV2/1-CBA [62], ssAAV2/8-cGPE [63], and scAAV2/8-hmGPE [64] delivered the G6Pase- α transgene to the liver and achieved extended metabolic correction of GSD-Ia symptoms, none completely normalized hepatic G6Pase- α deficiency. Sequence analysis of nucleotides -382 to -2864 of the human *G6PC* coding region identified multiple transcription factor binding sites that could be important gene control elements. Since a construct expressing human G6Pase- α under the control of the human *G6PC* promoter/enhancer at nucleotides -2864 to -1 exceeded the packaging capacity of a scAAV [48], Yiu *et al.* [65] constructed it in ssAAV (ssAAV2/8-hGPE). In addition to expression efficiency, Yiu *et al.* [65] examined the impact of age at the time of infusion, reasoning that the efficiency and persistence of gene transfer might be influenced by the rate of hepatocellular proliferation [97], which decreases with age. The *G6pc*^{-/-} mice were infused with a single fixed dose (1.2×10^{11} vp/mouse) of ssAAV2/8-hGPE at one of three ages (2 days, 2 weeks, and 4 weeks) and hepatic G6Pase- α expression assessed out to 24 weeks of age. Since the weight of the mice increased with age, this also resulted in decreased viral doses for the older mice, namely 6×10^{13} vp/kg in 2-day-old mice, 1.5×10^{13} vp/kg in 2-week-old, and 1.2×10^{13} vp/kg in 4-week-old.

In mice infused at age 2 days [65], hepatic G6Pase- α activity was 78% of control activity at age 2 weeks, declining 4.8-fold by age 4-weeks and another 2.5-fold by age 6 weeks where it leveled out for a further 18 weeks at 6.5% of wild type activity (Table 1), an overall 12-fold decline in G6Pase- α expression (Table 1). In mice infused at age 2 weeks, when hepatic cellular proliferation is declining, there was an initial 2.6-fold decline in hepatic G6Pase- α activity between ages 4 to 6 weeks (Table 1) which then stabilized at wild type levels out to 24 weeks, when estimated by both quantitative phosphohydrolase and enzyme histochemical analyses. Finally, in mice infused at age 4 weeks, when hepatic cellular proliferation has decreased dramatically, hepatic G6Pase- α activity was restored to a level 1.9-fold higher than the control littermates (Table 1). These findings are consistent with previous gene transfer studies [97] that show high rates of hepatic cellular proliferation are associated with loss of persistence of transgene expression and efficacy. However, other age-related factors at the time of infusion may also be relevant. Irrespective of the mechanism, the study indicates that infusion of older mice is more effective for persistent transgene expression in the liver for GSD-Ia gene therapy.

The mice infused with ssAAV2/8-hGPE at age 2 or 4 weeks, had hepatic G6Pase- α activity greater than or equal to wild type levels, exhibited normal levels of blood glucose, normal profiles of blood metabolites, normal levels of hepatic glycogen, and normal levels of hepatic fat [65]. Moreover they maintained normoglycemia over 6 hours of fasting. The total normalization of hepatic G6Pase- α deficiency indicates that *in vivo* glucose homeostasis can be adequately maintained by ssAAV2/8-hGPE-mediated gene therapy. Despite the differences in the vectors used by Koeberl *et al.* (scAAV) [64] and Yiu *et al.* [65] (ssAAV), both studies infused 2-week-old G6pc^{-/-} mice from the same source, with an rAAV2/8 vector at equivalent dosages ($1-1.5 \times 10^{13}$ vp/kg). Therefore, it is reasonable to use these studies to compare the efficacy of the minimal human *G6PC* promoter (nucleotides -382 to -1) [64] to the extended promoter (nucleotides -2864 to -1) [65]. Clearly some, if not all, of the additional regulatory elements in the extended promoter sequence improve efficacy and are critical for optimal *G6PC* expression *in vivo* in rAAV-mediated gene transfer for GSD-Ia.

This conclusion is further supported by comparing the efficacy of hepatic G6Pase- α transgene expression in G6pc^{-/-} mice mediated by either ssAAV2/8-hGPE or ssAAV2/8-CBA [65]. Both vectors efficiently deliver the transgene to the liver but the persistence of transgene expression directed by the two promoters differed markedly. While hepatic G6Pase- α activity in ssAAV2/8-CBA-infused G6pc^{-/-} mice underwent a 95-fold decline from age 2 weeks to adulthood, the activity in ssAAV2/8-hGPE-infused G6pc^{-/-} mice underwent only a 12-fold decline (Table 1). There was no evidence of antibodies directed against the G6Pase- α transgene in the infused GSD-Ia mice in either case [65]. However, an elevated hepatic CD8+ lymphocyte infiltration was only observed in mice infused with ssAAV2/8-CBA [65]. Notably the rise of lymphocyte infiltration correlated with the rapid decline in transgene expression. Whether a cell-mediated immune response is the sole or primary mechanism of inactivation remains to be determined, but the study provides another reason for favoring the native *G6PC* promoter/enhancer over ubiquitous regulatory elements.

4.2 Gene therapy in canine GSD-Ia

The GSD-Ia dog is a useful intermediary preclinical model. Carrier GSD-Ia dogs do not manifest hypoglycemia or other complications of GSD-Ia and were used as controls. In the first canine study Beaty *et al.* [98] investigated the effects of a neonatal infusion of ssAAV2-AB, an AAV2 vector carrying canine G6Pase- α under the control of an albumin promoter/enhancer. Three animals each received 3 different vector doses, at 1.6, 7.0, and 14×10^{11} vp/dog, that resulted in the restoration of 9%, 4.5%, and 9% of normal hepatic G6Pase- α

activity, respectively. Two of the dogs died within 39 days. Only the medium dose recipient, which expressed the lowest level of G6Pase- α activity, survived to 86 days. This dog exhibited improved liver histology along with improved serum glucose, cholesterol, triglyceride, and lactate levels. While a promising observation, the biochemical profiles are only representative of one animal, and there is no statistical support to conclude if this therapy could reliably correct canine GSD-Ia.

In a second study, gene therapy attained extended survival of the GSD-Ia dog. The trial consisted of three GSD-Ia dogs who were each infused at age 3 days with 10^{13} vp/kg of scAAV2/8-hmGPE [64]. All dogs became independent of dietary glucose therapy and survived to at least age 11 months. The infused dogs exhibited levels of hepatic G6Pase- α activity similar to those of the carrier dogs. Their fasting levels of blood glucose and lactate were similar to those of their unaffected, carrier littermates, and there were marked reductions in both hepatic glycogen and lipid contents compared to untreated GSD-Ia dogs. Another promising sign was the absence of any apparent somatic or cell-mediated immune response to the therapy.

A third study also attained extended survival of the GSD-Ia dog [89]. This protocol, performed on a single animal, used two serial infusions of two different rAAV vectors carrying the same transgene. The therapy consisted of 5×10^{13} vp/kg of ssAAV2/8-CBA at age 1 day, at which time dietary therapy was discontinued, followed by infusion of 1×10^{12} vp/kg of ssAAV2/1-CBA at age 5 months. The first infusion led to a transient metabolic correction but at 2 months post-treatment, the dog was no longer able to sustain normal blood glucose levels after 1 hour of fasting, and dietary glucose therapy was resumed. The transient relief of metabolic abnormalities from the first newborn infusion could result from the loss of persistence of transgene gene expression associated with the high rates of hepatic cellular proliferation [97] as well as the ssAAV2/8-CBA-elicited CD8⁺ lymphocyte infiltration similar to that observed with the mouse ssAAV2/8-CBA study [65] reviewed above. Following the second infusion at age 5 months, the dog was supported by dietary glucose supplementation for an additional month, before the dietary supplement was discontinued. The dog then continued to thrive with minimal metabolic abnormalities to age 23 months. When examined two months after the second infusion, blood glucose and lactate levels were normal, even following a 4 hour fasting. By age 23 months, hepatic G6Pase- α activity was 6.3% of wild type levels. In addition to improved glucose homeostasis, the infused dog also exhibited a more mild abnormality in liver histology and reduced hepatic glycogen storage, compared to young untreated GSD-Ia dogs.

Given these promising canine results it would be interesting to see if the ssAAV2/8-hGPE vector, that achieves total normalization of hepatic G6Pase- α deficiency in murine GSD-Ia [65], would be equally effective in the canine model. Clearly more study animals would also be needed to strengthen the findings.

5. Gene therapy for GSD-Ib

GSD-Ib represents 20% of the human GSD-I cases [1,2] and is less studied than GSD-Ia. In the only rAAV-mediated GSD-Ib gene therapy reported to date, Yiu *et al.* [66] undertook a 72-week study using ssAAV2/8-CBA-G6PT. The G6pt^{-/-} mice were infused at age 2 days with 6×10^{13} vp/kg and the dietary therapy was terminated immediately. The transgene was successfully delivered to both the liver and bone marrow (BM). Hepatic G6PT activity was 50% of wild-type levels at 2 weeks post-infusion but declined rapidly to reach 3% of wild-type levels at age 6 weeks, a level it then maintained out to 72 weeks. Despite the low hepatic G6PT activity, the infused animals were able to maintain normoglycemia throughout the 72-week study although their blood glucose levels were consistently lower than wild

type littermates. The serum metabolite profiles were also normalized, and the mice exhibited near normal growth with moderately reduced hepatomegaly. However, myeloid correction was transient. The loss in myeloid correction correlated with the rapid decline in G6PT expression in BM cells. All five treated GSD-Ib mice that lived over 50 weeks exhibited excessive accumulation of glycogen in their hepatocytes, marked hepatic steatosis, neutrophil infiltration, fibrosis, and hepatocellular injury reminiscent of non-alcoholic steatohepatitis [66]. Two mice developed multiple HCAs with one undergoing malignant transformation. While a promising start, challenges clearly remain. Using a native *G6PT* promoter/enhancer and infusing at a later age would be the most obvious next steps to improving the metabolic response, based on the GSD-Ia studies.

Correction of neutropenia and neutrophil dysfunction are critical aspects for GSD-Ib gene therapy that differentiate it from GSD-Ia. Neutrophils are terminally differentiated, short-lived, leukocytes that are replenished from the multi-potent hematopoietic stem cells (HSCs) in the BM [99,100]. Modification of neutrophil responses will depend on efficient gene transfer into the BM. Recent studies have demonstrated that a sustained transgene expression in HSCs can be obtained using scAAV vectors in conjunction with an HSC-specific promoter/enhancer [101,102]. These modifications are worthy of further consideration for GSD-Ib gene therapy. The question remains, however, whether a single vector will be able to balance the needs of hepatic and renal correction alongside myeloid correction or whether a multi-pronged approach may be necessary.

6. Expert opinion

GSD-Ia and GSD-Ib patients exhibit a deficiency in G6Pase- α and G6PT, respectively. In the liver these two proteins associate to form a functional G6Pase- α /G6PT complex [1,2] that catalyzes endogenous glucose production critical for interprandial glucose homeostasis and normal blood metabolite profiles. In the liver and kidney, dysfunction of these proteins also leads to excess glycogen storage and subsequent chronic organ dysfunction. Current dietary therapies can alleviate hypoglycemia but do not address chronic complications. Since both proteins are embedded in the ER membrane, gene therapy may offer the most effective long term therapy. Liver transplantation has shown promising outcomes in clinical studies [103–106], guiding initial efforts with rAAV-mediated gene therapy towards hepatic targeting. Through the use of mouse models, the most promising approach to GSD-Ia gene therapy has been with the use of an ssAAV2/8 containing the G6Pase- α coding sequence along with ~ 3 kb of the *G6PC* gene 5' flanking region [65]. This strategy results in a complete normalization of blood glucose levels, blood metabolite profiles, hepatic glycogen storage, and hepatic fat levels, in the absence of any apparent immune response. Given the difference in immune reactivity seen between small and large animal models [73–77], it is important to replicate these results in the canine GSD-Ia model. HCAs are a severe long-term complication of GSD-I [1,2]. The untreated G6pc^{-/-} mice and dog rarely survive to age 3 weeks, so it is not clear if HCAs develop in these models. Despite the study by Donsante *et al.* [85] showing an increased incidence of hepatocellular carcinoma in rAAV-infused mice, none of the GSD-I gene therapy studies that improved the metabolic defects have been designed to assess the development of HCA. This is a critical aspect to address in future studies.

Correction of renal disease in GSD-I has been less extensively studied. The available data suggest that rAAV2/9 may be the preferred choice for future renal gene delivery [67,68]. The current systemic delivery of transgene mediated either by rAAV2 [93] and rAAV2/1 [62] has resulted in uniform transgene expression across the kidney. However systemic delivery is probably a suboptimal route. In normal kidneys, the *G6PC* gene is expressed primarily in the proximal tubular epithelial cells [11]. A successful renal tubular epithelial

cell transduction has been obtained with an ssAAV2 vector using a catheter-based renal artery delivery system [107], and this approach warrants further investigation. More study of kidney transduction and renal tissue targeting through capsid modifications are also warranted.

GSD-Ib gene therapy is in its infancy. Only partial correction of metabolic profiles has been observed with the single construct studied to date [66]. However these vectors do not address the additional complications of neutropenia and neutrophil dysfunction [1,2]. Unlike hepatic cells, HSCs continue to proliferate and differentiate *in vivo* [99,100]. Therefore, an integrating, rather than episomal, rAAV vector may be required for stable transgene expression. Both scAAV2/1 and scAAV2/7 vectors, directed by a HSC-specific promoter/enhancer, have been shown to stably integrate into the HSC-derived progenitor cell chromosomes in a murine serial BM transplantation model in the absence of overt hematological consequences [101,102]. Studies are needed to confirm the lack of insertional mutagenesis with such approaches. There is also concern that the HSC-specific promoter/enhancer elements in these vectors will not function efficiently in the liver and kidney. Therefore it is important to understand more about the mechanism promoting integration before these findings can be applied to the design of GSD-Ib gene therapy vectors. BM transplantation has shown promise in one clinical treatment of GSD-Ib myeloid deficiency [108]. In animal studies transplanting G6pt^{-/-} BM cells into G6pt^{+/+} mice, the absence of G6PT expression in the BM and resident neutrophils led to abnormal myeloid function [109]. This suggests the transplanted BM cells impact overall myeloid function. Therefore, a dual strategy of gene therapy targeted at the liver and kidney, followed, when necessary, by BM transplantation, might be a reasonable first step clinically.

Beyond Ad and AAV directed gene therapy in GSD-I there has also been one study using a lentiviral vector based on feline immunodeficiency virus (FIV) [110]. These vectors are clinically attractive because of their wide tropism, sustained gene expression, and lack of association with human disease [111]. Grinshpun *et al.* [110] infused GSD-Ia mice with two serial doses of a FIV vector expressing human G6Pase- α under a liver-specific promoter, one at birth and the second at age 1 week. The mice expressed 56% of wild type hepatic G6Pase- α activity at age 6 months and exhibited normalized blood glucose levels, reduced hepatic glycogen storage, and improved survival. However, there was significant hepatomegaly, and kidney function was not normalized. The full potential of these vectors for GSD-I gene therapy remains to be determined.

There have been significant advances in rAAV gene therapy for both GSD-Ia and GSD-Ib. Preliminary outcomes are promising for significant improvement, if not complete resolution, of these disorders, but significant challenges remain before this approach is ready for clinical trials.

Acknowledgments

Declaration of interest

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Figure 1.

The G6Pase-α/G6PT complex maintains interprandial glucose homeostasis. G6Pase-α and G6PT are shown anchored in the membrane of the ER in contact with both the cytoplasm and ER lumen. The catalytic site of G6Pase-α lies in the ER lumen and the amino acids participating in G6Pase-α catalysis are highlighted. The spatial representations are illustrative only, for the complexes to be active, G6Pase-α must couple functionally with G6PT.

Figure 2.
GSD-Ia and GSD-Ib manifest overlapping, yet distinct, phenotypes.

Table 1

Relative hepatic G6Pase- α activity in G6pc^{-/-} mice infused with either ssAAV2/8-hGPE or ssAAV2/8-CBA at ages 2 days, 2 weeks, or 4 weeks [65]. Dietary therapy was immediately halted following infusion. Hepatic G6Pase- α activity was measured at the indicated ages using a quantitative phosphohydrolase activity assay [65] and expressed as percent of wild type activity.

Mice genotype Treatment	Age (weeks)	G6Pase- α activity (% wild type)	
		ssAAV2/8-hGPE 1.2×10^{11} vp/mouse	ssAAV2/8-CBA 4.8×10^{11} vp/mouse
Wild type	2–24	100	100
G6pc ^{-/-} Infusion at age 2 days	2	77.6	214.7
	4	16.2	11.6
	6–24	6.5	ND*
G6pc ^{-/-} Infusion at age 2 weeks	4	243.3	133
	6	87.6	31.3
	24	97.7	21.4
G6pc ^{-/-} Infusion at age 4 weeks	24	188.4	ND

* ND, not determined.