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Gene Therapy for Type I Glycogen Storage Diseases

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Abstract

The type I glycogen storage diseases (GSD-I) are a group of related diseases caused by a deficiency in the glucose-6-phosphatase-α (G6Pase-α) system, a key enzyme complex that is essential for the maintenance of blood glucose homeostasis between meals. The complex consists of a glucose-6-phosphate transporter (G6PT) that translocates glucose-6-phosphate from the cytoplasm into the lumen of the endoplasmic reticulum, and a G6Pase-α catalytic unit that hydrolyses the glucose-6-phosphate into glucose and phosphate. A deficiency in G6Pase-α causes GSD type Ia (GSD-Ia) and a deficiency in G6PT causes GSD type Ib (GSD-Ib). Both GSD-Ia and GSD-Ib patients manifest a disturbed glucose homeostasis, while GSD-Ib patients also suffer symptoms of neutropenia and myeloid dysfunctions. G6Pase-α and G6PT are both hydrophobic endoplasmic reticulum-associated transmembrane proteins that can not expressed in soluble active forms. Therefore protein replacement therapy of GSD-I is not an option. Animal models of GSD-Ia and GSD-Ib that mimic the human disorders are available. Both adenovirus- and adeno-associated virus (AAV)-mediated gene therapies have been evaluated for GSD-Ia in these model systems. While adenoviral therapy produces only short term corrections and only impacts liver expression of the gene, AAV-mediated therapy delivers the transgene to both the liver and kidney, achieving longer term correction of the GSD-Ia disorder, although there are substantial differences in efficacy depending on the AAV serotype used. Gene therapy for GSD-Ib in the animal model is still in its infancy, although an adenoviral construct has improved the metabolic profile and myeloid function. Taken together further refinements in gene therapy may hold long term benefits for the treatment of type I GSD disorders.

Introduction

The type I glycogen storage diseases, GSD-I, are a group of autosomal recessive disorders caused by a deficiency in the glucose-6-phosphatase-α (G6Pase-α) complex (Fig. **1**) [Chen 2001;Chou *et al*., 2002]. Currently, two enzyme activities, embedded in the endoplasmic reticulum (ER) membrane are implicated in GSD-I. One activity is a glucose-6-phosphate transporter (G6PT) that translocates glucose-6-phosphate (G6P) from the cytoplasm into the lumen of the ER. The second activity is a glucose-6-phosphatase catalytic unit (G6Pase, also known as G6PC) that hydrolyses G6P into glucose and phosphate [Chen 2001;Chou *et al*., 2002]. Early literature only recognized a single G6Pase activity. More recently a second activity was identified [Guionie *et al*., 2003;Shieh *et al*., 2003;Ghosh *et al*., 2004]. The original G6Pase is now more specifically identified as G6Pase-α, while the more recent activity is called G6Pase-β [Shieh *et al*., 2003;Ghosh *et al*., 2004]. Blood glucose homeostasis between meals is maintained by the G6Pase-α-G6PT complex. A deficiency of G6Pase-α causes GSD type Ia (GSD-Ia, MIM232200) and a deficiency in G6PT causes GSD type Ib (GSD-Ib, MIM232220).

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The expression of G6Pase-α is tissue restricted, the predominant sites of expression being the liver, kidney, and intestine [Nordlie and Sukalski 1985;Pan *et al*., 1998b]. In contrast, G6PT is expressed ubiquitously [Lin *et al*., 1998]. Both GSD-Ia and GSD-Ib patients fail to hydrolyze G6P to glucose and manifest a phenotype of a disturbed glucose homeostasis. The hallmark of all GSD-I is hypoglycemia following a short fast [Chen 2001;Chou *et al*., 2002]. This loss of glucose homeostasis leads to the accumulation of elevated levels of G6P in the cytoplasm. This in turn stimulates the alternative metabolic pathways involving G6P (Fig. **2**) which lead to the hypertriglyceridemia, hypercholesterolemia, hyperuricemia, and lactic acidemia that also characterize the clinical pathophysiology of all GSD-I. The elevated levels of G6P also lead to excessive accumulation of glycogen in the liver and kidney, which promotes progressive hepatomegaly and nephromegaly. Additional accumulation of fat droplets in the liver also contributes significantly to the hepatomegaly. Unlike the GSD-Ia patients, GSD-Ib patients manifest additional symptoms of neutropenia and myeloid dysfunctions [Beaudet *et al*., 1980;Gitzelmann and Bosshard 1993;Visser *et al*., 2002;Chou and Mansfield 2003] that are not obviously related to metabolism in the gluconeogenic tissues. In examining the potential roles of G6PT outside of the gluconeogenic tissues, a recent bone marrow transplantation study established that G6PT expression in the bone marrow is required for normal neutrophil and myeloid functions [Kim *et al*., 2006].

There is no cure for GSD-Ia or GSD-Ib and both are fatal within the first two decades of life if not treated [Chen 2001; Chou *et al*., 2002]. The current treatment for both GSD-Ia and GSD-Ib is a dietary therapy augmented by various conventional drugs. Infants typically receive nocturnal nasogastric infusion of glucose to avoid hypoglycemia [Greene *et al*., 1976]. Older patients eat uncooked cornstarch, which acts as a slow release carbohydrate, to prolong the length of euglycemia between meals [Chen *et al*., 1984]. GSD-Ib patients also receive granulocyte colony stimulating factor (G-CSF) therapy to restore myeloid functions [Schroten *et al*., 1991; Roe *et al*., 1992]. While these therapies are sufficiently successful to enable patients to attain near normal growth and pubertal development, the underlying pathological process remains uncorrected and patients continue to suffer from hyperlipidemia, hyperuricemia, hypercalciuria, hypocitraturia, and lactic acidemia [Chen 2001; Chou *et al*., 2002]. As a result, long-term complications such as short stature, osteoporosis, gout, renal disease, pulmonary hypertension and hepatic adenomas, that may undergo malignant transformation, still persist in GSD-I patients and become increasingly significant in older patients. In addition to these conditions, GSD-Ib patients are at a significantly higher risk of developing inflammatory bowl disease [Roe *et al*., 1986; Visser *et al*., 2000], while G-CSF therapy also increases the risk of splenomegaly [Calderwood *et al*., 2001]. A significant practical problem with this therapeutic approach is that despite the benefits of dietary therapy, its efficacy is frequently limited due to poor compliance.

Alternative therapeutic approaches to the GSD-I disorders are required. Both G6Pase-α and G6PT are hydrophobic ER-associated transmembrane proteins [Pan *et al*., 1998a;1998c; 1999] that must not only embed within the ER membrane in the correct conformation, but also couple with each other functionally, to hydrolyze G6P to glucose [Lei *et al*., 1996;Hiraiwa *et al*., 1999]. Attempts to express the proteins in a way that would allow their isolation in an active soluble form have been unsuccessful due to their hydrophobicity, ruling out protein replacement therapy as a current treatment option. An alternative therapeutic approach is gene therapy. Animal disease models of GSD-Ia [Lei *et al*., 1996; Kishnani *et al*., 1997;2001] and GSD-Ib [Chen *et al*., 2003] that closely mimic the human disorders are available and have been used to develop somatic gene therapies and are showing promise as more efficacious treatments for GSD-I.

GSD-Ia

G6Pase-α deficiency (GSD-Ia) is the most prevalent form of GSD-I, representing over 80% of GSD-I cases [Chen 2001; Chou *et al*., 2002]. The disease is caused by a deficiency in the G6Pase-α catalytic unit that hydrolyses G6P into glucose and phosphate in the terminal step of gluconeogenesis and glycogenolysis (Fig. **2**). Both human [Lei *et al*., 1993] and mouse [Shelly *et al*., 1993] G6Pase-α are encoded by single copy genes, spanning 10-12 kb of chromosomal DNA, and composed of 5 exons. In the human, the gene maps to chromosome 17q21 [Lei *et al*., 1994] and in the mouse it maps to chromosome 11. In both organisms, expression is limited primarily to the liver, kidney cortex, and intestine [Nordlie and Sukalski 1985; Pan *et al*., 1998c]. Human and mouse G6Pase-α proteins are highly hydrophobic, 357 amino acid glycoproteins, sharing 88% sequence identity, which includes the conserved amino acid residues participating in catalysis and the potential glycosylation sites [reviewed in Chou and Mansfield 1999]. In human G6Pase-α Asp^{96} was shown to be the acceptor for oligosaccharide chains [Pan *et al*., 1998c].

Human G6Pase-α is anchored in the ER by 9-transmembrane helices with the amino-terminus in the ER lumen and the carboxyl-terminus in the cytoplasm [Pan *et al*., 1998a;1998c] (Fig. **1**). The amino acids comprising the catalytic center of G6Pase-α include Lys^{76}, Arg^{83}, His^{119}, Arg^{170}, and His^{176}. His^{176} in G6Pase-α is the nucleophile that covalently bound the phosphate moiety forming the phosphohistidine-G6Pase-α intermediate during catalysis [Ghosh et al., 2002]. Of these residues, all but Lys^{76} (in helix-2), are predicted to lie on the luminal side of the ER (Fig. **1**), confirming the view that the active site of G6Pase-α is not accessible from the cytoplasm. Therefore, G6P hydrolysis is dependent upon transport of the cytoplasmic G6P across the ER membrane by G6PT [Chou *et al*., 2002;Chou and Mansfield 2003]. To be functional, G6Pase-α must couple with the G6PT [Lei *et al*., 1996;Hiraiwa *et al*., 1999].

GSD-Ia patients manifest a phenotype of disturbed glucose homeostasis, characterized by growth retardation, hypoglycemia, hepatomegaly, nephromegaly, hypertriglyceridemia, hypercholesterolemia, hyperuricemia, and lactic acidemia. Long-term complications include osteoporosis, gout, renal disease, pulmonary hypertension, and hepatic adenomas that may undergo malignant transformation. Renal transplantation in GSD-Ia patients normalized kidney function but failed to correct the metabolic abnormalities [Emmett and Narins, 1978; Chen and Scheinman, 1991; Gossmann *et al*., 2001], consistent with the primary role of the liver in glucose homeostasis. Presently, in patients who fail to respond sufficiently to dietary therapy, or who exhibit multiple liver adenomas and risk of malignant transformation, orthotopic liver transplantation is advocated [reviewed in Matern *et al*., 1999; Labrune, 2002]. In patients who present with, or have an impending, renal failure, a combined liver and kidney transplantation may be considered [Matern *et al*., 1999]. Liver transplantation appears to correct metabolic abnormalities in the short term, but there is insufficient experience yet to conclude whether it will be effective in the long term, especially in preventing the development of slowly progressing renal disease [Matern *et al*., 1999; Labrune, 2002]. In addition, liver transplantation is a highly invasive procedure, requires matched donors, carries complications inherent to long-term immunosuppressive therapy, and the risk of rejection. Therefore alternative treatment strategies are required.

Gene therapy for GSD-Ia

There are mouse [Lei *et al*., 1996] and dog [Kishnani *et al*., 1997; 2001] models of GSD-Ia. Both animal models are being used to further our understanding of the biology and pathophysiology of GSD-Ia and to develop novel therapies for this disorder. The GSD-Ia (G6Pase-$\alpha^{-/-}$) mice [Lei *et al*., 1996] were generated by gene targeting and manifest all of the

symptoms of human GSD-Ia - hypoglycemia, growth retardation, hepatomegaly, nephromegaly, hyperlipidemia, and hyperuricemia - with the exception of hyperlactacidemia. The originally identified GSD-Ia dog is a Maltese breed carrying a natural G450C/M121I G6Pase-α mutation. Transient expression assays show this mutation retains only 6.6% of wild-type activity [Kishnani *et al*., 1997]. More recently, because the Maltese breed is small in size, exhibits low survival rate of newborns, and has a small litter size, a new dog model was established by crossbreeding the carrier Maltase dog with Beagles [Kishnani *et al*., 2001]. Both canine models manifest all the typical symptoms of the human disorder, including hyperlactacidemia [Kishnani *et al*., 1997; 2001], but is less readily manipulable than the mouse model. The phenotypic similarity between the GSD-Ia mice carrying a null mutation and the GSD-Ia dog carrying 6.6% normal hepatic activity indicates that to correct the GSD-Ia disorder, more than 7% of normal activity must be restored in the liver.

In 2000, Zingone *et al*. used G6Pase-$\alpha^{-/-}$ mice to evaluate the feasibility of gene replacement therapy for GSD-Ia using an adenoviral (Ad) vector carrying murine G6Pase-α (Ad-mG6Pase-α) under the control of the constitutive RSV promoter. A single intravenous infusion of 2×10^9 plaque forming unit (PFU) of Ad-mG6Pase-α into the G6Pase-$\alpha^{-/-}$ mice at 2 weeks of age restored 19% of normal hepatic G6Pase-α activity, improved survival and growth of GSD-Ia mice, and transiently corrected the metabolic abnormalities manifested by these mice. However, the benefits were short-lived because of the rapid loss of the vector-mediated gene [Benihoud *et al*., 1999; Wilson 2001].

To achieve long-term correction of the GSD-Ia disorder, more stable transgene expression is required. Recombinant adeno-associated virus (rAAV) vectors are particularly attractive in this respect. The rAAV vectors have generally proven to transduce non-dividing cells efficiently, leading to long-term expression of the introduced recombinant gene [Carter and Samulski 2000; Tenenbaum *et al*., 2003; McCarty *et al*., 2004; Flotte 2004]. In addition they do not appear to induce immune responses to the vector sequences or the transduced cell. To date, there are no known diseases associated with rAAV which could limit its use in gene therapy. The AAV genome is a 4.7 kb single strand DNA which contains only two genes *rep* and *cap* (Carter 1992), and short inverted terminal repeats (ITRs). The rep gene encodes proteins involved in replication, while the cap gene encodes the capsid proteins. The ITR sequences are 145 bases long and are the only AAV sequences absolutely required in *cis* to function as the origin of replication, for packaging into AAV particles, and for efficient integration. The rAAV vectors have been constructed by deleting the rep and cap genes and replacing them with the gene of interest, leaving the ITR as the only viral DNA sequences retained in the rAAV vectors. The rAAV-mediated gene therapies have been investigated in both the canine [Beaty *et al*., 2002] and murine [Sun *et al*., 2002; Ghosh *et al*., 2006; Koeberl *et al*., 2006] models.

In the canine study Beaty *et al*. [2002] investigated the effects of a neonatal infusion of 3 GSD-Ia dogs of Maltase-Beagle crossbreeding with an AAV serotype 2 (AAV2) vector carrying canine G6Pase-α (cG6Pase-α) under the control of an albumin promoter-enhancer, expected to provide a liver-specific expression profile. The 3 animals each received a different dosage, namely 1.6, 7.0, or 14×10^{11} particles of AAV2-cG6Pase-α per dog and they died at 39, 86 and 20 days post-infusion, respectively, showing that survival was not linearly dependent on the viral dose. This strategy resulted in hepatic expression of 10.6% of wild-type G6Pase-α activity by infusion of 7.0×10^{11} particles/dog and 14.9% of wild-type activity by infusion of both 1.6×10^{11} and 14×10^{11} particles/dog. Therefore, the activity restored in the liver did not correlate with the dosage of rAAV2 particles administered. All 3 animals were found to be hypoglycemic at death, although it remains unclear if glucose therapy was terminated following infusion. The biochemical profile of the serum was examined in all three infused animals. The fasting glucose levels were normalized for two puppies that received the lower dose of AAV2-

cG6Pase-α, which suggests 10.6% of wild type G6Pase-α activity can be sufficient to normalize fasting glucose. However, the correction of cholesterol, triglycerides, and lactate was only achieved in the animal that lived to 86 days of age and only observed after 8 weeks of age. This dog also exhibited an improved liver histology compared to untreated controls. Since this animal had the lowest level of G6Pase-α activity restored in the liver, it suggests that the normalization of the GSD-Ia phenotype is dependent on the length of survival and, again, 10.6% of wild type G6Pase-α activity might be sufficient. While a promising observation, the number of animals investigated was small and therefore, it is difficult to conclude if the therapy could reliably correct this genetic disorder.

In the first murine study [Sun *et al*., 2002], neonatal infusion of G6Pase-$\alpha^{-/-}$ mice with a rAAV2 carrying murine G6Pase-α (AAV2-mG6Pase-α) was performed using a recombinant gene driven by a constitutive chicken β-actin promoter/CMV enhancer expected to be expressed efficiently in most tissues. Immediately following the AAV2-mG6Pase-α-infusion, glucose therapy was terminated in the mice, forcing them to depend solely on the recombinant gene expression. However, AAV2-mG6Pase-α alone failed to normalize hypoglycemia or sustain the life of G6Pase-$\alpha^{-/-}$ mice because the kinetics of rAAV2-mediated transgene expression was too slow [Thomas *et al*., 2004]. To address this, a two step regime strategy was examined [Sun *et al*., 2002] consisting of the use of two different viral vectors - the AAV2-mG6Pase-α and an Ad-mG6Pase-α [Zingone et al., 2000]. In the first step, a neonatal co-infusion of both vectors, 10^9 infectious units (equivalent to 4×10^{13} vector particles/kg) of AAV2-mG6Pase-α and 4×10^7 PFU of Ad-mG6Pase was implemented. This was followed by a second infusion, at 2-weeks of age, with a higher dose (5×10^9 infectious units, equivalent to 8×10^{13} vector particles/kg) of just AAV2-mG6Pase-α. The strategy, conducted on 10 animals, completely prevented premature deaths and corrected the metabolic abnormalities of the murine GSD-Ia disorder for the full 12 months of the study [Sun *et al*., 2002]. In contrast, untreated G6Pase-$\alpha^{-/-}$ mice supported only by glucose therapy rarely survived beyond weaning [Lei *et al*., 1996]. As anticipated, the G6Pase-α transgene was expressed in both the liver and kidney, and resulted in the sustained expression of a complete, functional, G6Pase-α system. Of particular significance was the finding that the Ad/AAV2-mG6Pase-α-infused animals grew normally, with only mild glycogen accumulation in the liver and the kidney.

While this two vector, two step therapy appeared promising, this particular regime was not clinically relevant because recombinant Ad-mediated gene transfer is associated with undesirable inflammation and cellular immune responses [Benihoud *et al*., 1999; Wilson 2001]. Moreover, while AAV2 is not considered pathogenic, and has not been implicated in known human diseases [Tenenbaum *et al*., 2003; McCarty *et al*., 2004; Flotte 2004], it does have a number of practical limitations also. For instance, in addition to the delayed onset of gene expression, the vector has low transduction efficiencies [Thomas *et al*., 2004; Gao *et al*., 2005]. Moreover, there is a prevailing pre-existing immunity in the general human population to the AAV2 serotype, which limits its value as a clinical gene therapy vector [Gao *et al*., 2005]. Therefore an improved strategy is needed.

Since the original work a number of other, novel AAV serotypes have been identified and adapted for use [Chao *et al*., 2000; Gao *et al*., 2002; Rabinowitz *et al*., 2002; Grimm and Kay 2003; Choi *et al*., 2005; Gao *et al*., 2005]. These new AAV vectors have improved potency, broadened tropism, and the ability to circumvent the pre-existing immunity to AAV2. Of particular value for these vectors has been the development of a transcapsidation method that enables the packaging of rAAV2 genomes within the capsids of these different AAV species [Gao *et al*., 2002; Rabinowitz *et al*., 2002]. These advances were exploited in the second [Ghosh *et al*., 2006] and third [Koeberl *et al*., 2006] murine GSD-I studies, that used AAV serotypes shown to direct efficient hepatic gene transfer [Rabinowitz *et al*., 2002; Loiler et al., 2003; Gao *et al*., 2002; Thomas et al., 2004].

In the study of Ghosh et al. [2006] rAAV serotype 1 (rAAV1) [Rabinowitz *et al*., 2002; Loiler et al., 2003] and rAAV serotype 8 (rAAV8) [Gao *et al*., 2002; Thomas et al., 2004] were evaluated. These different AAV serotypes display distinct tissue tropism, believed to be related to the distribution of their receptors on target cells. The primary receptors for AAV1 are alpha-2,3-linked, or alpha-2,6-linked, sialic acid which also transduces AAV6 [Wu *et al*., 2006] while the primary receptor for AAV8 is the 37/67-kDa laminin receptor which also plays a role in transduction of AAV2, AAV3, and AAV9 [Akache *et al*., 2006]. So different efficiencies in transducing the liver and kidney between rAAV1 and rAAV8 might be anticipated, based on the distribution of the receptors on the surfaces of the liver and kidney. In both vectors, the murine G6Pase-α was under the control of a chicken β-actin promoter driven by a CMV enhancer [Ghosh et al., 2006]. Because the volume of the virus that can be administered to a neonatal mouse is restricted by the size of the mouse, two parallel studies were conducted. One group of mice was infused once neonatally with 5×10^{11} particles (equivalent to 2×10^{14} vector particles/kg) of AAV1-mG6Pase-α (or AAV8-mG6Pase-α), while the second group was infused with two doses - 5×10^{11} particles (equivalent to 2.5×10^{14} vector particles/kg) of AAV1-mG6Pase-α (or AAV8-mG6Pase-α) in the neonatal period and again with 1.5×10^{12} particles of AAV1-mG6Pase-α (or AAV8-mG6Pase-α) when the animals were 1-week old. Again, immediately following AAV1-mG6Pase-α or AAV8-mG6Pase-α infusion, glucose therapy was terminated in the mice, forcing them to depend solely on the recombinant gene expression.

Three G6Pase-$\alpha^{-/-}$ mice received a single infusion of AAV8-mG6Pase-α and another 3 received the double infusion. In both groups the G6Pase-α transgenes were effectively delivered to the liver and the survival of the mice was markedly improved [Ghosh *et al.*, 2006]. However, there was little or no detectable transgene expression in the kidney with either treatment group. At 6 weeks post-infusion, the doubly AAV8-mG6Pase-α-infused mice, who had received the highest recombinant gene dose, had 20.3% of normal hepatic G6Pase-α activity, but only 0.3% of normal kidney activity. Since there is strong evidence to suggest that hepatic, as well as renal, G6Pase-α gene expression is required for longer term correction of GSD-Ia [Matern et al., 1999; Sun et al., 2002], the rAAV8-mediated therapy still did not fully address the long term goals and was terminated.

The single AAV1-mG6Pase-α-mediated infusion study, conducted on 12 animals, resulted in markedly improved survival of the infused mice [Ghosh *et al*., 2006]. G6Pase-α activities in the liver and the kidney were ~4% and ~3% of normal activity, respectively, and the activities persisted for the duration of the 48 week study. However, 4 of the 7 animals designated for longer term study died prematurely at 12, 13, 15, and 30 weeks post-infusion, suggesting that these levels of hepatic and renal G6Pase-α activities are borderline for the survival of the treated G6Pase-$\alpha^{-/-}$ mice.

The double infusion study of AAV1-mG6Pase-α, conducted on 9 animals, resulted in a sustained level of expression of the G6Pase-α transgene in both the liver and kidney [Ghosh *et al*., 2006]. The AAV1-mG6Pase-α-infused animals grew normally. There were no premature deaths of the infused mice, and no anti-G6Pase-α antibodies detected, even in the doubly-infused G6Pase-$\alpha^{-/-}$ mice that were not sacrificed until the end of the full study at 57 weeks. Similarly, in the livers of the doubly infused animals there were no histological abnormalities throughout the 57-week study. Biochemical measurement of microsomes isolated from the treated mice showed sustained restoration of approximately 11% of the normal liver G6Pase-α activity at 57 weeks post-infusion. All infused mice had normal plasma glucose, cholesterol, triglyceride, and uric acid profiles, indicating that glucose homeostasis was being adequately maintained. In the kidneys, biochemical measurements showed that the restoration of approximately 7% of the normal kidney G6Pase-α activity was sustained for the duration of the study. Pathology analysis showed that the normal kidney histology was maintained over

most of the study, although increased glycogen accumulation and the development of histological abnormalities including glomerulosclerosis and tubular dilatation, indicative of kidney disease did start to emerge at 57 weeks. Despite this, the serum levels of creatinine, an index of altered renal function [Ruilope *et al*., 2001], remained within the normal range. Since G6Pase-α is primarily expressed in the kidney cortex [Nordlie and Sukalski 1985; Pan *et al*., 1998b], 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 symptomatic dietary therapies for human GSD-Ia patients targeting the control of symptomatic hypoglycemia, result in good glucose homeostasis, but fail to prevent longer term kidney complications.

In the third murine study [Koeberl *et al*., 2006], conducted on 9 G6Pase-$\alpha^{-/-}$ mice of the same strain used in the studies of Ghosh *et al*. [2006], 1×10^{12} particles (equivalent to 2×10^{14} vector particles/kg) of a rAAV8 carrying the canine G6Pase-α under the control of the canine G6Pase-α promoter were administrated to 2-week-old G6Pase-$\alpha^{-/-}$ mice. During the 28-week study, the infused animals grew normally, exhibited normal serum cholesterol and triglyceride profiles, but had only partially normalized blood glucose levels. Hepatic G6Pase-α activity declined from 42% of wild type activity at 4-weeks post-infusion to 21% of wild type activity at 28 weeks, a 50% decline in only 24 weeks. While the levels of expression obtained were higher than those for the single AAV1 infusion of mouse G6Pase-α described above, the outcome of the two studies were similar. Two of the 9 AAV8-cG6Pase-α-infused mice died prematurely. Also consistent with the study of Ghosh *et al*. [2006], AAV8-cG6Pase-α-mediated gene transfer resulted in little or no renal G6Pase-α expression [Koeberl *et al*., 2006]. Since liver transplantation corrected metabolic abnormalities seen in GSD-Ia patients, the levels of hepatic G6Pase-α activity appear to correlate, in general, with phenotypic correction. However, hepatic G6Pase-α activity remained high in both studies, 21% of normal activity at 28 weeks post AAV8-cG6Pase-α infusion [Koeberl *et al*., 2006] and 11% of normal activity at 57 weeks post AAV1-mG6Pase-α infusion [Ghosh *et al*., 2006].

The need for dual, high dose infusions of the rAAV vectors for long term survival of G6Pase-$\alpha^{-/-}$ mice may reflect, to some degree, the state of the infused vector. Extrachromosomal forms of rAAV are the primary mediators of gene expression in the liver (Nakai *et al*., 2001) and these will become diluted or lost during the extensive hepatocyte cell division occurring in the rapidly growing neonatal and young mice. This may explain the 50% decline over 24 weeks in the AAV8-cG6Pase-α-mediated hepatic G6Pase-α expression in mice infused at 2 weeks of age [Koeberl *et al*. 2006]. Such an interpretation is also consistent with the finding of persistent long-term hepatic transgene expression mediated by rAAV8 in mature mice [Conlon *et al*., 2005; Nakai *et al*., 2005] and rat [Seppen *et al*., 2006] where there is a much lower rate of hepatic cell division. But extrachromosomal expression can not explain all of the observed effects, since the AAV1-mG6Pase-α-mediated hepatic G6Pase-α expression appeared to persist throughout the duration of the 48- to 57-week study [Ghosh *et al*. 2006]. Therefore it will be important in future studies to address the other significant differences between the two studies such as the age of the mice receiving gene transfer; the earliest time point analyzed for G6Pase-α expression; the serotype used; and the expression cassettes used in the vectors. In this respect, the differences in the promoters used between the studies may be particularly significant. The canine G6Pase-α in rAAV8 is under the control of nucleotides -1272 to -11 of the 5′-flanking region of the canine G6Pase-α gene. This would seem reasonable given that transient expression assays have shown that the minimal human G6Pase-α promoter is contained within nucleotides -234 to +3 of the 5′-flanking region [Lin *et al*., 1977]. However, the sequences that direct tissue-specific G6Pase-α gene expression *in vivo* have not been characterized in the human or the canine gene and it remains possible the canine G6Pase-α

promoter used lacked DNA elements necessary for persistent expression. What was demonstrated successfully, however, was that the canine G6Pase-α protein is sufficiently conserved across species that it can couple functionally with the murine G6PT.

The high doses used in both studies [Ghosh *et al*., 2006; Koeberl *et al*., 2006] were chosen in an effort to maximize the therapeutic potential because the G6Pase-$\alpha^{-/-}$ mice suffer from frequent hypoglycemic seizures [Lei *et al*., 1996]. However, a dose-response correlation should be performed in the G6Pase-$\alpha^{-/-}$ mice to determine if a lower dose of rAAV is equally effective in correcting the GSD-Ia disorder. It has been shown that the rAAV doses used in hemophilia A mice were not predictive of vector efficiency in hemophilia A dogs [Sarkar *et al*., 2006; Jiang et al., 2006] which are a useful preclinical model of scale-up for the therapeutic outcome in humans. Therefore a careful study of rAAV-mediated gene therapy should also be conducted in the GSD-Ia dog before the current guidelines for human clinical trials are met.

AAV vectors induce little or no innate immunity and the vectors do not contain any viral open reading frames, leaving the transgene product and the virus capsid as the only source of foreign antigens [reviewed in Bessis *et al*., 2004; Zaiss and Muruve 2005]. A humoral response directed against the G6Pase-α transgene was not observed in the infused G6Pase-$\alpha^{-/-}$ mice [Sun *et al*., 2002; Ghosh *et al*., 2006] but focal lymphocytic infiltrations in the liver and kidney were present in the AAV8-cG6Pase-α-infused mice [Koeberl *et al*., 2006], suggesting an inflammatory response to the AAV8 capsid. The anti-AAV antibodies can decrease the efficiency of *in vivo* gene therapy and prevent vector re-administration. In the phase 1/2 gene therapy clinical trials for hemophilia B, it was shown that T cell-mediated immunity to AAV capsid antigen caused destruction of AAV2-transduced hepatocytes [Manno *et al*., 2006]. Strategies to overcome humoral immunity to the AAV capsids have been reviewed by Zaiss and Muruve [2005] which include AAV capid alterations, alterative serotypes, and immunosuppression during primary exposure. The AAV capsid needed to deliver the therapeutic gene is present only transiently in the transduced cells being gradually degraded and cleared. Therefore, a short-term immunomodulatory regimen that blocks the response to capsid until these sequences are completely cleared from the AAV-transduced cells may permit long-term expression of the transgene.

In summary, the sustained hepatic G6Pase-α transgene expression mediated by rAAV1 and rAAV8 as well as renal G6Pase-α transgene expression mediated by rAAV1 raise the possibility that human GSD-Ia may be amenable to AAV-G6Pase-α gene therapy, although a number of key questions remain to be investigated before clinical trials could be contemplated.

GSD-Ib

The G6PT defects leading to GSD-Ib are less common than the G6Pase-α defects and represent approximately 20% of all GSD-I cases [Chen 2001; Chou *et al*., 2002; Rake *et al*., 2002; Chou and Mansfield, 2003]. The primary function of G6PT is to translocate G6P from the cytoplasm to the lumen of the ER where it is hydrolyzed to glucose by G6Pase-α (Fig. **2**). Both human [Marcolongo *et al*., 1998; Hiraiwa *et al*., 1999; Gerin *et al*., 1999] and mouse [Chen *et al*., 2003] G6PT are encoded by single copy genes, spanning 5.5-6.5 kb of chromosomal DNA, and composed of 9 exons. In the human, the G6PT gene maps to chromosome 11q23 [Annabi *et al*., 1998] and in the mouse it maps to chromosome 9.

The human G6PT gene encodes two alternatively spliced transcripts [Marcolongo *et al*., 1998; Hiraiwa *et al*., 1999; Gerin *et al*., 1999]. The primary transcript (G6PT) encodes a 429 amino acid protein, while the variant transcript (vG6PT), which contains an additional 66-bp in exon-7, encodes a 451 amino acid protein. The G6PT transcript is expressed in all tissues examined [Lin *et al*., 1998], in contrast to the G6Pase-α gene, although tissue specific splicing may occur. For example, the vG6PT transcript is expressed primarily in the brain, heart, and

skeletal muscle [Lin *et al*., 2000]. Both G6PT variants function as transporters and can couple with G6Pase-α to form an active G6Pase-α complex [Hiraiwa *et al*., 1999; Lin *et al*., 2000]. Both G6PT (Fig. **1**) [Pan *et al*., 1999] and vG6PT [Lin *et al*., 2000] are hydrophobic, 10 transmembrane domain, ER proteins, with a similar topology. To be functional, the G6PT must embed within the ER membrane in the correct conformation not only to transport G6P, but also couple functionally to the hydrolytic enzyme G6Pase-α. Neither of the G6PT proteins can be expressed in a active soluble form, thus protein replacement therapy is not an option for GSD-Ib either.

GSD-Ib has a similar clinical course to GSD-Ia and patients manifest a disturbed glucose homeostasis characterized by fasting hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, lactic acidemia, and growth retardation. In addition, GSD-Ib patients manifest neutropenia along with myeloid dysfunctions in Ca^{2+} mobilization, respiratory burst, and chemotaxis [Beaudet et al., 1980; Gitzelmann and Bosshard 1993; Visser *et al*., 2002; Chou and Mansfield 2003]. Oral and intestinal mucosal ulcerations are common in GSD-Ib, and most patients also suffer from chronic inflammatory bowel disease [Roe *et al*., 1986; Visser *et al*., 2002]. Long-term complications include renal disease and hepatic adenomas that may undergo malignant transformation. GSD-Ib patients receiving liver transplantation have exhibited improved metabolic profiles, confirming the role of hepatic G6PT in glucose homeostasis. However, the consequence transplanted liver on the myeloid dysfunction remains controversial. In two transplant patients, neutropenia was resolved, at least for the first 3 and 4 years after transplantation [Martinez-Olmos *et al*., 2001; Adachi *et al*., 2004], but in the other three patients, neutropenia persisted [Lachaux *et al*., 1993; Matern *et al*., 1999]. The neutropenia and myeloid dysfunctions manifested by GSD-Ib patients are not obviously related to G6P metabolism in the gluconeogenic tissues of the liver, kidney and small intestine, and point to a potential role of G6PT in bone marrow. Bone marrow transplantation experiments between wild-type and G6PT-deficient mice were conducted to determine if G6PT expression in the bone marrow is linked to myeloid functions [Kim *et al*., 2006]. The study showed that neutrophils lacking G6PT have intrinsic defects in their respiratory burst, chemotaxis, and Ca^{2+} mobilization. The study established that while the metabolic dysfunctions are due to the loss of G6PT in the liver, kidney and intestine, the myeloid dysfunctions are due to the loss of G6PT expression in the bone marrows and neutrophils. Therefore, to correct all of the abnormalities associated with GSD-Ib, a functional G6PT must be expressed in multiple tissues.

Gene Therapy for GSD-Ib

The GSD-Ib disorder has only been described in humans and there are no naturally occurring animal models for the disease. A G6PT-deficient ($G6PT^{-/-}$) mouse model of GSD-Ib was generated by gene targeting [Chen *et al*., 2003]. The resulting $G6PT^{-/-}$ mice share the same phenotype as human GSD-Ib patients manifesting a disturbed glucose homeostasis as well as myeloid dysfunctions. The bone and spleen of $G6PT^{-/-}$ mice are developmentally delayed and accompanied by marked hypocellularity and elevation of myeloid progenitor cell frequencies in both organs. There is a corresponding dramatic increase in G-CSF levels in GSD-Ib mice which mirrors the findings in human GSD-Ib patients [Chen *et al*., 2003]. The mouse study also shows that in addition to transient neutropenia, a sustained defect in neutrophil trafficking may underlie the myeloid deficiency in GSD-Ib.

Of the currently available viral vectors, adenovirus [Legrand *et al*., 2002; Mizuguchi and Hayakawa 2004] and AAV [Carter and Samulski 2000; Tenenbaum *et al*., 2003; Flotte 2004; McCarty *et al*., 2004] are two of the most efficient at transducing nondividing cells and both tranduce liver efficiently. *In vitro* studies had suggested the Ad vectors might also be effective with bone marrow cells [Mitani *et al*., 1994] and embryonic stem cells [Mitani *et al*., 1995].

More recent results suggest that AAV1 might be effective with primitive murine hematopoietic stem cells [Zhong *et al*., 2006]. However, there has been no information on the ability of recombinant adenovirus or AAV to deliver a transgene to myeloid tissues *in vivo*.

Yiu *et al*. [2006] first evaluated the feasibility of gene replacement therapy for GSD-Ib by infusing an Ad vector carrying human G6PT (Ad-hG6PT) under the control of the constitutive RSV promoter into $G6PT^{-/-}$ mice. Because fetal liver is a hematopoietic organ in the first 10 postnatal days [Wolber *et al*., 2002], two parallel studies were conducted to ensure that Ad-hG6PT-mediated gene transfer delivered the transgene to gluconeogenic as well as myeloid tissues. One group of mice was infused neonatally with 5×10^7 PFU of Ad-hG6PT, the second group was infused with 3×10^8 PFU when the animals reached age 2 weeks. Similar results were obtained. Ad-hG6PT-infusion restored significant levels of G6PT mRNA expression in the liver, bone marrow, and spleen. At 1 and 2 weeks post-Ad-hG6PT infusion, microsomal G6P transport activity in the liver reached 38% and 29%, respectively of normal hepatic activities and the GSD-Ib mice receiving the recombinant virus exhibit improved growth; normalized serum profiles of glucose, cholesterol, triglyceride, uric acid and lactate; reduced hepatic glycogen storage; and improved hepatomegaly. These changes were all consistent with the reconstitution of a functional G6Pase system in the gluconeogenic tissues. In addition, the Ad-mediated gene transfer also improved neutropenia; normalized serum G-CSF levels; improved bone and spleen development; lead to increased cellularity of the bone and spleen; and normalized myeloid progenitor cell frequencies in both tissues. These findings are consistent with the reconstitution of G6PT activity in the myeloid cells, demonstrating that recombinant adenovirus can deliver a transgene to the bone marrow *in vivo*.

In contrast to the G6PT reconstitution within the liver, bone marrow, and spleen, effective adenovirus-mediated G6PT transduction of the kidney did not occur, and the typical GSD-Ib characteristics of kidney enlargement associated with abnormal glycogen deposition, continued to develop [Yiu *et al*., 2006]. Therefore, correction of the full phenotype of GSD-Ib will require co-transfection of an adenoviral vector carrying G6PT with a second G6PT carrying vector capable of transducing the kidney, or the use of a single vector with a liver, kidney, bone marrow and spleen transduction potential.

The Ad vector used by Yiu *et al*. [2006] is further limited by the short-term expression of the transgene [Benihoud *et al*., 1999; Wilson 2001] and effective gene therapy requires sustained expression of the transgene. Helper-dependent adenoviral vectors deleting most or all of the viral protein encoding sequence [Alba *et al*., 2005; Jozkowicz and Dulak 2005; Palmer and Ng 2005; Bangari and Mittal 2006] which are significantly less toxic may be more attractive in the future. The AAV vector, which has been shown to correct the GSD-Ia disorder, is also attractive. Further investigation should identify a more comprehensive gene therapy vector that will achieve long-term correction of metabolic and myeloid dysfunctions in GSD-Ib or that a transgene can be repeatedly administrated in the absence of adverse immune responses. In summary, the effective use of gene therapy to correct metabolic imbalances and myeloid dysfunctions in GSD-Ib mice holds promise for the future of gene therapy in humans.

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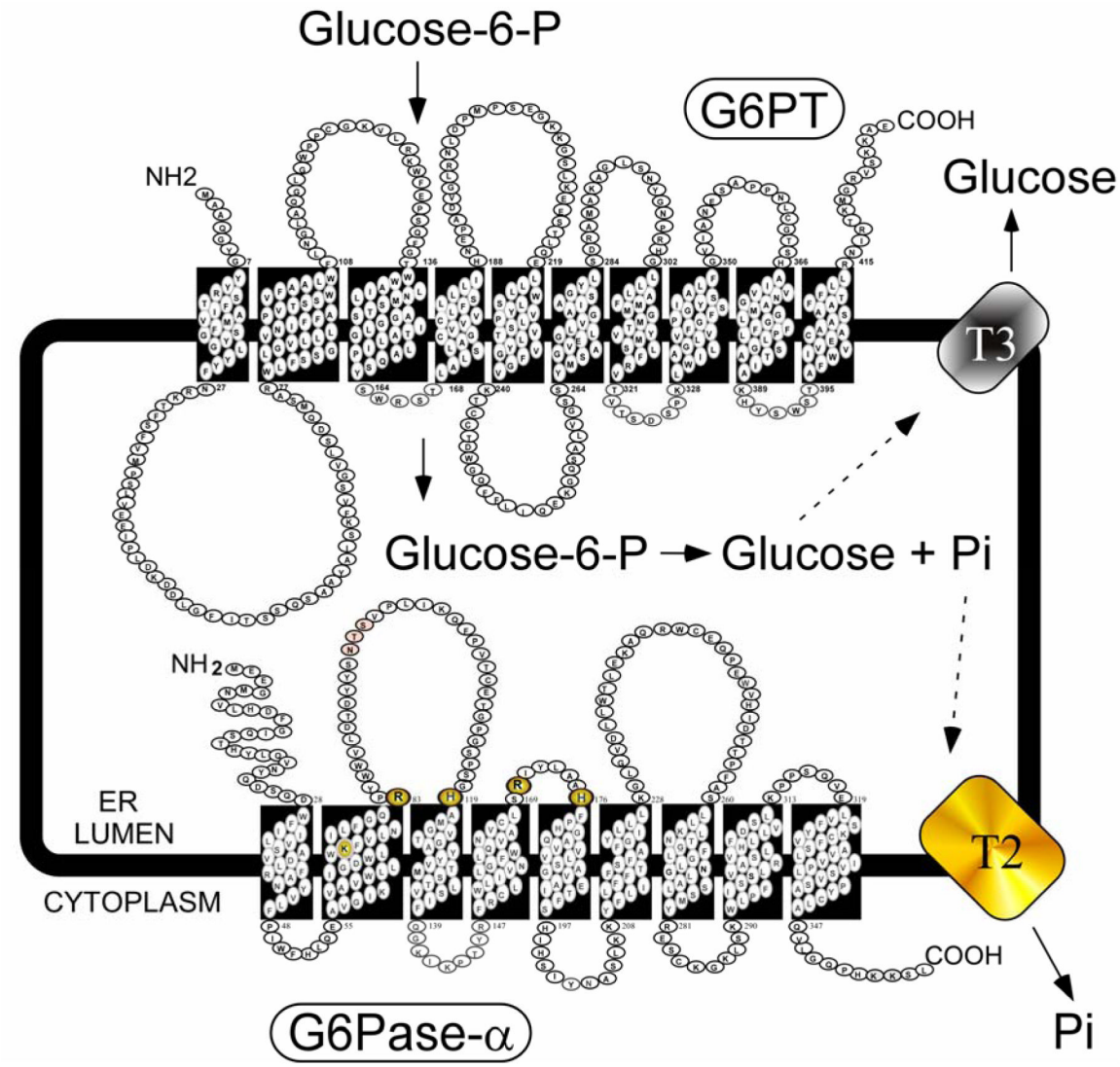

Fig. (1).

The G6Pase-α system, an enzyme complex essential for glucose homeostasis. The components, G6Pase-α, G6PT, a putative phosphate transporter (T2), and a putative glucose transporter (T3), are shown anchored in the membrane of the ER in contact with both the cytoplasm and ER lumen. The spatial representation is illustrative only; the proteins must couple functionally and probably exist as a multi-protein cluster. Amino acids participating in G6Pase-α catalysis are highlighted.

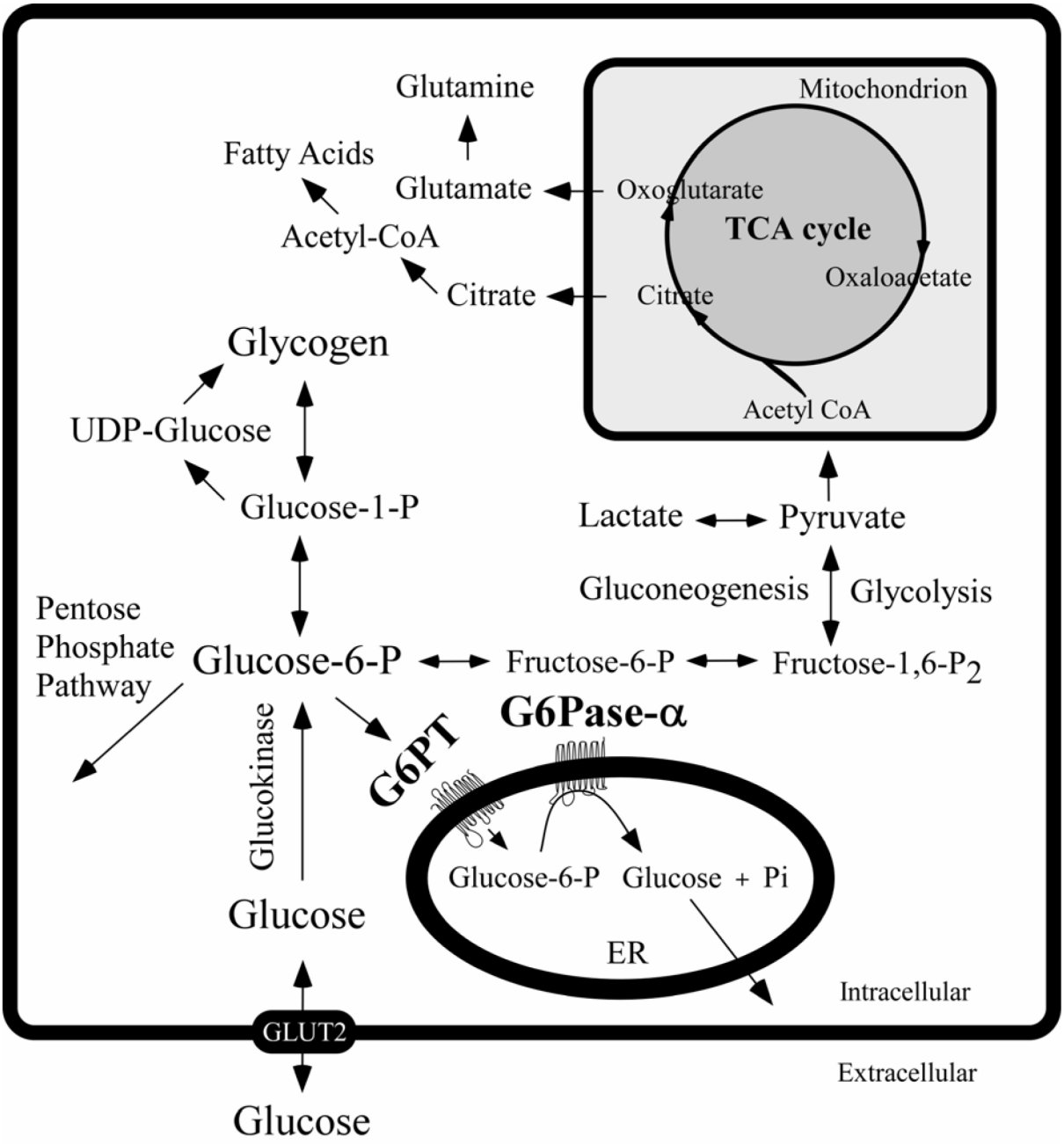

Fig. (2).
The primary anabolic and catabolic pathways of G6P in the liver. The G6Pase-α and G6PT components of the G6Pase-α system are shown embedded within the membrane of the ER. The GLUT2 transporter, responsible for the transport of glucose in and out of the cell, is shown embedded in the plasma membrane.