EXPERIMENTAL TREATMENT STRATEGIES FOR METABOLIC LIVER DISORDERS AND CHARACTERIZATION OF THE MURINE MODEL OF GLYCOGEN STORAGE DISEASE TYPE IA

By

SUSAN VICTORIA ELLOR

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To the families affected by GSD:
May a timely, safe and effective cure come of conscientious science.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

-/- or j/j	Homozygous negative
+/- or n/j	Heterozygous
2-AAF	2-acetoaminofluorene
AFP	***α***-fetoprotein
APS	Ammonium persulfate
BMDC	Bone marrow derived cell
BMSC	Bone marrow derived stem cell
bp	Base pair
CNS	Crigler Najjar Syndrome
CK	Cytokeratin
DNA	Deoxyribonucleic acid
ds	Double stranded
ECM	Extracellular matrix
FBS	Fetal bovine serum
G6Pase	Glucose-6-phosphatase
GFP	Green Fluorescent Protein
GSD	Glycogen Storage Disease
HGF	Hepatocyte Growth Factor
HOC	Hepatic oval cell
HSC	Hematopoietic stem cells
IFN	Interferon
IHC	Immunohistochemistry
IMD	Inherited Metabolic Disorder

i.p.	Intraperitoneal
IV	Intravenous
KO	Knock out
LRP	Low density lipoprotein receptor-Related Protein
NRL	Normal rat liver
nt	Nucleotide
OCT	Optimal cutting temperature
OLT	Orthotopic liver transplant
O/N	Overnight
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHx	Partial hepatectomy
RBC	Red Blood Cell
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Room temperature
rtPCR	Reverse transcription PCR
RT-PCR	Real Time PCR
ss	Single stranded
TEMED	Tetramethylethylenediamine
Tx	transplant
UGT	Uridine diphospho glucuronyl transferase
WT	Wild type

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Susan Victoria Ellor

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Metabolic disorders of the liver manifest in a large number of ways, and have an equally wide spread of impact on an afflicted patients life. Many different avenues for treatment are under investigation, including direct administration of the deficient protein or cofactor, replacement of the aberrant gene by way of viral vectors, and even stem cell repopulation of the target organ. The highly regenerative nature of the liver provides more benefit than hindrance for investigations on potential curative therapies. Still, this deceptively complex organ is merely a part of an extremely intricate whole organism; thus the organism, the disease, and the proposed treatment must all be understood completely before there is hope for releasing a safe and effective cure.

Of the many liver metabolic disorders in existence, those most conducive to novel curative strategies tend to be of simple, well-characterized monogenic origin. The animal models of two such disorders were evaluated for their basic potential to benefit from combination *ex vivo* engineered stem cell therapy. The hyperbilirubinemic Gunn rat model of Crigler Najjar Syndrome was initially examined for hepatic reconstitution capacity from various cell infusions. We discovered that the therapeutic index for monocrotaline, administered to potentiate infused

cell repopulation, is too small for practical use in this model. This finding is important for future investigations of the model, indicating the use of the more costly alternative, retrorsine.

The hypoglycemic mouse model of Glycogen Storage Disease type 1a (von Geirke's disease) was also investigated for potential gains with novel treatment.

CHAPTER 1
INTRODUCTION AND OVERVIEW

The Liver

Anatomy of the Liver

Structure of the hepatic organ

The liver is the largest parenchymal organ in the human body, weighing between 1.4 and 1.6 kg and accounting for approximately 2% of the total body weight of a full grown healthy adult. Based on its surface features, the human liver has 4 lobes. The left and right lobes are most apparent upon laparotomy, and from an anterior view (Figure 1-1a) right and left hemispheres are visibly tethered at the falciform ligament. A visceral view (Figure 1-1b) of the organ exposes the remaining caudate and quadrate lobes.(1) From this same visceral view, the left lobe is still visible to the left of the ligamentum venosum and ligamentum teres. The caudate and the quadrate lobes are clearly divided by the transverse fissure (or porta hepatis), and the right sagittal fossa separates these two lobes from the right lobe. Though fairly similar in appearance, the adult rat liver weighs in around 7 to 8 g, and accounts for more than double the fraction of body weight as it does in the human (approximately 5%). The rat also has an additional 5^{th} liver lobe,(2) which accounts for a portion of the increased relative mass.

The hepatic vasculature is quite conserved between the rodent and the human; in both cases the organ is fed simultaneously by a vein as well as an artery. The portal vein supplies over 60% of incoming blood to the organ. Coming from a vein, this blood is oxygen poor, but is instead rich in nutrients having passed along the intestinal epithelium. The second afferent vessel, the hepatic artery, provides the remaining 40% of the liver's blood intake.(2) Contrary to the portal vein, the hepatic artery carries blood which is oxygen-rich, but relatively low in nutrient content. Along side the blood vessels but with an opposing direction of flow, the

excretory ducts of the biliary tree allow for the transport of bile into the duodenum. The ‘portal triad’ is thus the vascular bundle consisting of the vein, hepatic artery and biliary tree.(3)

Microarchitecture

The liver’s lobes are divided into hexagonal lobules, which surround their own central vein, and are flanked by 6 portal triads. The lobule itself consists of cords of hepatocytes which radiate from the central vein toward the portal triad (Figure 1-2).(4) The liver’s microvasculature is made up of 4 sinusoids of endothelial cells which line each cord of hepatocytes. Essentially, blood enters the liver through the portal vein and hepatic artery, continues among the lobules via the portal triads, and flows through the parenchyma in direct contact with each hepatocyte as it moves along a cord and ultimately drains into the central vein.(3) As this is occurring, the bile canaliculus provides a route of excretion for and metabolites produced by the hepatocytes. The bile canaliculi empty into the canal of Hering, which is the terminal portion of the bile network within the portal triad. The liver’s architecture can be described in three general ways. The “classic” lobule (Figure 1-2)(4) is the unit most frequently referenced for histology. The lobule contains the portal triads positioned at the hexagonal points, with the hepatic cords radiating out from a unique central vein. Alternatively, blood flow from one portal triad to the surrounding central veins outlines what is designated the portal lobule. Finally, while the classic lobule is most easily identified under the microscope, the liver acinus is the true functional unit of the liver (Figure 1-3).(3)

The various cell types that comprise the organ include hepatocytes, bile ductular epithelial cells, sinusoidal and vascular endothelial cells, fat containing stellate cells, debris cleaning macrophages known as Kupffer cells and, in times of injury, liver progenitors known in rodents as hepatic oval cells.(3) Hepatocytes account for approximately 90% of liver weight and carry out the organ’s biochemical functions, including the production of bile. Hepatocytes are large

(30-40uM) polygonal cells with a high abundance of both smooth and rough endoplasmic reticulum.

The hepatocytes that lay between two particular central veins may be divided into three zones based on their position in the cord in terms of proximity to the central vein. Zone 1 includes hepatocytes surrounding the portal triad and receives the greatest concentration of nutrients; Zone 2 is composed of inter-zonal hepatocytes; and Zone 3 consists of poorly oxygenated hepatocytes nearest to the central vein (Figure 1-3) (4). Within the liver acinus, blood flows through sinusoids from Zone 1 to Zone 3 and then enters the central vein. On the contrary, the bile moves in the antiparallel direction, from Zone 3 to Zone 1.(3) Interestingly, Zone 3 hepatocytes often have an elevated DNA content, with 4 to 16 chromosome sets, rather than the standard diploid 2. Many of these cells are bi-nucleate and above average insize. To the contrary, Zone 1 hepatocytes are smaller and usually diploid with a single nucleus.(5)

The liver's macroarchitecture as seen histologically does not truly reflect the dynamic functional units of the liver. Indeed, the macro and microcirculation, including the biliary tree, are far more intricate as functional units than a two-dimensional illustration can represent. Instead, full three-dimensional tissue analysis provided by modern layered imaging techniques have only recently begun to unlock the true physiology of the hepatic lobule.(3)

Function

Biological homeostasis

Although it is a single organ, the liver performs a multitude of diverse bodily functions. For its role in homeostasis, the liver metabolizes amino acids, lipids, carbohydrates and serum proteins as nutrient rich blood trickles through the parenchyma. One of the key functions in homeostasis is the conversion of glucose into glycogen. In one respect, this prevents glucose from building up in the blood and causing damage to tissues; alternatively, the interprandial

metabolization of such glycogen reserves are critical for restoring blood glucose levels when food is not readily available. The production of the most abundant plasma protein, albumin, allows for the maintenance of the colloid osmotic pressure of the blood. The liver also produces other important plasma lipo- and glycoproteins, such as prothrombin, fibrinogen, and the nonimmune α- and β-globulins. Furthermore, the liver plays a role in amino acid metabolism through deamination and formation of excretable urea.

Storage

Most obviously, the liver is a very important storage site for much of the body's blood volume. In fact, its intricate vasculature and overall size make the liver the largest blood storage organ in the body. At any given moment, the adult human liver holds 25% of total cardiac output, which equates to roughly 1.5L of blood.

The liver is also important for the storage and conversion of several important vitamins acquired as blood filters through the parenchyma. Stellate cells contain lipid pools within which they store lipophilic vitamin A. It also has a pivotal role in vitamin D metabolism, enabling the circulating form of vitamin D (25-hydroxycholecaliferol) to be converted by the kidney to its active form. Without this modification, patients would develop rickets and impaired bone mineralization. Separately, the liver utilizes vitamin K to produce important clotting factors. Bleeding disorders often result from a decrease of corruption in the liver's use of vitamin K.

The bulk of the body's iron stores is also located within the liver. The storage and metabolization of iron is critical for maintaining iron homeostasis in the blood. Iron overload can result in hemochromatosis and severe liver damage; not enough, and a patient if vulnerable to anemic malaise.

Blood detoxification

Another critical function of the liver is detoxification of the blood, and with 1.5L coursing through the complex web of microvasculature, the organ serves as an excellent filter for eliminating the presence or overabundance of unwanted compounds. Many chemical xenobiotics are modified and/or removed by the liver's numerous enzymes, such as alcohol dehydrogenase (ADH), cytochrome-P (CYP) and isoforms of uridine diphosphoglucuronate glucuronosyl-transferase (UGT). In particular, the liver serves to convert hydrophobic drugs to a more water soluble form, which aids in their excretion by the kidneys.

Endocrine functions

The liver does not directly produce hormones, however, it does modify hormones released by other organs in order to alter their activity. Specifically, it acts on Vitamin D and thyroxin through metabolism, but acts to regulate pituitary output of growth hormone by releasing growth hormone-releasing hormone. The liver is also a predominant site for degradation of insulin and glucagon, which goes on to affect blood glucose regulation.

Exocrine functions

The liver also has the critical function of bile production. Bile is comprised primarily of conjugated bilirubin, and is important for intestinal absorption of nutrients and elimination of cholesterol. As it is produced, bile travels through the biliary tree in a direction antiparallel to the flow of blood. The hepatic bile ducts empty into the, where bile is stored and eventually drained to act as a detergent in the duodenum.

Regeneration and Repair

Under normal conditions, only 1 in every 20,000 hepatocytes is in the process of mitotic division at any one time; but hepatocyte division is the major driving force behind the unique phenomenon of liver regeneration. Liver regeneration is a common term to explain the

compensatory hyperplasia which occurs in response to mild to severe hepatic injury. Surgical resection of one or more lobes or exposure to destructive agents, such as hepato-toxins or hepatotropic viruses, are both insults that are well known to induce the compensatory response. Figure 1-4 is a replicate of a drawing by Higgins and Anderson, representing the growth of the remaining liver lobes after ⅔ partial hepatectomy.(6)

Within 15 hours of partial hepatectomy (PHx) in the rat, the cyclin D1 pathway induces hepatocytes to transition from the G0 resting phase of the cell cycle into G1. The first cells to undergo DNA syndissertation are the hepatocytes in the peri-portal regions, and proliferation gradually spreads to the hepatocytes surrounding the central vein. Unlike hepatocytes, which display a wave of DNA syndissertation from periportal to pericentral, NPCs across the lobule exhibit simultaneous DNA syndissertation. Remarkably, by the tenth day following PHx, the mass of the liver is typically restored to normal. Figure 1-5 is a graph by Michalopoulos and DeFrances, (1997)(7) which represents the temporal replication of individual hepatic cell types during the course of hepatic regeneration induced by ⅔ PHx.

Stem Cell Theory

Emergence of the Progenitor Hypothesis

Factors that govern stem cell differentiation potential are not understood completely, and so there is a widening interest in the isolation, characterization, and therapeutic potential of stem cells that reside in adult tissues for the purpose of tissue repair and regeneration.

Common Origins

While progenitor cells have been isolated in various forms throughout many of the body's tissues, one attractive hypothesis that has emerged is that of a common facultative stem cell. This omnipotent progenitor is posited to remain within the body from the time of embryogenesis, and through life maintain the potential to gain stepwise specificity and repopulate the body's tissues.

Bone marrow is fairly well accepted as a holding place for these common progenitor cells until they are recruited to damaged tissue.(8,9) Once localized to their target tissue, these stem cells receive the appropriate signals that determine their fate with regard to differentiation.(10)

Transdifferentiation *vs.* fusion

Not only is there evidence that organ-targeted stem cells are at least sometimes derived from distantly archived progenitor, but numerous reports have also demonstrated that stem cells in one organ or tissue contributing to regeneration of another organ or tissue after targeted transplantation. This phenomenon, known as 'transdifferentiation' was reported by Malouf *et al.* (2001), when stem cells from adult rat liver were transplanted into damaged cardiac muscle where they became functional myocytes *in vivo*.(11) Remarkable phenotypic plasticity has also been described for cells from adult brain, skeletal muscle and neuronal tissue.(12-14)

In 2002, Terada *et al.* and Ying *et al.* spurred a transdifferentiation debate, noting earlier reports that found such changes occurring from adult stem cell fusion with pluripotent ES cells. Both authors based their arguments on the data showing embryonic or adult stem cells fusing to create tetraploid cells.(15, 16) In 2003, two papers in Nature further described bone marrow cells fusing with hepatocytes to produce phenotypically different liver cells.(17, 18) A major concern regarding the implications of such an event was whether or not fused cells could suffer from genetic instability, and/or if these cells were more prone to cancerous transformation.

The question remains whether or not fusion was accountable for all lineage changes observed, and if cell fusion had been misinterpreted as transdifferentiation. While possible in some circumstances, fusion most likely cannot explain all cases of phenotypic change. Adult stem cells can adopt new fates *in vitro* without ES cells present, which suggests environmental influences on cell lineage. The fusion phenomenon may be driven artificially by particular experimental conditions, or it may be a natural useful process that coexists together with cell

transdifferentiation. Whatever the cause, cell fusion debate continues along with a stream of bi-directional evidence.

Stem Cells for Hepatic Reconstitution

The ⅔ partial hepatectomy model in rodents highlights the liver's exceptional restorative capacity. Indeed, the rodent models of carbon tetrachloride (CCl4) or allyl alcohol (AA) exposure demonstrate remarkable resilience to chemical hepatotoxic insult, as proliferation of mature hepatocytes leads to 'regeneration' of the necrotic region. However, when hepatocytes are functionally compromised and/or unable to divide, the liver is also known to call upon stem cells as a backup for regeneration.

Hepatic Oval (Stem) Cell

The reservoir of naturally occurring 'resident' hepatic progenitors, known as the Hepatic Oval Cell (HOC) (8, 19-21) is activated when liver regeneration is needed, but hepatocyte replication is impaired. Though they were first posited in the 1950s (22) HOCs are fairly new to the accepted stem cell repertoire, and there is much work to be done to fully understand their origins, characteristics, and control mechanisms for emergence and fate determination.

Morphology

Oval cells are small cells approximately 10um in size (23) with little cytoplasm, and a large oval-shaped nucleus. They have similarities to bile ductular cells with regards to their isoenzyme profiles, expressing certain keratin markers (e.g. CK-19), and gamma-glutamyl transpeptidase (GGT); yet HOCs may also express high levels of alpha-fetoprotein (AFP). Monoclonal antibodies, such as OV6, OC.2 and BD1, have also aided in their identification and characterization.(21)

Physiological origin

The origin of the HOC has been a subject of much ongoing debate. Some believe that oval cells exist in very small numbers in the periportal region of the liver lobule, and that they emerge from that hidden niche upon liver damage.(24, 25) In particular, histological studies have suggested that resident liver stem cells may exist as epithelial cells within the canal of Hering (16). Others extend beyond this possibility and suggest that HOCs do derive from the infamous 'common progenitor' that rests at a distance prior to recruitment.(8, 27, 28)

There is reason to suspect that HOCs originate according to such a phenomenon, as bone marrow derived stem cells (BMSCs) have repeatedly shown ability to change into liver cells when environment permits.(8, 27, 28) Moreover, after hepatic transplantation, BMSCs were shown to transiently populate the hepatic oval cell pool, and then further differentiate into mature hepatocytes. Whether BMSCs must pass obligatorily through the HOC stage is not decidedly known, but there appears to be at least two mechanisms for bone-marrow based hepatic reconstitution. In those cases where donor-derived hepatocytes are scattered throughout the hepatic parenchyma, it seems likely the cells had entered via the circulation and intercalated as hepatocytes directly into the existing liver cords. (29) However, in the cases where injury precipitates extensive ductular response, as in rodent oval cell proliferation, the stem cells appear to enter the parenchyma as oval-like cells, and subsequently expand and differentiate producing clusters of donor derived hepatocytes.(28)

Recruitment

In terms of their emergence, oval cells are seen to increase in number when hepatocyte proliferation is suppressed.(8, 28) Though the existence of a naturally occurring hepatic progenitor would seem to hold tremendous therapeutic value, their potential for use in clinical interventions is currently limited because hepatic injury is required for sequestering these cells in

transplantable numbers. These reparative cells are most often found in the portal region of the hepatic lobule in patients with compromised liver function, as in the case of alcoholic liver disease, non-alcoholic fatty liver disease, chronic viral hepatitis, and cirrhosis. In general, the more advanced the disease the greater appearance of hepatic progenitors,(23) however several key questions still remain regarding the molecular cues that initiate oval cell proliferation and lineage specific differentiation.

In the laboratory, these cells must be induced to appear in formerly healthy animals. For example, when mice are feed with 3,5-diethoxycarbonyl-1,4-dihydro-collidine (DDC) in a standard chow at a concentration of 0.1%, the chronic damaging effect of DDC to the liver results in suppression of hepatocyte proliferation and expansion of the hepatic oval cell compartment.(30) These cells are induced to appear in rats via administration of 2-acetylamino fluorine (2AAF) in dissolvable pellet form, followed by PHx 9-11 days prior to isolation.

Another current obstacle to using HOCs in the clinic is that human hepatic progenitors are not identical to the 'oval cells' found in the rodent models.(31, 32) While their similarities suggest that data from one may apply to the other, the exact similarities and differences must be thoroughly teased out in the laboratory. Initial studies of rodent HOCs showed a low engraftment rate,(9) which may be attributed to low seeding efficiency, or to the population dynamics and differentiation potential of rodent BMSCs. Such human studies have not been undertaken because there is not yet a way to practically obtain these cells for therapeutic use; prospective healthy donors do not visibly harbor these cells, and afflicted patients are already making use of whatever progenitor population they have been able to summon. Future clinical employment of resident hepatic progenitors will rely on a technique to increase the appearance

and efficacy of these cells in the afflicted patient, or an alternative means to summon these cells within a healthy donor liver in the absence of hepatic injury.

Bone Marrow Derived Stem Cells

Studies both *in vivo* and *in vitro* have repeatedly shown that Bone marrow derived stem cells (BMSCs) can become mature and functional hepatocytes in a permissive environment (1, 18). Moreover, studies of liver disease in animal models have clearly shown that BMDC transplantation may hasten the process of liver regeneration, reduce fibrosis, improve liver function and boost the overall survival rate.(17, 33, 34)

In a study by Theise *et al.* (2000), liver tissue from cross-gender bone-marrow transplant recipients contained between 4 and 43% hepatocytes and cholangiocytes of donor origin. This lent strong evidence that extrahepatic circulating stem cells, probably of bone marrow origin, could contribute to substantial hepatic reconstitution in humans.(28) Though transdifferentiation was found to replenish large numbers of hepatic parenchymal cells, engraftment is not always permanent. This transient donor cell contribution was recently observed in a young female GSD1a patient who had received autologous bone marrow and saw clear yet temporary improvement in glycemic control (personal communication with Dr. David Weinstein).

Despite decades of study on the subject of bone marrow cells and hepatic repair, the precise *in vivo* mechanisms by which this transition takes place are still under debate.(35) Nevertheless, while mechanistic studies will reveal how to manage and utilize these clever cells most efficiently, the mystery of how they accomplish what they do should not suspend their use in the current clinical setting if such use confers more benefit than risk to the sick patient.

Hematopoietic Stem Cells

Lagasse *et al.* (2000) showed that Hematopoietic Stem Cells (HSCs) also have hepatic reconstitution potential; regenerating up to 40% of the organ when provided in large numbers,

but also showing measurable reconstitution from as little as 50 sorted cells (36) however more recent review of the literature suggests that hematopoietic cells realistically contribute little to hepatocyte formation under either physiological or pathological conditions.(37)

Inherited Metabolic Disorders of the Liver

Metabolic processes linked to liver function are so integral to the body's welfare, that the corruption of a single enzymatic gene can trigger debilitating illness. This often inherited phenomenon is classically illustrated by the disorders the proposed initiative seeks to treat. A wayward nucleotide base pair within the coding region for the G6Pase leads to Glycogen Storage Disease type 1a (GSD1a). Similarly, such a base error in the UGT gene leads to Crigler Najjar Syndrome type 1 (CNS1).

Crigler Najjar Syndrome

Many infants develop jaundice shortly after birth, as an immature liver learns to cope with a plethora of metabolic duties. In the vast majority of cases, jaundiced babies respond well to a few sessions of phototherapy. During these sessions, they are placed mostly unclothed beneath a blue 'bili-light' which emits a wavelength that is able to penetrate the skin and break-down the subsurface intracapillary bilirubin into excretable form. When this systemic 'yellowing' persists however, a more serious pathology may underlie the symptom.

Infants with Crigler-Najjar syndrome type 1 (CN1) have a homozygous aberration in the gene (UGT-1A1) encoding diphosphoglucuronate glucuronosyltransferase. This enzyme is responsible for the conjugation of serum bilirubin into an excretable form. While exposure to phototherapy does transiently help to reduce their severe jaundice, the symptoms rapidly return because their bodies can not eliminate excess bilirubin.

For the first step of bilirubin metabolism, hemoglobin from red blood cells is broken down to heme and globin. Heme is converted to bilirubin, which is carried by bloodstream albumin to

the liver. There most bilirubin is attached to a glucuronide before excretion in the bile. This "conjugated" bilirubin is called direct bilirubin; Total serum bilirubin equals direct plus indirect bilirubin.(38) Normally, conjugated bilirubin is excreted into the bile and stored in the gall bladder or transferred directly to the small intestines for bacterial breakdown and elimination.

Corruption of the UGT-1A1 isoform prevents this conjugation, so bilirubin is left in the hydrophobic form with no way for the body to eliminate it. Natural albumin breakdown continues to release bilirubin into the system, where it accumulates in the blood. While total bilirubin is normally 0.3 - 1.1 mg/dL, levels near 12mg/dl indicate a palliative controlled condition in the CNS patient. In less well-managed situations, the CNS patient may face the toxic effects of hyperbilirubinemia with levels between 17 and 30mg/dL, and in times of 'crisis' (acute stress and/or illness) they may even register as high as 50mg/dl total bilirubin, at which time death is eminent.(39)

Today's treatment involves abundant exposure to specialized bright-blue lighting of a wavelength that breaks down subcutaneous bilirubin. Exposure is extended when levels are particularly elevated, as often occurs during times of stress or battle with even mild infection. In addition to invoked 'crises', the blood-levels become increasingly difficult to suppress as the skin naturally thickens with age, and despite consistent light exposure, baseline total bilirubin is known to increase at approximately 0.8 mg/dl/year.(40) Failure to control bilirubin levels potentiates kernicterous – neurotoxicity that occurs as bilirubin accumulates in nerve terminals and glial cells (29). Brain damage, and/or permanent disability are a frequent repercussion. This disorder results in hepatomegaly and fibrosis, which worsens in early adult life. In common with GSD, the only 'curative' treatment available for CNS is liver transplant, though a 10-year-old girl with CNSI was treated fairly successfully by hepatocyte transplantation.(42)

Dr. Chowdhury of Einstein College of Medicine has spearheaded CNS biomedical research. In 1990, Chowdhury identified the truncated protein which causes hyperbilirubinemia in the Gunn rat, and in 1991 the molecular basis of the rodent enzyme deficiency was described.(43, 44) Then in 1998, the Chowdhury group used retrovirus administration in Gunn rat liver with notable lasting success.(45) In collaboration with Chowdhury, Kren, B. T. *et al.* showed correction of the UGT1A1 genetic defect with site-specific nucleotide replacement using an RNA/DNA oligonucleotide designed to promote endogenous repair of genomic DNA.(46)

Glycogen Storage Disease

'Glycogen Storage Disease' includes a group of recessive inherited disorders stemming from a defect in glycogen breakdown. When the body takes in sugar, it uses what it needs to for current activities, and stores the excess in the liver and muscle as glycogen. Glycogen is comprised of a 1-4 linked glucose chain with a 1-6 linked branch point every 4 to 10 residues. (47) After a few hours of fasting, circulating post-prandial glucose is low, and the body typically converts the glycogen energy stores back into glucose. The first step in this process, glycogenolysis, results in glucose-6-phosphate, which must be further dephosphorylated by Glucose-6-phosphatase (G6Pase) to create usable glucose for metabolism. Failure to do this leads to the severe and chronic hypoglycemia characteristic of GSD1a.(47) Also known as von Gierke disease, GSD1a was first described in 1929,(48) and was characterized as a glycogen metabolism disorder later that same year.(49) Since hepatic glycogen functions as a reservoir for glucose, the inability to access these stores results in excessive glycogen accumulation and interprandial hypoglycemia (1940 Thannhauser, 1952 Cori, Cori). GSD1a arises when a gene mutation is present in the coding region of the active site for G6Pase - the key enzyme in homeostatic regulation of blood glucose. G6Pase is translocated from the cytoplasm to the

endoplasmic reticulum lumen where it catalyzes hydrolysis of G6P to glucose and phosphate. (50)

Affected individuals are often recognized within a day or so of birth. Treatment is immediately indicated, and consists of frequent feedings to maintain blood-sugar levels within target range (70-140mg/dl).(51) Neglect can potentiate hypoglycemic seizures and brain damage. Compounding glycogen stores and heightened stress-hormones from erratic glucose levels cause damage to a patient over time – even with of meticulous care. Hepatomegaly from stockpiled glycogen, hypercholesterolemia, hyperuricemia, lacticacidemia and diminished growth are among typical GSD1a pathologies. Though present at birth, the more insidious symptoms typically manifest in the early 20's, including hypertension, hepatic adenoma, proteinuria, renal stones, altered creatinine clearance, and eventually renal failure.(52) Though the mechanism remains elusive, hepatic adenomas often develop in early adulthood, and patients must be constantly monitored for this possibility.(53)

Patients with GSD1a rely upon frequent meals, and ingestion of uncooked cornstarch to provide a constant supply of carbohydrate (53, 54) to manage their disease. They must also avoid fruits, juices, corn-syrup and dairy, as the complex sugars within these foods require G6Pase for breakdown, and can worsen hyperlipidemia and hepatic glycogen accumulation. Failure to comply with treatment regimens often leads to hyperuricemia, lactic acidemia, gout, osteoporosis and worsened pathology of the liver and kidney. Moreover, improper management of the disease can rapidly lead to hypoglycemia which may be fatal (54). Even with careful management, metabolic instability poses a lingering threat to both the short and long term health of the patient.(55, 56)

Beyond palliative care of frequent feedings and a specialized diet, orthotopic liver transplant (OLT) is currently the only 'cure' for the disorder.(57, 58) This comes with the severe drawbacks of lasting immunosuppression, and likelihood of eventual donor-organ failure. While liver transplantation corrects the primary hepatic enzyme defect, the extrahepatic manifestations of GSD often complicate post-transplantation management.(59) This added risk keeps many physicians from prescribing liver transplantation, unless organ failure or malignancy makes it necessity.(54, 59)

The blaring need for a treatment that is superior to mere 'management' and eventual organ disposal has lead therapeutic investigations to a promising new level. Numerous advances in gene-bearing vectors and cell handling techniques have inched clinical trials for curative treatments into view.

Dr. Janice Chou of the NIH has been advanced much of our understanding of the molecular and mechanistic origins of the disorder. With molecular research beginning in 1993, her lab cloned and characterized murine G6Pase cDNA and identified the 5 exon gene (60) in an animal model. Shortly thereafter, mutations specific to GSD1a were identified, and her lab began pre-clinical work on gene therapies. Chou's lab has taken a predominantly AAV vector-based approach to treating GSD, and has shown promising correction of the disorder in mice.(52, 61)

General Treatment Options for Liver Inherited Metabolic Disorders (IMDs)

Orthotopic Transplantation

In the present day, orthotopic liver transplant (OLT) remains the most commonly used and most effective treatment for the majority of life-threatening liver diseases. Although OLT is highly effective, the 5 year patient survival rate is 74% (62) and the risk of rejection and/or other complications ensures that the procedure is not taken lightly. Moreover, OLT is expensive, the number of viable donors does not nearly meet the demand for transplantable organs, and the post

surgical immune suppression has severe side effects that are more poorly tolerated by patients with certain metabolic disorders.

Hepatocyte Transplantation

Transplantation of “normal” hepatocytes into the enzyme-deficient liver seems to be a logical means of approaching Inherited Metabolic Disorders (IMDs). Many different rodent models have been ‘cured’ of their enzyme deficiency by this strategy, but a very long bridge must be crossed to bring such advances in the laboratory to the human clinic. In terms of therapeutic cell transplantation, one of the largest differences between mice and men is that researchers can selectively work with an inbred strain, so heterozygous or homozygous wild-type donor cells would not induce an immune response. In human patients, transplanted cells would come either from the patient himself, be genetically corrected *ex vivo* and then returned to the same patient, or they would be from a hepatocyte donor. In the latter case, the allogenic transplantation would require ongoing immunosuppression, as without, the innate and adaptive immune response clears donor hepatocytes 7 to 10 days.(63) With adequate immune suppression however, hepatocytes can provide a means to prolong life while a patient waits for a suitable whole liver transplant. Moreover, hepatocyte transplantation does not prohibit future gene therapy or orthotopic liver transplantation, should subsequent intervention become necessary. Still, liver disease remains a pandemic problem in all of its forms, despite the availability of transplantable liver cells. History lends some explanation as to why some roadblocks remain.

In 1976, hepatocyte infusion via the portal vein resulted in the reduction of plasma bilirubin levels in the Gunn rat model of type 1 Crigler-Najjar syndrome.(64) This prompted hype for what was hoped to be the arrival of a true cure for liver disease; however it was not until 1992 that the very first hepatocyte transplantation was achieved in the clinic.(65) Hepatocytes transplanted via splenic injection to patients with liver cirrhosis were detected in the spleen up to

six months post infusion, but little could be deduced in terms of clinical implications beyond a confirmation of detectable engraftment.

Following success in the Watanabe rabbit model of heritable hyperlipidemia, the maiden clinical venture using hepatocytes to correct a metabolic disorder was done on five human patients with the disease. These patients served as self-donors, undergoing left lateral segment resection to yield hepatocytes for *ex vivo* manipulation. The hepatocytes were transduced with the LDL receptor prior to reimplantation.(66) Because these cells were derived from their eventual recipient, there was significantly less cause for concern over possible immune rejection. Though 2 years out this trial also demonstrated no safety concerns with tumor development or infection, the level of detectable transgene expression was beneath 5% at just 4 months post-transplantation.

Undoubtedly, the largest roadblock to clinical hepatocyte transplantation is the unpredictable degree of lasting and functional engraftment. Nonetheless, certain metabolic disorders may still be amenable to this therapy, as even a small percentage of hepatic reconstitution may be able to produce sufficient enzyme for at least partial mitigation. In 1998, Fox *et al.* reported marked partial amelioration of CNS1 in a ten year old girl with poor response to phototherapy. Prior to hepatocyte transplantation, the girl had developed acute kernicterous following an infection and, even after recovery, she required 10-12 hours of phototherapy to maintain plasma bilirubin in the range of 24 to 27 mg/dL. Though in a more severe clinical case such as this it may seem that a patient has little to loose, there are a number of immediate and life-threatening complications that can arise from bolus portal infusion of hepatocytes, including portal-vein thrombosis and resultant liver injury, portal hypertension, vascular hemorrhage, and pulmonary embolism resulting from passage of cells to the lungs. None of these occurred with

the 10 year old girl, and elevation of serum alanine amino-transferase (ALT) was only transient (a few days at most) following cell delivery; most likely resulting from short-term hepatic ischemia.(42) Eleven months after receiving 7.5 billion hepatocytes by portal vein infusion, the patient required just 6 to 7 hours of daily phototherapy and her total serum bilirubin remained greatly reduced at approximately 14 mg/dL; a third of which was conjugated by active UGT-1A1.(42)

Another study published seven years later repeated this same procedure on a 9 year old boy with CNS1. Again, 7.5 billion hepatocytes were infused by portal vein and the patient was placed on an immunosuppressive regime. Although nocturnal phototherapy was still prescribed, compliance with this and the immunosuppressive therapy was not adequate. The conclusion of this study was that allogenic hepatocyte transplantation alone was not sufficient to correct CN1, and that strict family compliance was likely the greatest factor hampering the outcome.(67)

Greater success was reported by an Italian group which used hepatocyte transplantation to treat a 47 year old woman with GSD1a. She too was infused with cells via the portal vein, but received only 2 billion hepatocytes (compared with 7.5 billion in the Crigler Najjar studies). At 9 months post-infusion, the patient was eating a normal diet and could maintain normoglycemia for 7 hours between meals; a remarkable improvement from the average 3-4 hour intervals.(68)

These apparent clinical victories are still somewhat in question, as the actual longevity of the transplanted hepatocytes has not been clearly determined in human patients. Rodent models have shown lifelong functional hepatocyte engraftment within hepatic chords, (42, 69), but it will take far longer than the rodent lifespan to determine if these cells enjoy such permanence for the human recipient.

Stem Cell Transplantation (Practical)

The potential and practical aspects of stem cell transplantation for liver metabolic disorders are similar in many ways to hepatocyte transplantation. Indeed, bolus injection of cells into the liver does pose the same immediate risks to health as does injection of hepatocytes, however there is the added uncertainty regarding control of their differentiation. Greater detail on stem cells and their relation to the liver is given in the above subsection "Stem Cells for Hepatic Reconstitution" and is expounded in the context of liver metabolic disorders in Chapter 2.

Gene Therapy

Viruses have evolved over time with great proficiency for infecting specific types of target cells and delivering nucleic acid such that their host may propagate new virions. In a very short time period, scientists have taken this brilliantly basic product of mother nature, and modified it to infect for purposes other than replication. To date, bridled versions of retrovirus, adenovirus, adeno-associated virus (AAV), simian virus (SV40) and others have been explored for their use as tools for various applications in gene therapy. Certainly, each viral type has its own unique set of aptitudes and limitations, which often involve such characteristics as host-genome integration vs. extra-chromosomal gene delivery, immunogenicity both systemic and localized, the native capacity to successfully transduce the targeted tissue, and the amenability to modified tropism. Indeed, as the search for the 'perfect' vector continues, there is a fair amount of interest in the creation of chimeric viral-vector systems,(70) which could combine the advantageous properties of multiple vector types while eliminating some of the detractants. Whether the vectors applied are minimally engineered, or completely revamped hybrids, the suitability of any given one most likely differs by application.

Adenoviral vectors

Adenoviral vectors are non-enveloped, linear double-stranded DNA viruses in the family *Adenoviridae*. Virions measure 90-100nm in diameter and are icosahedral in shape with surface spikes that aid in host-cell attachment (Figure 1-6).(71) After binding to the cell receptor via the viral knob domain, the αv integrin co-receptor stimulates endocytosis of the virion. Once into the cell, the adenoviral life cycle is separated into two phases. Genes activated during the early phase are responsible for expressing mainly non-structural, regulatory proteins. Genes activated during the late phase of the adenovirus life cycle are responsible for production of structural proteins that new virions will use to house their genetic material.(71)

Naturally, subtypes of adenovirus affect a large range of animal hosts as well as humans, and most commonly cause upper-respiratory and throat infections.(72) Many of the attributes that make adenovirus a good pathogen also make it a desirable gene therapy vector, such as broad infectivity amongst cell types and the ability to efficiently transduce both dividing and non-dividing cells. However, effective as it is at transduction, the vector DNA is non-integrating, (71) which compromises transgene stability long-term. Although a non-integrating vector has the benefit of carrying a much lower risk of oncogene activation, the drawback is that the expression of the transgene becomes diluted in a replicating cell population. This would ordinarily call for repeated administrations of the vector, but the adenoviral vectors elicit a strong immune response, often with inflammation of the tissue expressing the transgene.(52) This immunogenic response can lead to rapid clearance of infected cells and pose an immediate risk to the patient. In particular, systemic administration of these vectors, such as by intravascular (IV) injection may elicit a toxic and potentially lethal reaction.

Upon administration to research mice, blood-pressure rapidly drops as Kupffer cells are activated, and further on destroyed.(73) Interestingly, even systemic infusions of the virus are known to deliver the transgene primarily to the liver.(74) Rapid hepatic recruitment of CD11b-positive leukocytes occurs following the hyperactivation of inflammatory cytokine and chemokine genes in the liver, such as interferon-inducible protein-10 (IP-10), macrophage inflammatory protein-2 (MIF-2), interleukin-6 (IL-6) and tumor necrosis factor-$\boldsymbol{\alpha}$ (TNF$\boldsymbol{\alpha}$).(73) While the modified gutless 'helper-dependent' adenoviruses do not induce T lymphocyte-mediated cytotoxicity, the capsid still provokes the acute toxicity described above; unfortunately the capsid is also crucial for delivery of transgene.

Two separate studies from the laboratory of Janice Chou used 2nd generation adenoviral vectors to successfully ameliorate the symptoms of G6Pase-alpha deficiency in the mouse model of GSD1a. Despite its initial efficacy, the adeno-delivered transgene activity was only temporary, reaching 19% of wild-type G6Pase-/- levels 7 days post-infusion, but falling to under 1% by 70 days out.(74) This directly demonstrated the immune response that has since been more thoroughly detailed.(73) Two years later, this same group published another investigation for which they co-infused AAV-mG6Pase and Ad-mG6Pase to neonatal mice, followed by a second infusion of AAV-mG6Pase at 2 weeks of age.(52) This therapy reportedly provided G6Pase-activity to both the liver and the kidney (also affected) for the duration of the 12 month study. Since rAAV-mediated transgene does not reach full expression until 5–7 weeks post-infusion, the adenoviral vector provided a rapid transgene expression to neonatal mice. There was no sensitization observed against the second AAV infusion, nor towards the transgene product.

Unfortunately, strong immunogenicity hampers the opportunity for continued infusions of adenoviral vector, as allowing a second exposure could elicit a host-response magnitudes more aggressive than what was initially evoked. Some more recent studies have sought to protect vectors from a preexisting immune response by reducing protein–protein interactions, which may reduce vector uptake by Kupffer cells.(75) For now however, the use of adenoviral vectors is limited and less appealing with the availability of more amenable alternatives.

Adeno associated viral vectors (AAV)

AAV vectors are small (~ 25nm), non-enveloped single-stranded DNA viruses from the parvovirus family. They are icosohedral virions with a pore at the center of the 5-fold symmetry and protruding spires surrounding the 3 fold symmetry (Figure 1-7).(76) The virion DNA of the recombinant virus (rAAV) is very simple, and has just two open reading frames, Rep and Cap. The Rep reading frame consists of four genes encoding proteins required for the viral life cycle; Cap contains the genes encoding the capsid proteins: VP1, VP2 and VP3.(76)

While wildtype AAV integrates at a very specific locus in human chromosome 19, the recombinant AAV vectors lack the Rep protein needed for integration. Because of this, AAV vectors transfer their genome to the host nucleus where it is instead maintained as an episome. In place of the usual rep and cap genes, the vector is engineered to contain the appropriate promoter and gene-of-interest between the inverted terminal repeats (ITR).(76)

While the genetic code enhoused by rAAV vectors is merely half that of the wild-type counterpart, the transduction efficiency is much greater because these recombinant virions already contain dsDNA and thus are not delayed by the rate limiting step of DNA duplication. (77)

AAV also infects many cell types, both dividing and non-dividing. While many studies have explored it’s potential for treating muscle and eye disease, AAV has also shown promise

for transduction of a very broad range of tissues, as well as correction of liver IMDs. In particular, AAV vectors have shown promising results through multiple trials seeking to restore G6Pase-activity to the mouse and dog model of GSD1a.(61, 77) In terms of its clinical appeal, AAV has the significant benefit of low immunogenicity; in fact, most people harbor some form of AAV without any symptoms. The major drawback to this vector is that it has a limited DNA carrying capacity (70) so only smaller gene cassettes can be transferred. Additionally, because the DNA transferred by the recombinant virions does not become integrated into the host genome, transgene expression is not always stable over time in the presence of cell turnover; although this effect seems to correlate with vector dosing and serotype.(77)

Simian Virus 40 (SV40) vectors

SV40 is also an attractive, though lesser known candidate for gene delivery. The virus is a member of the family polyomaviridae. Its genome exists as a single molecule of supercoiled, circular double-stranded DNA. The viral genome encodes viral protein 1 (VP1) which is a central structural component of the capsid. It is not uncommon for fragments of host genes to also be contained within the capsid. SV40 virions are enclosed in a capsid of about 49 nm diameter, but are non-enveloped, and have icosahedral (T = 7) symmetry. Surface projections are small, and the exterior of the virus appears rough (Figure 1-8).(78)

Early associations with tumorigenicity of the native virus stalled its therapeutic development, but researchers are beginning to realize its potential in adapted form. Assets of SV40 vectors include high titer replication, broad spectrum infectivity (dividing and resting), stable integration into cellular DNA, and minimal immunogenicity. Uniquely, these vectors are also able to bypass the cells' antigen processing apparatus, and they seem to be able to activate expression of their own capsid genes in trans.(79) Dr. David Strayer of Thomas Jefferson University, PA has been forefront in advancing SV40's potential therapeutic potential. Beyond

likely applications in *ex vivo* transduction of missing gene, Dr. Strayer is researching its value as an tool against HIV infection.(80)

Lentiviral vectors

Lentiviral vectors are enveloped, single-stranded RNA viruses in the family retroviridae. Virions measure 80-100 nm in diameter and have small surface projections covering the envelope (Figure 1-9).(81) Natively, lentiviruses have 4 main genes coding for virion proteins gag, pro, pol, and env. There are additional genes responsible for replicative functions for the syndissertation and processing of viral RNA, although these genes vary somewhat depending upon the viral subtype (e.g., HIV-1 with vif, vpr, vpu, tat, rev, nef).

The attributes which make it a desirable gene therapy vector include high-titer replication, high transduction efficiency, large transgene capacity, stable expression of transgene, and low immunogenicity. They are also capable of transducing dividing and quiescent cells.(82) The engineered viral system yields virus that cannot replicate without a eukaryotic producer cell line, such as HEK293FT, and is thus contained in the infected host cell as an integrated provirus. This system has been extensively modified to remove risks associated with the HIV based virus. The potential for homologous recombination that produces full-functional HIV virus is infinitesimal.

A major limitation to using lentiviral vectors for gene therapy in the liver is the strong induction of the type 1 interferon response (IFN-alpha,beta).(82, 83) Following injection into the spleen, the vector particles enter the complex vasculature of the liver and begin infecting hepatocytes. However, prompt elicitation of the type 1 IFN response results in destruction of infected cells and rapid clearance of remaining vector. The speed and degree of this response is thought to be attributed to the immunogenic function of the spleen and liver, with the hepatic kupfer cells particularly positioned to intercept such an invasion. (82, 83)

Counter measures to immune elicitation

In concert with findings on the livers IFN1 elicitation detailed above,(82) Brown also revealed a cleaver new approach to reducing the immunological side effects of gene therapy by using microRNA to knock down transgene expression in immune cells.(84, 85) In this recent study, he showed that immune sensitization to vector borne-transgene can be bypassed by tagging the transgene with a sequence complementary to a hematologic lineage-specific microRNA (miRNA). This was demonstrated with self-inactivating lentiviral vector bearing the tagged GFP cassette. While mice given untagged lenti-GFP experienced immune-mediated clearance of signal in under two weeks, mice given miR42-tagged lenti-GFP maintained signal within 10-20% of hepatocytes for over 4 months, with the liver's immune Kupffer cells no longer expressing GFP.(84) To the great encouragement of patients with liver IMDs, this work seems to have overcome what was thought to be a sizable roadblock to liver directed gene therapy, and may have broader applications for vectors with otherwise limiting immunogenicity.

Non-viral means of gene delivery

Alternatively, non-viral means of gene-delivery are also under development. These nonviral systems often incorporate portions of viral vectors, such as 'naked plasmid' constructs, to allow for transfer of transgene. The most apparent benefits of a non-viral mediated approach include low host immunogenicity and the simplicity of large scale production. A major obstacle to the use of any system wherein the exogenous DNA is maintained epigenetically is that the transgenes are not replicated along with the host cell DNA, and thus the fraction of cells transduced and producing transgene declines in proportion to cell division. However, with great persistence in the area of vector design, some of the more recent non-viral systems are now reporting reliable transduction efficiencies approaching those of viral vectors.

Naked DNA has been used in several trials via direct intra-tissue injection of DNA plasmid constructs or even simple PCR product. While direct injection of naked DNA has not been greatly successful, other methods for targeting these molecules have produced slightly better results.(86) In general, cellular membranes are quite resistant to the passive entry of large polar molecules, such as DNA and protein, because of the hydrophobic interior of the phospholipid bilayer. The method of electroporation works around this obstacle by employing an electrical current to briefly shift the phospholipids of the target cell membrane and allow for the passage of densely charged nucleic acid into the cell.(87) A disadvantage of this technique is the risk that it poses to the cells. The electrical current can easily damage targeted cells beyond repair, and of greater concern, the permeability that electroporation confers to the membrane is in no way selective; thus, materials other than the desired transgene may move freely in and out of the cell for a brief period before the pores have resealed. At the least, this threatens to throw off intracellular solute concentrations and precipitate cell death; at worst, this technique could allow the introduction of other elements, nucleic acid included, into the cell that were not intended for transfer.

Another technology developed for the purpose of gene shuttling is the ‘Gene Gun’. This ballistic tool uses high-pressure gas to literally shoot DNA-coated gold or tungsten particles directly into the cells. First developed in the 1980’s at Cornel University as a means to genetically toughen plant crops,(88) the gene gun now has broad implications in fields from infection control to horticulture.

In common with the non-integrating viral vectors, the major drawback of any of the mainstream techniques for naked DNA transfection is that the transgene is not necessarily duplicated upon cell division. Even if the primary transfected cells maintain the nucleic acid

within the nucleus and in tact, only one of the two daughters resulting from the first division actually retains the transgene, and thus the strength of its expression becomes diluted over time in any tissue with moderate cellular turnover.

Combined Stem Cell-Gene Therapy

Many research teams have now begun moving in the direction of engineered stem-cell therapy. This alternative to systemic viral administration has a number of advantages in terms of dosing (with respect to both virus, and gene), as well as eliminating the chance that a genetic element may engraft in an undesired location (ex. the gametogenic organs). Undesired expression of transgene can be prevented by a tissue-specific promoter. However, this still does not eliminate the chance that millions of viral particles are integrating into the DNA of as-many cells. The chance for hazardous integration of virus into *ex vivo* transduced cells still exists - at the same time, rather than juggling concerns over proto-oncogene activation throughout the body's systems, cells fated to engraft in a fairly contained location does dim concern of unmanageable metastasis.

A paper by Nguyen *et al.* (2006) described successful employment of *ex-vivo* engineered hepatocytes for treating CNS upon reimplantation.(89) While hepatocytes existent in a sick patient may indeed be harvested, gene-modified and reimplanted, they bear with them the disadvantage of long-term subjection to the disease phenotype; this includes chronic and substantial hepatic injury (be it in the form of excessive glycogen storage, or unremitting elevated bilirubin). With this recent *ex vivo* transduction study in mind, we had set out to employ the very same strategy of cell harvesting, correction and reimplantation using both parenchymal and progenitor cell types for comparison.

Transplanted stem cells had a growth advantage relative to surrounding liver tissue in two ways: First, they bore the transgene needed for normal metabolism and, upon differentiation in

the liver, the exogenous enzyme activity would keep them healthy (i.e. not storing mass excessive amounts of glycogen). Secondly, unlike cells that have existed in the liver, transplanted stem cells are harvested from the shielded environment of the bone marrow prior to transplantation, and have thus evaded direct exposure from the cellular pathologies associated with the disease.

A potential complication is that a vector's ability to confer sustained expression is known to vary from one tissue/cell type to another; for instance, certain stem cells - especially of embryonic origin,(90) are known to silence the expression of lenti-derived transgene. This natural survival advantage poses a frustrating hurdle for research that would otherwise benefit from the useful qualities of the vector. This hurdle is being dissembled in the basic-research arena, and will gradually be averted with intelligent modification to the vector backbone.

The ability to cure a disease by harvesting a patients own cells and correcting them *ex vivo* prior to return could have the potential to eliminate any number metabolic disorders – simply by variance of the vector-borne transgene. The unification of cell- and vector-based therapy could therefore evade some of the concerns that apply when either therapy stands alone. Not only could the control of gene and vector dosing be more refined in the *ex vivo* system, but patients would no longer have to choose between battling their chronic ailment, or the consequences of allogenic tissue replacement. As the corrective therapies are streamlined, patients with IMDs will receive a chance at a full life-span, and a tremendous reduction in the need for specialized medical care.

When to Intervene with a Medical Procedure

The stage at which a therapeutic intervention is employed to combat an ongoing disease is determined by many patient-specific factors. In the most common cases where the doctor is

presented with a sick patient, the timing and treatment strategy must be decided by weighing the costs and benefits of a procedure in relation to the patients condition.

In some cases where the risk or cost of a therapeutic intervention is extraordinary, intervention may be delayed until life is otherwise unsustainable. In less extreme cases, a therapy that stands to greatly improve quality of life may be utilized when the cost/risk of intervention just surpasses the cost/risk of not pursuing the treatment.

In recent years the ability to diagnose genetic disease even before a patient is born has expanded the avenues for treatment tremendously. Such early testing is of particular value in cases where both parents are known carriers of a given gene defect, and fear a low quality of life for their future child should (s)he express the heritable disease phenotype. When presented with an afflicted fetus, there are an even greater number of questions contemplated prior to any intervention. Indeed, the decision to intervene with a 'curative' therapy at all is made by weighing the risks and benefits to the primary patient (the fetus) as well as the procedural risks to the mother. The severity of the disease at birth, and its amenability to treatment post-partum are also considerations for determining whether or not to treat an unborn child. If the decision to intervene is made, then the next decision must be at what stage of development to treat. This too is determined by multiple factors, including the size of the fetus and thus the accuracy of treatment infusion, the potential for damage as time goes on in the absence of treatment, and the physical and emotional condition of the mother. Though the complexities range greatly based on application, the decision of whether or not to treat, how to treat and when to treat should be the result of ongoing cost benefit analysis with the patients wellbeing foremost in mind.

Dissertation Specific Aims

The three preliminary branches of our research investigations are addressed by the specific aims. Many new opportunities for scientific exploration, some interesting, and others necessary,

arose during the pursuit of these aims (Figure 1-10). Indeed, while a vast spread of interrelated investigations produced of our many experimental findings from a vast spread of investigations, those of most immediate worth to new related investigations came not from the pursuit of a specific aim, but rather from the directed evolution of our work with the animal models. Beyond what could be gained by the pre-planned objectives, our work has uncovered significant new details with regards to experimental protocols for liver reconstitution of the hyperbilirubinemic Gunn rat, and has greatly enriched our understanding of the mouse model mouse model of GSD1a. The publication of both sets of findings will be of great value to investigators preparing to begin their work with these models.

Specific Aim 1 Employ Current Protocols for Liver Repopulation in the Gunn Rat Model of Crigler Najjar Syndrome

It is accepted that bone marrow derived stem cells can differentiate into hepatocytes and bile duct epithelium.(8, 9, 28, 36) This study was designed to examine the aptitude for a mixed population of BMDCs to confer enzyme activity to the deficient rodent liver. Whole bone marrow was compared against hepatocytes and oval cells for their potential to reconstitute and correct the liver UGT1A1 enzyme deficiency of the homozygous negative ($Gunn^{j/j}$) rat.

Phenotypically normal male 'Gunn' rats donated these cells, while $Gunn^{j/j}$ females siblings were prepared for cell implantation by administering monocrotaline (MCT) followed by partial hepatectomy (PHx). The cell type most capable of engrafting and functioning in the recipient liver was expected to produce the greatest sustainable reduction in bilirubin levels, signifying correction of the disease.

Specific Aim 2 Explore the Potential for *Ex vivo* Transduction with Vector bearing the UGT1A1 or G6Pase-alpha gene cassette

Both lentivirus and the lesser known SV40 have potential as therapeutic vectors for correcting genetic defects. While the 'perfect' vector awaits discovery, attributes of both vectors

make them very appealing as gene-shuttles to an enzymatically-deficient liver. Here, we sought to identify which of these vectors is better at conferring stable transgene activity to cells involved in regenerating a diseased liver. Selection markers, RT-PCR, and immunohistochemistry were used to explore vector infectivity and transduction efficiency *in vitro.*

The latter part of the investigation was designed to explore the relative potential of different combination of cell and vector types for correcting the enzyme deficiency. Hepatocytes, Oval cells, and Bone Marrow Derived Cells (BMDCs) were to be transduced *ex vivo* with either engineered viral vector prior to reimplantation into monocrotaline-treated recipients. In this case, the cell:vector combination capable of engrafting and functioning in the recipient liver was expected to produce the greatest sustainable reduction in bilirubin levels, signifying correction of the disease.

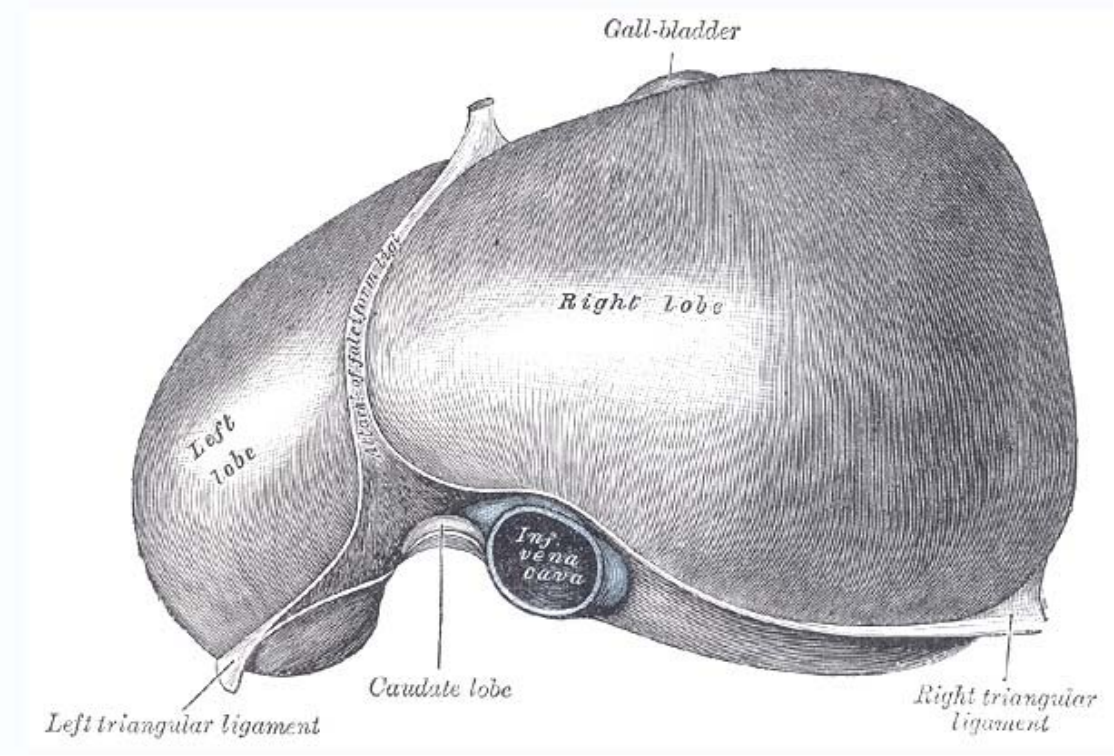
Specific Aim 3 Explore the Therapeutic Potential for Treating Inherited Metabolic Disorders *In Utero* by Infusion of Stem Cells or Lentiviral Vector

The strategies which most often come to mind for correcting a metabolic defect involve infusion of genetically engineered cells or therapeutic viral vectors to the individual who has presented with the disease. In turn, the individual is typically envisioned as an adult, child or even infant who has come to the clinic with an evident dysfunction. In reality however, our limitations have ever less to do with size or robustness of the individuals in need of treatment, but are instead merely dependent on our ability to accurately diagnose them. With the modern day availability of amniocentesis and high-precision robotics, we now have the technical capacity to deliver treatments directly to a developing fetus while still in the womb.

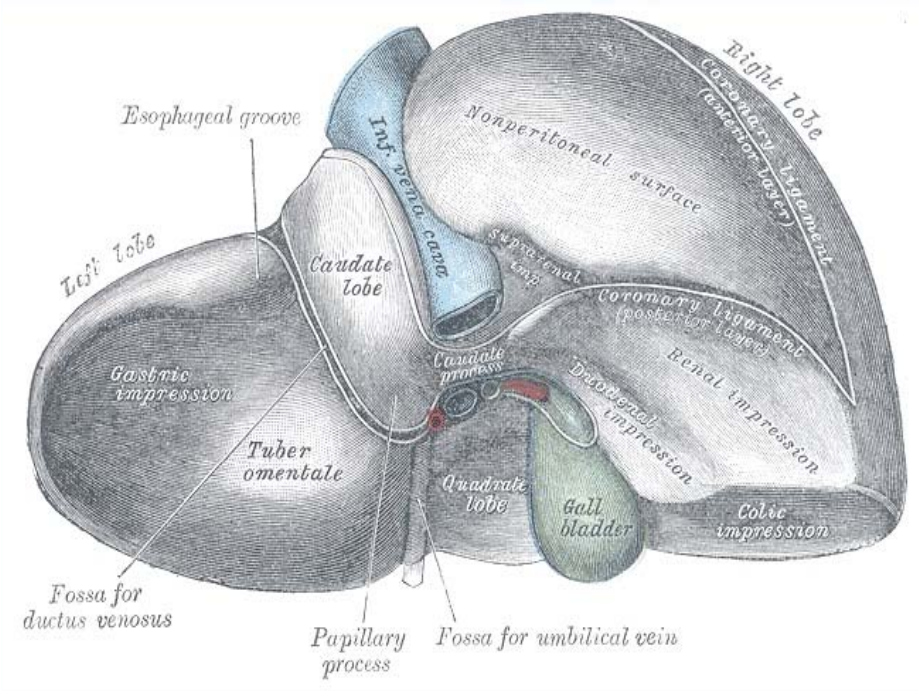
By intervening at this very early stage, patients who are otherwise destined to enter the world with very challenging physical limitations could be spared a great deal of hardship and

instead only know a ‘normal’ life. This is certainly the case patients identified *in utero* to have an inherited metabolic disorder caused by a single wayward gene.

While we were in the midst of developing our protocols for rearing a full colony of robust GSD1a adult mice, this investigation sought to determine whether we could reliably provide intrahepatic transgene activity to mice *in utero* via direct infusion of lentiviral vector or bone marrow derived cells into the developing mouse liver. Both of these modalities employed GFP as a visible marker of both location and penetrance of the therapeutic vehicle.



A



B

Figure 1-1. Views of the human liver. A) The superior surface of the liver. B) Posterior and inferior surfaces of the liver. 20th Edition of Gray's Anatomy of the Human Body, 1918

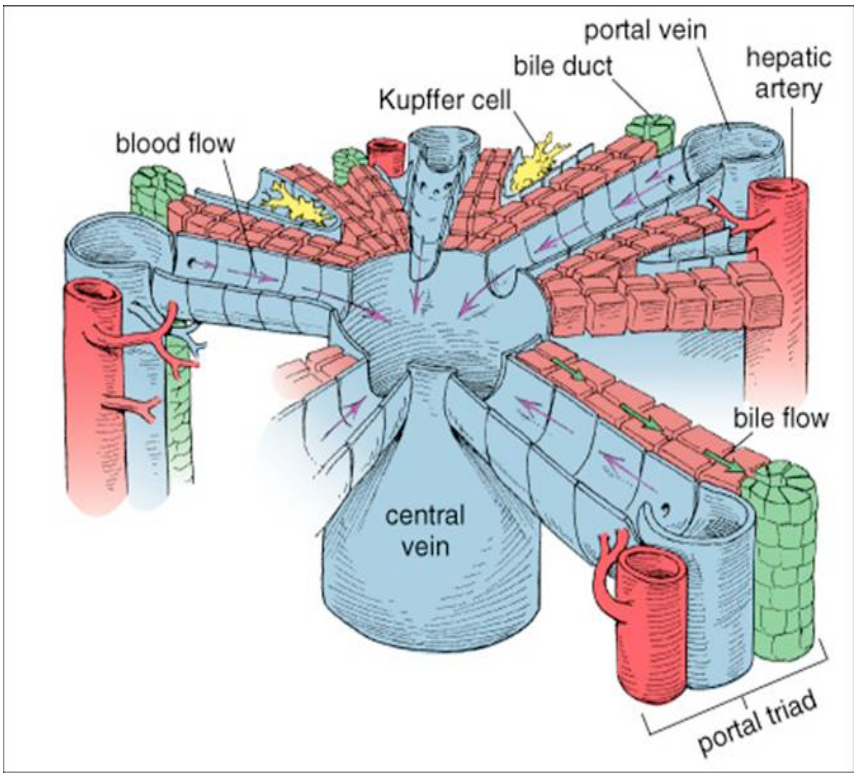


Figure 1-2. Hepatic microarchitecture and blood flow. Red arrows indicate blood flow and green arrows indicate the direction of bile flow. Copyright ©2003 M. H. Ross All rights reserved.

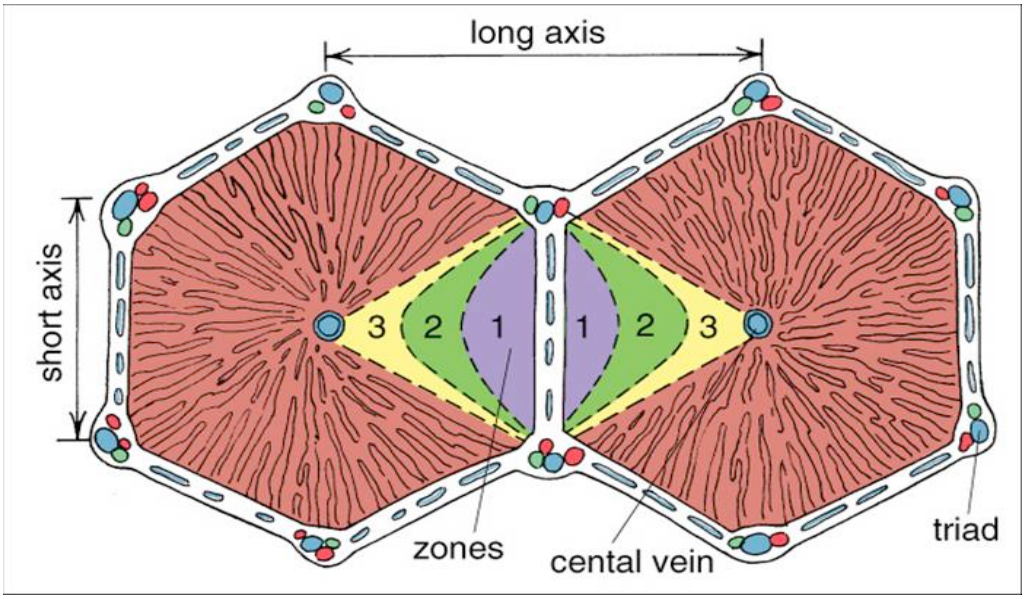


Figure 1-3. Liver acinus. The three zones of hepatocytes graded by distance from the lobule central vein. Copyright ©2003 M. H. Ross All rights reserved.

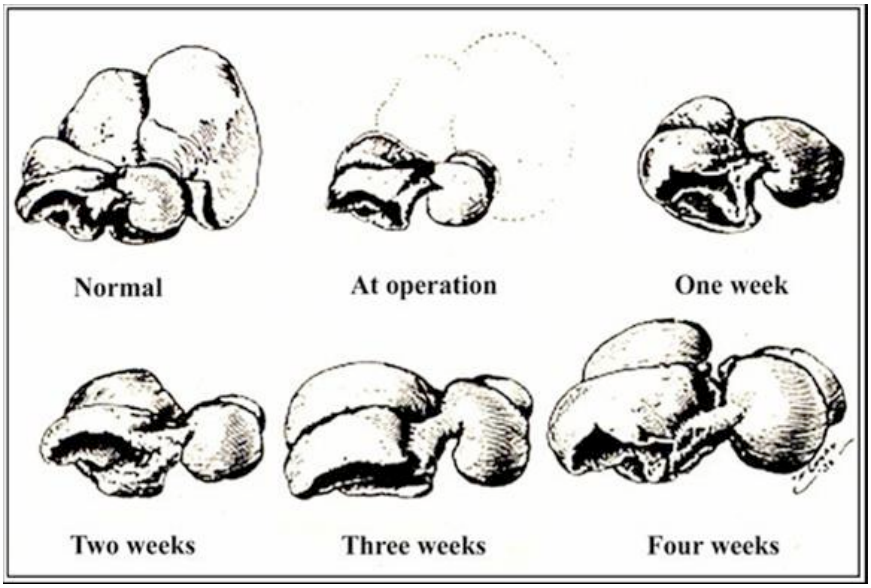


Figure 1-4. Graphic representation of growth of remaining three liver lobes after ⅔ partial hepatectomy in the rat. Compensatory hyperplasia results in the liver regaining original tissue mass in approximately 10 to 14 days. Copyright ©1931 American Medical Association. All Rights Reserved.

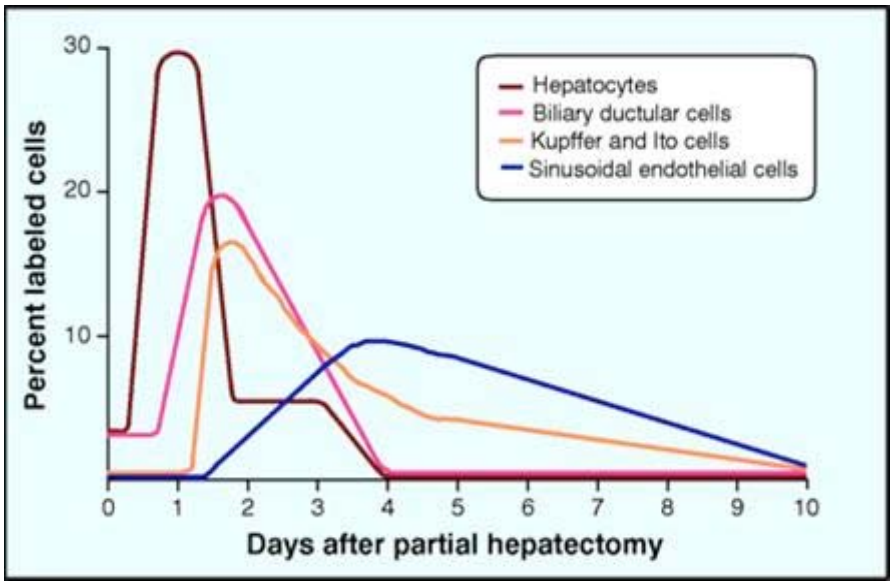


Figure 1-5. Graph of the amount of various resident hepatic cells within the cell cycle during the time following ⅔ partial hepatectomy. Hepatocytes represent the driving source behind hyperplasia-based liver regeneration. Copyright ©1997 AAAS. All Rights Reserved.

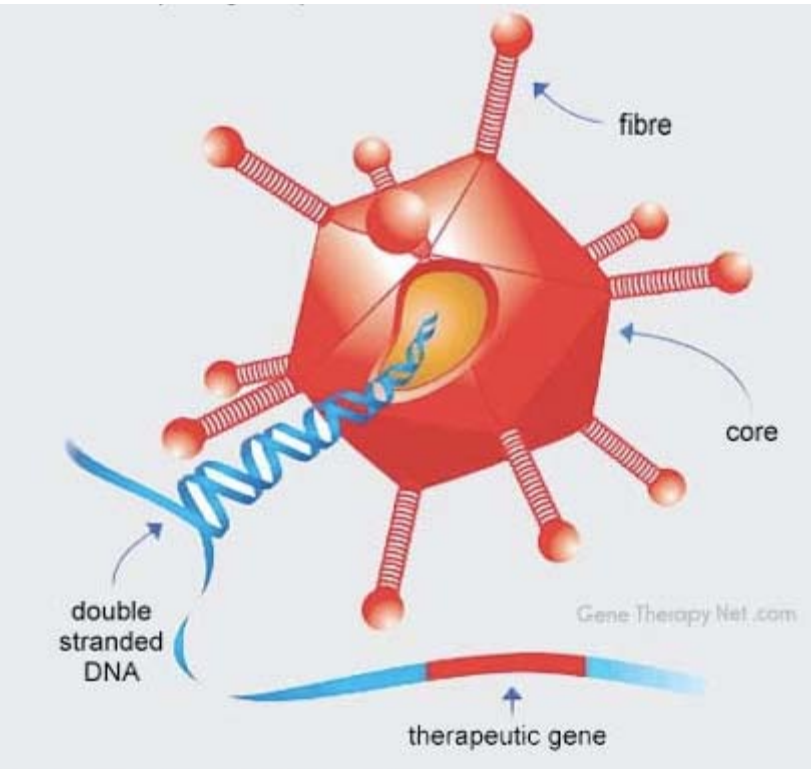


Figure 1-6. Adenoviral particle organization Copyright © 2007-2009 by Gene Therapy Net (49)

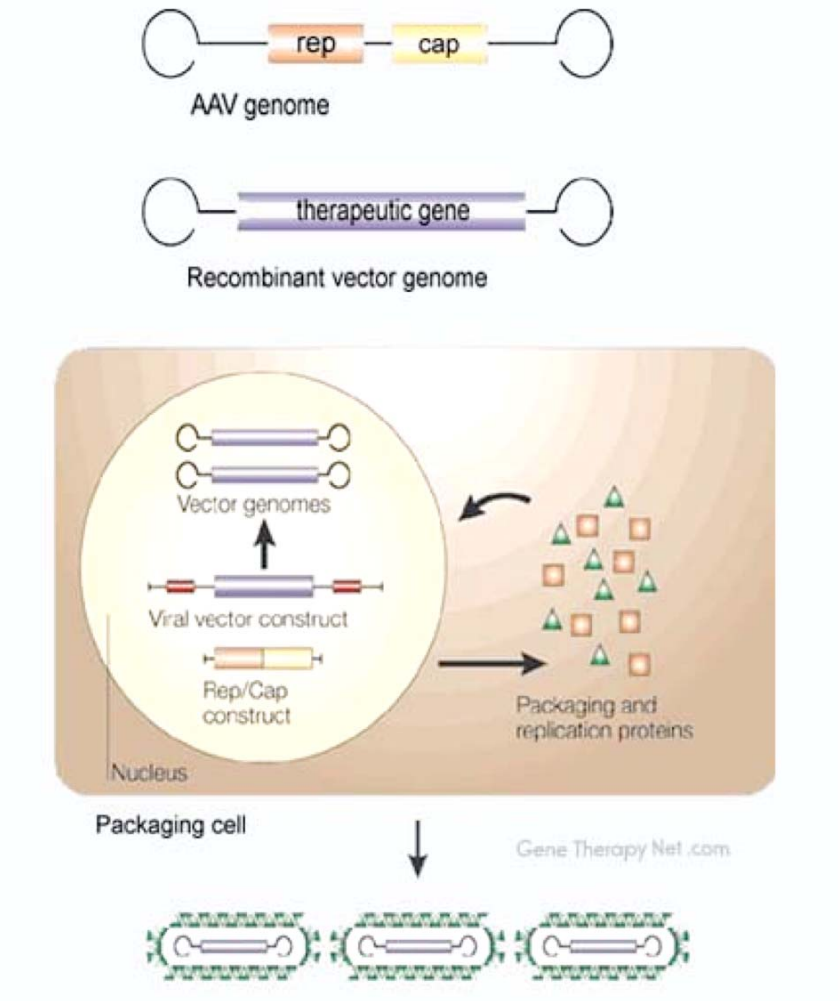


Figure 1-7. Producing recombinant AAV vectors Copyright © 2007-2009 by Gene Therapy Net (54)

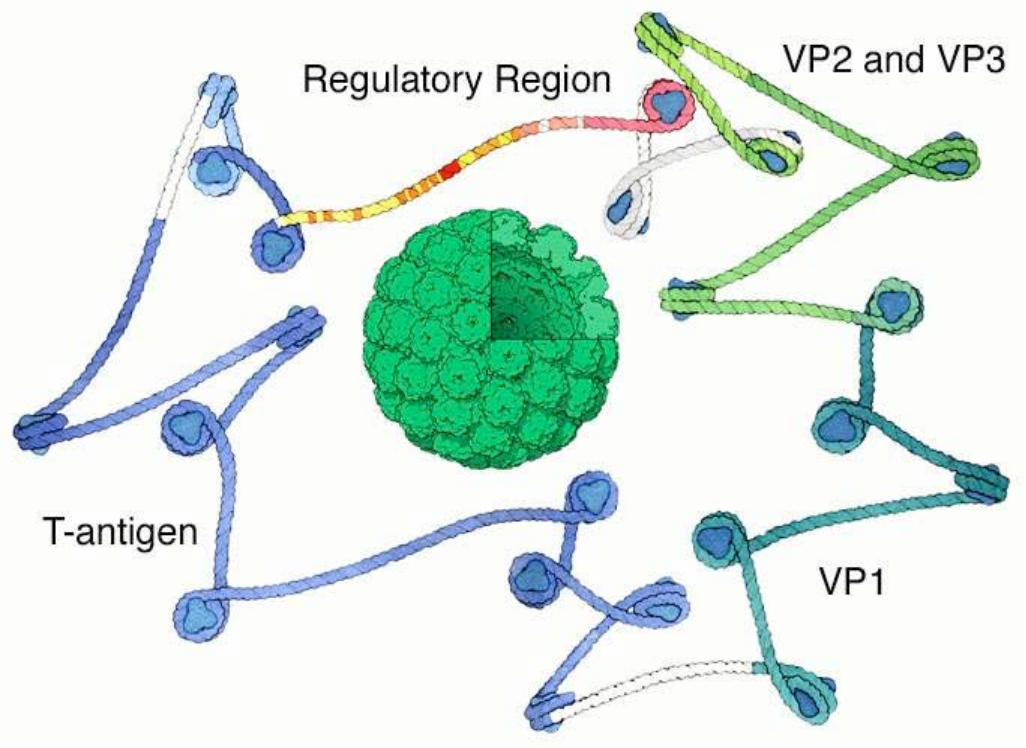


Figure 1-8. SV40 DNA (mini-chromosome) and its 360meric capsid. November 2003 Molecule of the Month by David S. Goodsell (public domain).

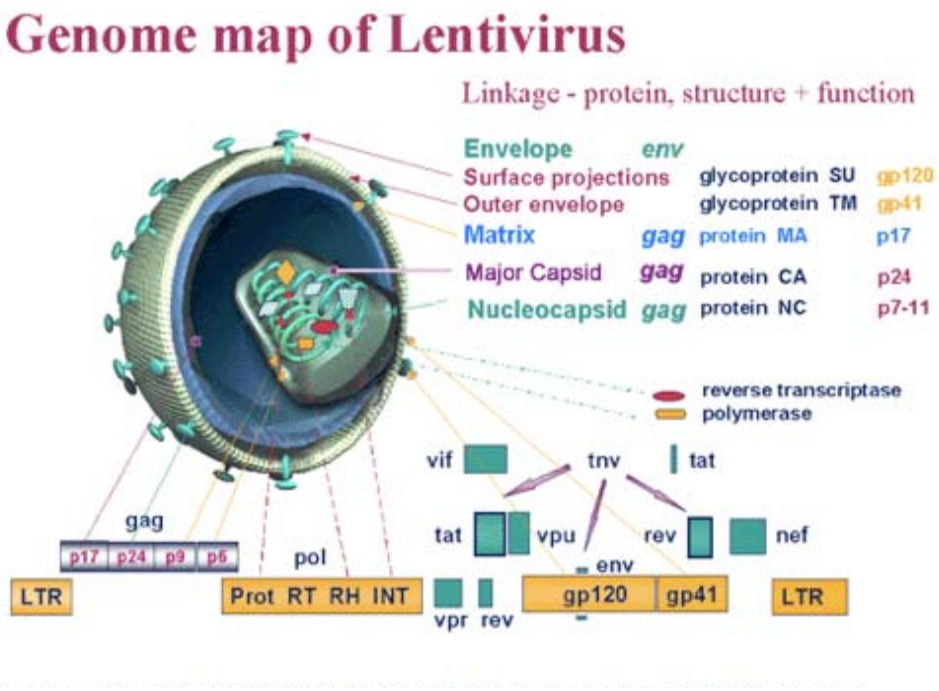


Figure 1-9. Structural illustration and genome map of lentivirus. Copyright © 2006 from ICTVdB - The Universal Virus Database, version 4. ( )

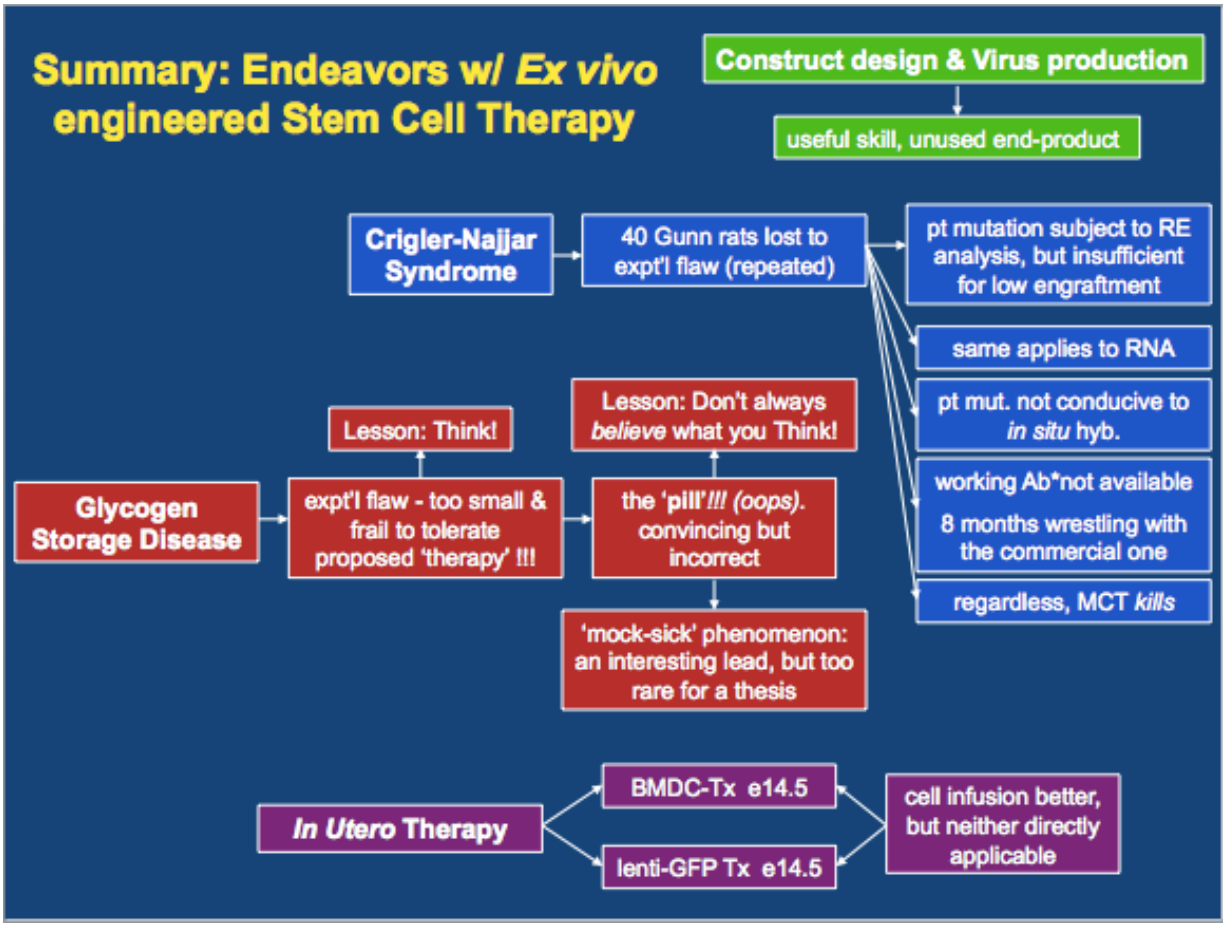


Figure 1-10. Many avenues of exploration with lessons learned *en route* to the doctoral degree. Bearing in mind the obstacles that had not been foreseen in the research outlined, our studies evolved to address some of the more fundamental qualities of the research models that had heretofore been overlooked.

CHAPTER 2
REVIEW PUBLICATION: STEM CELL THERAPY FOR INHERITED METABOLIC DISORDERS OF THE LIVER

Abstract

Modern medicine has conquered an enormous spectrum of health concerns, from the neonatal to the geriatric, the chronically ill to the acutely injured. Among the unmet challenges remaining in modern medicine are inborn disorders of metabolism within the liver. Such inherited metabolic disorders (IMDs) often leave an otherwise healthy individual with a crippling imbalance. As the principal regulator of the body's many metabolic pathways, malencoded hepatic enzymes can drastically disrupt homeostasis throughout the entire body. Severe phenotypes are usually detected within the first few days of life, and treatments range from palliative lifestyle modifications to aggressive surgical procedures. While orthotopic liver transplantation is the single last resort 'cure' for these conditions, research during the past few years has brought new therapeutic technologies ever closer to the clinic. Stem cells, therapeutic viral vectors, or a combination thereof, are projected to be the next, best, and final cure for IMDs, which is well-reflected by this generation's research initiatives.

Introduction

The liver is a remarkable organ in both form and function. Indeed, it stands alone in the mammalian world with a lasting capacity to self-regulate its mass in proportion to the needs of the body; a feat accomplished by a mechanism of compensatory hyperplasia.(80) Postnatally, the liver is responsible for maintaining homeostasis through a myriad of processes; some involving detoxification, and others, syndissertation. The liver processes consumed substances, sustains reserves of iron and other vitamins and minerals, it detoxifies alcohol, drugs, and other noxious chemicals, and removes inhaled poisons, such as exhaust, smoke, and volatile chemicals that accumulate in the bloodstream. As a production plant, the liver makes bile for digestion of lipids,

stores energy by stockpiling glycogen, and manufactures albumin, blood clotting factors, and a host of other key plasma proteins.(91) A single misprint in the genetic coding of a liver enzyme could completely destroy its capacity to carry out any one of these functions and, if left unchecked, often threatens the life of the afflicted patient.

Inherited metabolic disorders (IMDs) represent an array of conditions tied to one of the liver's many functions.(92) Monogenic disorders are often inherited through an autosomal recessive mechanism, resulting from a single coincident misprint in each parents' genetic code (93-95) Both parents of an afflicted child are effectively proven carriers of the recessive gene, and together bear a one in four risk of passing on the disease to each future offspring. Individual IMDs are exceptionally rare but, taken together, they are suspected to account for up to 20% of illnesses among full-term neonates without prior known susceptibility. In fact, an estimated 1:5000 newborns are afflicted with 1 of > 70 IMDs, and a clinician is likely to see a number of such patients throughout the course of his career.(96)

The average family is unlikely to have heard of any IMDs. Familiar or otherwise, it is an overwhelming situation for any family to deal with such a diagnosis in their own child. In addition to coming to terms with a decreased quality life, parents are often faced with a daunting treatment regime. Generally, these treatment regimes must be employed immediately upon diagnosis in order to minimize any impact on early mental and physical development. Less-severe IMDs can be managed by treating the symptoms of the disease. IMDs that involve more-critical liver functions must be dealt with much more aggressively, and often necessitate liver transplantation.

Palliative Treatment of IMDs

In cases where the disorder may be managed prior to or in lieu of risky procedures, a palliative approach involving diet or lifestyle modification is often the preferred course of action.

Phenylketonurics, for example, follow a strict dietary regime whereby they avoid foods rich in phenylalanine, thus minimizing intake of a molecule that is improperly metabolized, and avoiding buildup of the neurotoxic metabolite.(97) Other enzyme defects can be managed by supplementation with cofactors or vitamins that either enhance the residual activity of a partial defect, or bolster an alternate metabolic pathway. Infants suffering from carnitine deficiency usually present with hypoketotic hypoglycemia. While life-threatening cardiac malfunction can result from neglect of the symptoms, this outcome is easily avoided by adding L-carnitine to the diet.(98) Exogenous detoxifying agents are often prescribed to patients with deficient endogenous detoxification capacity. For example, urea-cycle defects that lead to hyperammonemia can be treated with ammonium detoxicants that enhance the elimination of nitrogen. This treatment combined with arginine supplementation has proven to be quite effective in lowering ammonia levels in patients with carbamyl phosphate synthetase, ornithine transcarbamylase, argininosuccinate synthetase, and argininosuccinate lyase deficiencies.(99, 100)

Solution-Based Treatment of IMDs

When such relatively simple measures are unavailable or insufficient to maintain health, physicians look to more direct treatment strategies. Exogenous supply of the deficient enzyme, known as enzyme replacement therapy has proven quite effective for treating a handful of enzyme deficiencies. For example, patients with Gaucher’s disease and other lysosomal storage disorders are able to benefit from this type of supplementation.(101) Nonetheless, it has been less successful for liver-specific pathologies.(102) Glycogen-storage disease results from nonfunctional glucose-6-phosphate dehydrogenase. During the last step in gluconeogenesis, the energy intermediate glucose-6- phosphate is actively transported into the cytoplasm, where the normal enzyme frees glucose for use in sustaining metabolism.(54) Therefore by necessity,

glucose-6-phosphate dehydrogenase activity is localized within hepatocyte endosomes, thus administering the enzyme systemically is ineffective for restoring its function. Even when enzyme replacement therapy is therapeutically effective, it does not provide a cure from the endogenous disorder, and the patient is left with lifelong dependence on pharmaceuticals.

Disorders like Gaucher's exemplify the true potential of cell-based therapies, as bone marrow transplantation could directly and permanently eliminate the disease by restoring enzyme function to the monocyte population. Many metabolic disorders are manageable for a period of time, but often bear a predictable need for more drastic future treatment. Patients with Crigler Najjar syndrome (CNS) lack functional uridine diphospho-glucuronosyl transferase, an enzyme necessary for bilirubin metabolism.(103) Afflicted individuals are unable to convert bilirubin to the excretable hydrophilic form, and it accumulates to toxic levels. As with glycogen-storage disease, the enzyme missing in CNS is also meant to function within cellular endosomes, rendering enzyme replacement therapy ineffective. Instead, CNS patients are prescribed a unique therapy involving abundant sun exposure and nights spent unclothed beneath a bright blue light. In addition to the natural ultraviolet rays, this light produces a wavelength that can penetrate the skin and break down bilirubin as it flows through subdermal microcapillaries, effectively replacing the role of the missing enzyme. Although this treatment is often sufficient through childhood, it becomes less so as the patient's skin naturally thickens with age; eventually a threshold is reached whereupon a healthy donor liver is imperative to escape lethal bilirubin accumulation.(40)

Liver Transplantation

The most insidious of metabolic syndromes do not always afford a patient time to mature, but instead require immediate intervention for life to be sustained. In the case of neonatal hemachromatosis, palliative care is insufficient and patients are generally transplanted as soon as

a suitable donor organ becomes available.(104) Since its inception in 1963, orthotopic liver transplantation has been the ultimate solution for most patients refractory to palliative or solution-based management.(105) A healthy donor liver will restore endogenous enzyme production, effectively repairing the metabolic disorder. This aggressive approach is not without pitfalls and, taken together, the scarcity of available organs, risk of surgical complications, expense, indefinite immunosuppression, and eventual rejection, lessen enthusiasm for this means to a cure.

Viral-based Gene Therapy

As new treatment options emerge, it becomes clear that the most effective therapies often bear the highest risks. This has driven research toward strategies that utilize minimally invasive techniques. Preclinical advances during the last decade have finally brought realistic hope for curing metabolic liver disorders without the complications inherent in orthotopic transplantation. Vector-based gene therapy has made headlines as a potential means for correcting imbalances at the genetic level. Research on plasmid vectors as potential vehicles for gene replacement is also underway, but the current generation of such vectors produces only transient gene expression.(106) Viral vectors such as adeno associated virus (AAV) maintain their construct-bearing genome episomally for a variable period post-transduction, but also lose expression as cells replicate endogenous DNA. To the contrary, vectors such as lentivirus are able to integrate the transgene into the cellular genome, resulting in transduced cells that produce the encoded factor indefinitely.(107) While this may eliminate the need for repeated administration of the vector, it also carries with it a series of very real risks. Genetic dosing and regulation of the transgene product are of foremost concern in cases where the enzyme activity levels should be restrained. The spread of vector and thus gene expression are also difficult to control when the

virus is administered to the body directly, and despite the availability of tissue-specific promoters, the potential effects of systemic integration must also be considered.

The dogma that the vast majority of the human genome was unimportant has recently come under scrutiny. The discovery of several genetic control mechanisms sandwiched between the coding regions of DNA has called into question the wisdom of allowing viral vectors to randomly integrate into the genome. Retrotransposons, regulatory micro RNAs, and other control elements that had once flown beneath our radar are now gaining due recognition. These elements, as well as oncogenic sites, are important to consider while weighing the suitability of viral-based gene therapy. Such a remedy relies on a degree of luck with regard to integration site. These are, of course, only of concern if the vector is able to successfully transduce cells. In the case of lentivirus, for example, target cells can produce a family of proteins known as apolipoprotein B mRNA– editing enzymes.(108) These proteins can both limit transduction of cells, and trigger an immune response to viral antigens. With so many details regarding the safety and dynamics of systemic transduction yet to be mastered, cell-based therapies do stand out as the imminent solution to IMDs.

Hepatocyte Transplantation

While hepatocytes are generally quiescent, the ability to rapidly enter the cell cycle offers a means to respond to chemical and physical insults. In the absence of inhibition, acute liver injury induces a state of massive compensatory hyperplasia among resident hepatocytes.(6) Indeed, transplantation studies in mice have shown a remarkable proliferative capacity over a span of several serial transplantations; a youth-sustaining feat that is suspected to originate from persistently high telomerase activity.(109, 110)

In the case of IMDs, compensatory hyperplasia yields a futile expansion of endogenous cells producing the malencoded enzyme. Fortunately, when the nature or extent of the

dysfunction overtakes its capacity for self-repair, liver function may still be regained by procedures such as organ or cell transplantation. The amount of active enzyme needed to sustain health varies by metabolic function. Therefore, the easiest disorders to correct are those that respond well to replacement of a relatively small portion of liver by cells bearing functional gene. In such cases terminally differentiated hepatocytes may be useful for cell-based enzyme replacement.

One of the earlier records of such an experimental treatment was detailed in the 1976 article by Matas and colleagues,(64) using the rat model of CNS (familial hyperbilirubinemia). When immunosuppressed Gunn rats lacking the enzyme uridine diphosphate glucuronyl transferase were infused with normal hepatocytes via the portal vein, plasma bilirubin concentrations stably decreased, ameliorating clinical symptoms of the disease.(64) The viability of this procedure was verified a year later when Groth and colleagues (111) reproduced the curative effect of mature hepatocyte transplantation in the same animal model. Two decades later Fox *et al.* (42) also saw success using hepatocyte infusion to treat humans with CNS-1. This reported achievement, supported by ongoing studies in the preclinical setting, would seem to validate this simple strategy.

In these models that employ partial hepatectomy followed by donor cell infusion, endogenous hepatocytes still outnumber the healthy donor cells by two to three orders of magnitude. Following proportional expansion *in vivo*, the minor contribution of healthy donor cells would provide some functional enzyme, but the level of enzyme activity needed to produce a normal phenotype is different for each metabolic disorder. Indeed, most animal models have shown that a two-thirds partial hepatectomy alone is not sufficient to promote engraftment of healthy donor hepatocytes in numbers that would be clinically useful.(112) Therefore, a selective

advantage must be intrinsic to the transplanted cells for this therapeutic approach to be effective for a majority of IMDs. The mouse model for hereditary tyrosinemia type 1 represents one disorder in which the transplanted hepatocytes possess a substantial proliferative disadvantage. Unable to produce fumaryl acetoacetate hydrolase, resident hepatocytes succumb to the toxicity of tyrosine catabolites. Upon withdrawal of the therapeutic drug 2(2-nitro-4 trifluoromethyl-benzoyl)-1,3 cyclohexane dione, the genetically defective hepatocytes begin to die, while healthy transplanted cells proliferate freely.(112, 114)

While engraftment may be regarded as a rate-limiting factor in the outcomes of cell transplantation, simple collagenase pretreatment of the liver can induce localized destabilization of the extracellular matrix, creating an environment in which transplanted cells can more easily take up residence.(113) A majority of the animal cell transplantation models requires pretreatment of the recipient liver with an antiproliferative agent, such as retrorsine or monocrotaline prior to a partial hepatectomy and cell transplantation.(112, 114) Such pretreatment is not desirable in humans, as the antiproliferative agents are also carcinogenic.

As an alternative to chemical inhibition, Chowdhury's group replaced pharmacological pretreatment of the liver with site-directed irradiation. This combination of insults also conveyed a selective advantage to the cell transplant. Neither partial hepatectomy nor irradiation to support engraftment at therapeutic levels.(115) Laconi's group (116) published one of the few accounts suggesting that, given enough time, donor rat hepatocytes are able to substantially repopulate the retrorsine-treated liver. Should this strategy be replicable, clinical application would still be unlikely because of the carcinogenic potential of retrorsine mentioned previously.

Additionally, obtaining hepatocytes for transplantation face many of the challenges associated with finding a donor organ. Both live and cadaveric sources are scarce, and the

requirement for a match in major histocompatibility complex between donor and recipient limits options still further. Autologous cell transplantation appears to be the most viable therapeutic strategy. However, without *ex vivo* modification, the patient's own cells are of little use. The replicative capacity of mature hepatocytes is greatly diminished *in vitro*, requiring a significant amount of cells for transplantation. Cultured hepatocytes also lose metabolic function rather quickly.(117) Taken in combination, these obstacles lessen enthusiasm for hepatocyte cell therapy.

Liver Development

Liver stem cell–based therapies will likely require *in vitro* recapitulation of at least a portion of the normal liver development process. Embryological studies have mapped the differentiation of early endodermal stem cells to both the hepatocytic and bile ductular cell lineages. Proliferation of undifferentiated endodermal cells associated with the developing ventral foregut followed by migration to the septum transversum, results in contact with precardiac mesoderm, which affects lineage determination of the liver stem cell population. Recent studies suggest that cells analogous to these very early epithelial progenitor cells may persist in the bone marrow of adults.(8, 28, 118) For the purpose of cell-based therapies of IMDs, a bone marrow– derived cell would hold the most promise from the standpoint of accessibility, although complicated *in vitro* processing would likely be required to move these cells to a differentiation state appropriate for transplantation. a-Fetoprotein (AFP) is the first lineage-specific marker expressed by developing foregut cells. AFP expression is soon followed by expression of classical hepatoblast markers, including albumin. It is generally accepted that at least a portion of the bipotent liver progenitor cell population is AFP+, so it is likely that cells suitable for therapies will derive from cells at this differentiation state. As development proceeds, further lineage commitment is evidenced by expression of more mature markers, such

as transferrin receptor, c-CAM, H.2, and H.4. While it is difficult, though not impossible, to alter the terminal lineage of cells expressing these markers, they still possess a robust proliferation capacity.(119) This fact leaves open the possibility to use cells already committed to the hepatocyte lineage for cell therapy. As organogenesis advances, the biliary tree and hepatic plates are formed, each adopting a marker expression profile associated with the terminally differentiated phenotype.(120) Proliferation capacity becomes more limited and suitability for use in cell therapy is diminished (Figure 2-1).

Bone Marrow Derived Hepatocytes

Literature of the late 20th century contained reports that bone marrow–derived stem cells could develop down hematopoietic, endothelial, and epithelial lineages. Their participation in liver regeneration, however, had not been shown. In 1999, Petersen *et al.* (8) demonstrated that bone marrow–derived cells could give rise to hepatocytes following liver injury in lethally irradiated rats that had received a bone marrow transplant.(8) This finding was confirmed when Y-chromosome–positive hepatocytes were detected in the livers of female patients who had received bone marrow transplants from male donor.(28, 121-123) Results of these studies broadened the possibility of cell-based therapies for the treatment of IMDs of the liver, as the bone marrow would represent a readily available source of liver progenitor cells.

In support of this notion, Grompe's research group began the Millennia with a report that challenged several paradigms of cell-mediated liver regeneration. His team employed the fumaryl acetoacetate hydrolase mouse model of tyrosinemia to investigate the potential for cell-mediated rescue from an inherited enzyme deficit. They were able to demonstrate restoration of a normal phenotype by intravenous infusion of bone marrow cells.(124) These findings, that healthy, syngenic, bone marrow–derived cells were able to remediate a metabolic deficiency, were not new; rather, what stirred controversy was the claim that only the rigorously purified

Sca-1+ hematopoietic stem cells gave rise to hematopoietic and hepatic regeneration.(36) This completely debunked the notion that the three germ layers were set early in embryogenesis, and that transdifferentiation among them was not possible.

While conflict grew among research groups trying to identify which subpopulation actually bore true therapeutic potential, enthusiasm for an extrahepatic source of liver stem cells was transiently diminished for another reason entirely. Shortly following these publications, Wang *et al.* (18) and Vassilopoulos *et al.* (17) reported that the genetically wild-type hepatocytes that replaced the diseased cells in the tyrosinemia model were not the product of bone marrow cell transdifferentiation, but rather the product of cell fusion. Extensive karyotyping revealed severe aneuploidy consistent with the combining of genomes between donor and recipient cells. The following years were witness to a slew of reports supporting either the transdifferentiation or fusion theory of bone marrow–derived liver cells.

Undoubtedly, a complete understanding of post-transplantation events is required to maximize the effectiveness of therapies based on bone marrow–derived cell transplantation. However, scientists with a more clinically driven mentality will quickly point out that the most important aspects of developing a clinical therapy using bone marrow–derived cells is the evaluation of safety and the effectiveness in treating disease. Several studies during the past few years have clearly demonstrated beneficial participation of bone marrow–derived cells in the repair of liver injury.(33, 125-127) Thus, bone marrow remains a promising source of material for cell-based therapies regardless of the mechanism by which the restoration of normal liver function is achieved.

To date, only one other study implicates bone marrow as a source of liver progenitor cells shown to correct an IMD *in vivo*; nonetheless, interest in the research arena is now gathering

momentum. In 2006, Misawa *et al.* (128) used desialylated bone marrow cells for treatment of Wilson's disease in a rodent model. The major finding of this study was that the interaction between the asialoglycoprotein receptor on hepatocytes and the desialylated bone marrow cells increased the rate of parenchymal engraftment. Large nodules of bone marrow–derived hepatocytes were identified within recipient livers, and survival was improved in the transplanted groups.

In 2007, Yan *et al.* (129) published the results of a Chinese study that demonstrated that peripheral blood mononuclear cells mobilized from the bone marrow of hepatitis B– infected patients could be enriched for early hepatocyte markers and transplanted back into patients. This autologous transplantation resulted in functional hepatocytes that sustainably improved liver function in transplant recipients. Similar studies have also shown improved liver function in patients following autologous bone marrow cell therapy.(130-132) While these studies do not deal directly with IMDs, they do verify that bone marrow–derived cells are capable of engrafting into the liver, and giving rise to functional hepatocytes.

Future studies will undoubtedly focus on specific bone marrow cell subpopulations that show enriched potential for engraftment and hepatocyte differentiation within the recipient liver. Ratajczak and colleagues have been at the forefront of this field for the past half-decade. In 2004, Ratajczak's laboratory identified a subpopulation of CXCR4+ bone marrow cells that are mobilized by granulocyte colony-stimulating factor and migrate in response to an stromal derived factor-1 gradient. Circulating among the pool of peripheral blood mononuclear cells, this particular subpopulation expressed markers for muscle (Myf-5, Myo-D), neural (GFAP, nestin) and liver origins (CK19, AFP).(118) Additional studies by Ratajczak *et al.* have identified an extraordinary cell population within the bone marrow.(133) Termed very small embryonic-like,

these cells are extremely small (2 um) with almost no cytoplasm. They express CXCR4 along with several embryonic stem cell markers, including Oct-4. Ratajczak hypothesizes that these cells remain dormant in the bone marrow following embryogenesis and may persist into adulthood as epithelial progenitors. The suitability of these cells as transplantable elements for the treatment of liver IMDs remains to be determined.

Of course, the propensity of stem cells for *ex vivo* expansion provides for replenishment of cell stocks available for transplantation. Indeed, in contrast to donor hepatocytes, stem cells are not nearly so limited by tissue origin, nor numbers available at harvest. Jiang *et al.* (134) published a 2002 report in which multipotent adult progenitor cells from a variety of murine tissues maintained multilineage capacity through >70 passages *in vitro* (134). A 2006 article by Tamama's group highlights the benefits of epidermal growth factor-induced cell expansion for this purpose, reporting that proliferation was bolstered in multipotent adult progenitor cells derived from human bone marrow.(135)

Veyrat-Masson *et al.* (136) critiques a wide assortment of studies on mesenchymal stem cells, which have produced an array of collective outcomes.(136) In their evaluation, Veyrat-Masson *et al.* urge scientists to look beyond the differences in study observations, and suggest that they first scrutinize more fundamental questions; foremost of which is whether or not expanded mesenchymal stem cells populations truly offer a proportionally enhanced therapeutic capacity, and whether there is notable benefit to using human serum in place of the standard fetal calf serum for purification and/or amplification of mesenchymal stem cells.

Clearly, many details involved in bone marrow cell to liver differentiation remain to be described. While there is strong evidence that bone marrow cells can aid hepatic reconstitution,

intra-hepatic progenitors remain the most extensively studied stem cells for the treatment of IMDs.

Hepatic Oval Cells

Distinct from hepatocytes and nonparenchymal cells, the liver is able to deploy a population of intrahepatic progenitors when hepatocyte proliferation is inhibited. The bipotential hepatic ‘‘oval’’ cell has been characterized both in rodent models and in human subjects.(137, 138) Investigators have recently begun to include these cells in transplantation studies (138, 139).

While a liver-specific stem cell may seem like the most likely candidate for cell-based therapies for IMDs, several issues lessen their clinical value. The largest obstacle that would need to be overcome is accessibility. Oval cells are extremely rare in healthy liver, most often associated with chronic hepatic injury in humans (Figure 2-2). In rodents, oval cell proliferation is induced upon chronic injury, or by blocking endogenous hepatocyte proliferation prior to chemical or physical injury.(19, 139) Whether donor-derived or autologous, obtaining oval cells in sufficient quantity for transplantation would require a significant volume of chronically injured liver, precluding live humans as a source.

Given this serious clinical drawback, the oval cell stands to be the greatest beneficiary of the previously mentioned *ex vivo* expansion strategy. Hepatic oval cells have been characterized and maintained in culture systems. Clonal subpopulations are morphologically homogeneous, being small polygonal cells with a large ovoid nucleus and scant cytoplasm (103). They propagate with ease under certain culture conditions, and can maintain a unique combination of markers including AFP, CK19, and albumin.(140)

The ability to culture cells between isolation and transplantation provides a host of advantages. Culturing allows for amplification of a small number of isolated cells to a volume

sufficient for transplantation. It also provides an opportunity to control the differentiation state of the cells, providing either highly proliferative bipotential progenitors or lineage-committed progenitors with less proliferative potential.

In 2002, Dr. Sell's team (138) reported an elegant strategy for modulating the lineage development of rodent oval cells in culture. They had suspected that non-transformed oval cell lines might exhibit a malleable differentiation state. Indeed, they found that oval cells grown in standard media on a feeder-layer of embryonic fibroblasts steadfastly maintained an immature phenotype; however, those grown without the feeder layer committed to the hepatocyte lineage. In contrast, oval cells cultured on Matrigel adopted a biliary-epithelial phenotype. Significantly, because these cells were derived without using carcinogens, such bipotential liver progenitor cell lines would be suitable for cell-based therapies.(141)

Three-dimensional culture systems have also been used for the *ex vivo* expansion of oval cell populations. Suh *et al.* (142) developed an oval cell culture system using a three-dimensional collagen scaffold impregnated with hormone factors and cytokines. Three weeks of tracking albumin secretion and rates of ammonia detoxification showed that scaffold-cultured oval cells achieve higher levels of hepatocyte function than those grown on two-dimensional tissue culture plates. Such innovations in biomaterial engineering are setting the foundation upon which clinicians will test the feasibility of *ex vivo* expansion. For oval cells specifically, systems that benefit expansion, genetic manipulation and genotypic stability *ex vivo* may lessen the risks associated with their use in the correction of IMDs.

Liver Progenitor Cells from Bone Marrow

With an enormous body of literature illustrating their capacities, our understanding of oval cell potential is currently more agreed upon than their origin. Some suggest that oval cells arise by dedifferentiation of resident hepatocytes.(143) A majority of experts still find that a bone

marrow origin of oval cells is at least conceivable. In support of this theory, *in vitro* studies have revealed that bone marrow cells may express hepatic oval cell markers.(144) *In vivo* studies have often employed female animals, lethally irradiated and rescued with allogenic male bone marrow cells detected by simple survey for the Y-chromosome. As mentioned previously, Petersen *et al.* (8) used a mitotic inhibitor to block proliferation of endogenous hepatocytes in female rats that had been transplanted with male bone marrow carrying a genetic marker. Physical injury was subsequently used to induce a proliferative response in the oval cell population. The resulting hepatic repair did, in fact, include hepatocytes that displayed both Y chromosome as well as the donor marker. The laboratories of Grampe, Theise, Petersen and others have repeatedly observed donor hepatocytes in the livers of BM transplant recipients, reinforcing the notion that bone marrow may act as a functional reservoir for liver progenitors.(8, 9, 28, 124, 145) Whether or not these bone marrow–derived hepatocytes transitioned through an oval cell phase was not addressed by a majority of these studies.

In search of solid evidence, Shafritz, Dabeva and colleagues designed a study in which lethally irradiated female rats were transplanted with bone marrow from males carrying a genetic marker, and then subjected to three models of oval cell activation and expansion. Results from this model suggested that it was not the bone marrow–derived oval cells, but rather the endogenous liver progenitors that provided the injured liver with the vast majority of expanding oval cells.(25) Using a serial transplantation model, Grompe's research group deduced that hepatic oval cells do not originate from bone marrow but from the liver itself. In this two-step experiment using the fumaryl acetoacetate hydrolase mouse model, the team reported that primary recipients of fah (+/+) hepatocytes become almost entirely reconstituted with donor derived hepatocytes reflective of their strong proliferative advantage. However, chemically

induced oval cells did not express the donor marker. Secondary transplant recipients of bone marrow also displayed the standard oval cell response to injury, but genetic analysis revealed that very few oval cells bore the original donor marker. With both dedifferentiation and bone-marrow origin ruled out, the group concluded that nonparenchymal cells of the liver were, in fact, the source of emergent oval cells. While in total these findings reinforced the value of oval cells for therapeutic liver repopulation, it contested a bone-marrow origin for the progenitors detected.(30)

In opposition to the studies conducted by Dabeva and Grompe, Petersen's laboratory conducted a study wherein rats were pretreated with a chemical agent that inhibited the liver resident oval cell reaction prior to bone marrow transplantation.(139) Following establishment of the chimeric BM, these rats were subjected to an oval cell–inducing injury. Up to 20% of the resulting oval cells carried the BM donor marker. It does appear that BM may give rise to oval cells, although whether or not this happens under normal circumstances remains to be determined.

The previously mentioned studies are just a sampling of liver stem cell studies. While the debate rages on about the true origin of these cells, opinions lose relevance without proof that the dynamics in animal models are, in fact, shared by potential human beneficiaries. Early investigations suggest that if the human liver does respond in a manner similar to rodent models, isolation of such a progenitor should be translatable to the clinic. Rather than join the speculation, an Italian group recently tackled this central question by isolating and characterizing a hepatocyte progenitor population from human liver. This cell type was found to display hepatic, osteogenic, and endothelial potential, which indeed surpasses the plasticity of the rodent analog. Most importantly, the human progenitor proved its clinical usefulness by successfully

reconstituting the severe combined immunodeficient mouse liver post-xenotransplantation. (146) The reaffirmation of such a progenitor in man, capable of isolation and hepatic reconstitution, opens all possible doors for their use in treating metabolic liver disease.

Conclusion

Where once the fate of those with hepatic IMDs was inevitable, there are now emerging options that stand to provide a means to a true cure without the need for orthotopic transplantation. Researchers in the fields of gene therapy work feverishly to produce suitable vectors that safely and sustainably transfer the desired gene to the correct tissues at the right dosage. Unfortunately, a 'perfect vector' is quite a ways down the road. In a different court, parallel research on cell transplantation has begun to provide a glimpse of the true capabilities of hepatic repopulation. The potential for cellular infusion, be they autologous or allogenic, progenitor or committed, will be realized when methods are optimized for administering an appropriate cell population to a niche that permits engraftment (Figure 2-2).

Perhaps the most useful studies are those that address the basic tenets of the natural mechanisms of liver repair. Ultimately, our capacity to correct IMDs will most benefit from a detailed characterization of pathways that mediate transdifferentiation and hepatic regeneration. This level of understanding will ease the transition from complex animal models to clinical application. While the earliest years of the 21st century witnessed great advances in research toward therapeutic potential, controversies remain. Indeed, it is likely that the best therapeutic approach will vary for each IMD, and specific strategies will need to be tailor fitted depending on requirements for each disorder.

Acknowledgments

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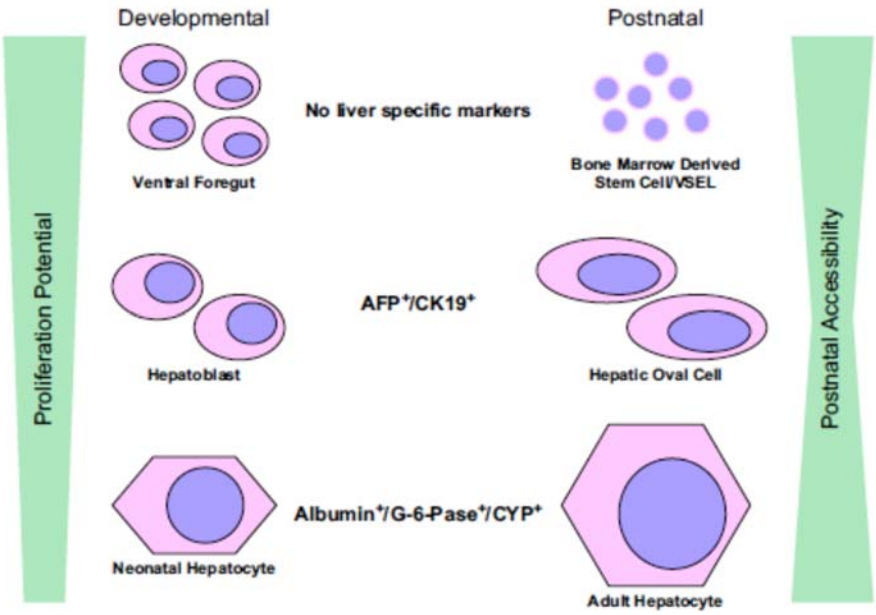

Figure 2-1. Potential material for liver cell therapy. Transplantable cells for the correction of liver metabolic disorders may be considered from among several differentiation states. These cell populations may be considered analogous to well characterized stages of liver development. Accessibility, expandability, and suitability for genetic manipulation must be considered when selecting cells for therapeutic strategies. AFP 5 a-fetoprotein positive; VSEL 5 very small embryonic-like.

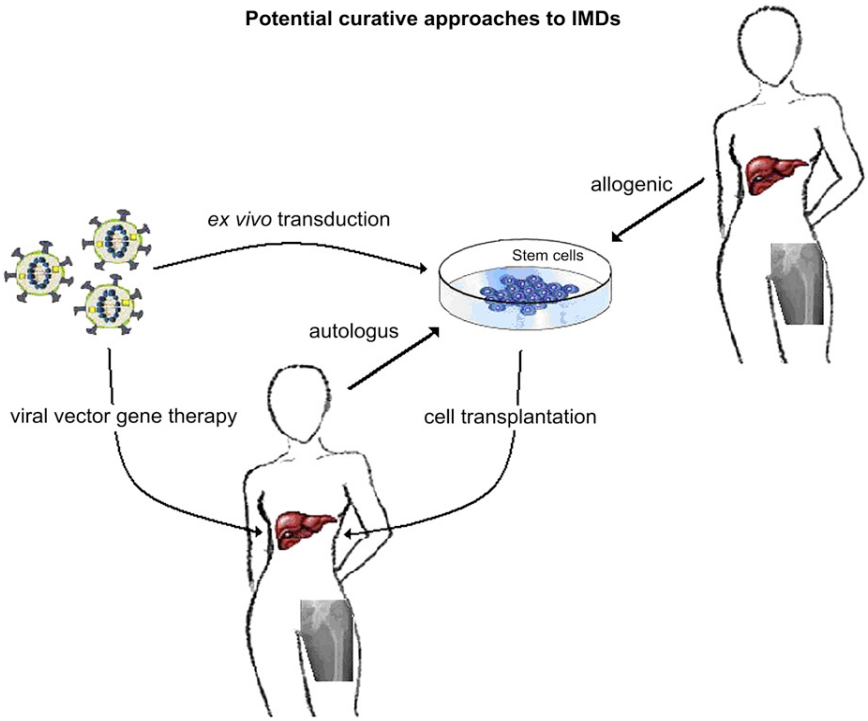

Figure 2-2. The curative approach to inherited metabolic disorders (IMDs) may evolve in many forms. Gene therapy could offer healthy copies of the corrupted gene directly to the patient via systemic infusion of a tissue-specific vector. Stem cell research is closing in on means to repopulate tissues from which a disease originates. For IMDs, transplantable stem cells will derive from a healthy allogenic donor, or from the patient, provided the cells are genetically corrected *ex vivo*.

CHAPTER 3
RESEARCH PUBLICATION: A DETAILED CHARACTERIZATION OF THE ADULT MOUSE MODEL OF GLYCOGEN STORAGE DISEASE 1A

Susan Ellor[1], David A. Weinstein[2], Thomas D. Shupe[1], Max Salganik[3], Dana Pintilie[1] and Bryon E. Petersen[1]

1. Department of Pathology, Immunology and Laboratory Medicine and The Program for Stem Cell Biology and Regenerative Medicine, University of Florida College of Medicine, Gainesville, Fla., USA 2. Division of Pediatric Endocrinology, Department of Pediatrics, University of Florida College of Medicine, Gainesville, FL, USA 3.Department of Molecular Genetics and Microbiology, Powell Gene Therapy Center, University of Florida College of Medicine, Gainesville, FL, USA

Abstract

Glycogen storage disease type 1a is caused by a genetic defect in the hepatic enzyme glucose-6-phosphatase (G6Pase-alpha), which manifests as life-threatening hypoglycemia with related metabolic complications. A G6Pase-alpha knockout mouse model was generated in order to study potential therapies for correcting this disorder. Since then, gene therapy studies have produced promising results, showing long-term improvement in liver histology and glycogen metabolism. Under existing protocols however, untreated knockout pups seldom survived weaning. Here, we present a thorough characterization of the G6Pase$^{-/-}$ mouse, as well as the husbandry protocol for rearing this strain to adulthood. These mice were raised with only palliative care, and characterized from birth through 6 months of age. Once knockout mice have survived the very frail weaning period, their size, agility, serum lipids and glycemic control improve dramatically, reaching levels approaching their wildtype littermates. In addition, our data reveal that adult G6Pase$^{-/-}$ mice are able to mate and produce viable offspring. However liver histology and glycogen accumulation do not improve with age. Overall, the reliable production of mature G6Pase$^{-/-}$ mice could provide a critical tool for advancing the GSDIa field, as the availability of a robust G6Pase$^{-/-}$ adult offers a new spectrum of treatment avenues that

would not be tolerated by the frail pups. Most importantly, our detailed characterization of the adult G6Pase$^{-/-}$ mouse provides a crucial baseline for accurately gauging the efficacy of experimental therapies in this important model.

Abbreviations: Glucose-6-phosphatase (G6P); Glycogen Storage Disease type 1a (GSDIa); knockout (KO); wildtype (WT)

Keywords: Glucose-6-phosphatase; GSD1a mouse model; Glycogen Storage Disease; hypoglycemia; metabolic disorder

Introduction

Glycogen storage disease type Ia (GSDIa) is a metabolic disorder resulting from mutation of the gene encoding for hepatic glucose-6-phosphatase (G6Pase-α).(61) The disorder results from homozygous recessive inheritance, and it is currently estimated to have an incidence of 1:100,000. (59) The G6Pase enzyme is now well understood as a phosphohydrolase that catalyzes the terminal step in gluconeogenesis and glycogenolysis. (147, 148) The absence of hepatic G6Pase activity impairs endogenous glucose production, and hypoglycemia develops with fasting. As a result, GSDIa is commonly diagnosed within the first few months of life upon recurrence of severe hypoglycemia and seizures.(149-151) Lactic acidemia is also present during the neonatal period, and severe hepatomegaly often leads to a protruding abdomen. Adolescents with the disease often have short stature, delayed puberty, hyperlipidemia, hepatomegaly and nephromegaly, accompanied by recurrent hypoglycemic attacks. Even with improved treatment, long-term complications remain common (149-151)

A detailed characterization of the molecular origins of GSDIa (60) allowed for the creation and recognition of GSDIa animal models (152). Dr. Janice Chou created the G6Pase-knockout mouse in 1993 (60), and this strain has since been widely used as a gold standard for studying GSDIa in the laboratory. While a naturally occurring dog model for G6Pase deficiency also exists (153), the long

lifespan and high level of husbandry required for maintaining these animals has resulted in continued interest in the mouse model.

Gene therapy experiments in both of these animal models have produced clear and promising results in terms of both hepatic glycogen accumulation and histology.(154) However, the results of these studies were interpreted without regard to untreated control animals of similar age. For this reason the prior studies in mice could not evaluate the potential for intrinsic gains in health due to maturation. In response to this gap in our knowledge, we set out to characterize the G6Pase$^{-/-}$ mouse as it transitions through the neonatal period to maturity. Here, we demonstrate that afflicted pups are not only capable of surviving the very fragile weaning period, but that they also experience the same age-related physiological improvement often reported by human GSDIa patients.(155) In particular, mice were consistently observed to improve glycemic control, serum profiles, physical activity and mobility, and showed normal reproductive capacity in both genders. These changes occurred in lieu of any genetic treatment, suggesting that biochemical and behavioral adaptations may contribute to the improvements described in animals that receive gene therapy. We also show that the widely-expressed G6Pase-β isoform does not undergo up-regulation in muscle tissue to compensate for the lack of G6Pase-α in the liver.

Experimental Procedures

Mouse Genotype Determination

C7/B6 mice bearing a G6Pase-α knockout (KO) allele (a gift of Dr. Janice Chou, NIH) were bred in-house and in accordance with IACUC approved guidelines. All pups were genotyped on the 3d day of life by PCR of DNA from tail snips, using the following primers: wildtype, 5'-AAGTCCCTCTGGCCATGCCATGGG-3' and 5'-CCAAG CATCCTGTGATAA CTC-3'; knockout, 5'-ATACGCTTGATCCGGCTACCTGCC-3' and 5'-CATTTGCACTGCCG GTAGAACTCC-3'. PCR was performed using the protocol previously outlined by Chou *et al.* (2007).(156)

Phenotypic Characterization

Mice ranging in age from 0 to 6 months were evaluated continuously with regards to the pathophysiology of G6Pase-deficiency. Measured parameters included weight, blood glucose, cholesterol, triglyceride, uric acid levels, liver- and kidney:body weight ratio, glycogen accumulation (liver), G6Pase-α activity (liver), and G6Pase-β activity (muscle). Body mass was measured with a pocket scale sensitive to 0.1g. Blood-glucose was checked (tail-bleed), no less than every 8 hours, using the Freestyle Flash glucose meter (Abbot Diabetes Care, Inc., Alameda, CA). All animal work was done in accordance with the regulations set by the Institutional Animal Care and Use Committee.

Pre-weaning Care

Pups with a glucose reading below 70mg/dL received 25-150ul of 15% glucose solution by dorsal subcutaneous injection. The exact volume was determined based on the severity of hypoglycemia. Onward from 14 days of age, pups received ad libitum access to standard mouse chow (2018S Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, Indianapolis, IN), as well as Nutragel® high calorie food supplement (Bio-Serv S4798-TRAY, Bioserv Corporation, San Diego, CA). Afflicted pups that survived the standard weaning period (21 days) were allowed to remain with the mother until day 30.

Post-weaning Care

Both standard chow and Nutragel® were available at all times, and glucose was added to the drinking water (5% final solution) daily. Weights were charted throughout the life of the animal. 100-200ul of 15% glucose solution was administered subcutaneously if blood glucose was between 70 and 30mg/dL; for lower readings, 200-300ul glucose was administered IP.

Sacrifice and Tissue Collection

Mice were weighed, and then euthanized via cervical dislocation following sedation with isofluorane. Muscle was immediately excised from the long bones of the hind limbs, followed by removal of liver and kidney. Collected tissues were partitioned into blocks for fixation/paraffin embedding, fresh-frozen Tissue-Tek® O.C.T. (Optimal Cutting Temperature) embedding, and microfuge tubes for immediate snap freezing in liquid nitrogen. Blood samples for analysis of lipid and uric acid (UA) content were was collected via heparin-treated syringe and transferred to a Capijet® blood collection tube. Samples were placed on ice and submitted to a CLIA approved clinical lab.

Histology

Paraffin-embedded liver and kidney tissue sections were cut to 6uM thickness, and sections were stained with Hematoxylin and Eosin (HandE), Masson's Trichrome or Periodic Acid Schiff (PAS) reagent for histological evaluation, visualization of fibrosis, and glycogen quantitation, respectively. Aperio image analysis software was used to quantify the intensity of PAS staining. Statistical significance was determined by ANOVA.

Phosphohydrolase Activity Assay

The phosphohydrolase activity of both isoforms of G6Pase was measured in liver and muscle tissue as per the Glucose-6-Phosphatase Activity Assay described by Alfred Harper.(157) In brief, 150mg tissue was pulverized in liquid nitrogen and suspended in 6 mL 0.1M citrate buffer. For test samples, 100ul G6P-buffer was added to 100ul of homogenate. For control solutions lacking G6P, 100ul tissue homogenate received 100ul of simple citrate buffer. The blank solution contained 100ul citrate buffer plus 100ul G6P solution, without any tissue. Reaction mixtures were incubated 15' at 37 °C, which is the optimal temperature for the β-isoform, and equally acceptable for Glc-6-Pase-α. (158) 2 mL of 10% trichloroacetic acid was added to each, and solution was centrifuged 10' at 12,000xg.

1ml supernatant plus 5 mL .002M molybdate solution was added to fresh tubes for experimental and G6P-free control samples. 1ml PO_4 standard solution plus 5 mL .002M molybdate was added to fresh tubes for blank solution. 1ml reducing reagent was added to each tube at 1' intervals, and samples were incubated 15' at room temperature. Samples were transferred to 1ml cuvettes for spectrophotometry at 700nm, with readouts between 1 and 20 arbitrary units. Each preparation represents an individual mouse, with at least three independent preparations used per assay. Micromoles phosphate liberated is found by Equation 1-1:

A700 for [(experimental – G6Pase-free control)/blank] x (0.5uM PO4) x (2.2ml total rxn vol) x [(1000/(15*2.5)]. Units are uM PO4/min/g tissue. (1-1)

Quantitative Real Time rt-PCR Analysis of G6Pase-β

G6Pase-β mRNA was quantified by real-time rt-PCR in an Applied Biosystems 7500 Real-Time PCR System using gene-specific Taq-Man Gene Expression Assays (Applied Biosystems), and then normalized to β-actin mRNA. The following probes were used, all purchased from Applied Biosystems: G6Pase-β, Mm00616234_m1; G6PT, Mm00484574_m1; GRP78, Mm00517691_m1; GRP170, Mm00491278_g1, caspase-3, Mm00438045_m1, and β-actin, Mm00607939_s1.

Western Blot Analysis of G6Pase-β

Protein from mouse thigh-muscle was retrieved from the inorganic fraction of the TRIzol RNA isolation procedure. Samples were resolved by electrophoresis through a 12% polyacrylamide-SDS gel and trans-blotted onto polyvinylidene fluoride membranes (Millipore Co., Bedford, MA). The membranes were incubated overnight with the polyclonal rabbit anti-Glc-6-Pase-β antibody targeting amino acids 71-114 of the human G6Pase-β (Chou Lab, NIH), and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (Kirkegarrd and Perry Laboratories, Gaithersburg, MD). The immunocomplex was visualized using the SuperSignal West Pico Chemiluminescent substrate from Pierce.

Results

Mice that are heterozygous or homozygous for the G6Pase-α gene are generally indistinguishable at all stages in life. However, mice that are homozygous null for G6Pase-α present with low birth weight, quickly develop severe and unremitting hypoglycemia, and gradually display increasing hyperlipidemia and osteodysmorphism. These problems worsened as the animals approached weaning. Their relative body mass also fell from 28% to 45% undersize. Without additional care at this point, weanlings inevitably died of hypoglycemia. These observations are in agreement with previously published findings.(52, 77,159)

Knockout Mice Undergo Intrinsic Physiological Improvement Post-weaning

Mice treated palliatively with glucose injections, glucose-fortified water and food supplementation had a 60% survival rate through weaning. Beyond this point, mice underwent significant transition, normalizing to various degrees in many disease-related parameters. While bodyweight of KO pups averaged 1/3rd below normal, KO adults finished growing just 5% beneath the median mass for healthy siblings. KO mice also showed marked improvement in mobility and activity. Liver-to-body weight improved, but remained above normal range, and kidney-to-body weight did not change with relation to normal animals (Figure 3-1). While KO pups averaged 677 mg/dL total cholesterol and 1720mg/dL triglycerides prior to weaning, these numbers fell to a mean of just 215 mg/dL total cholesterol and 205 mg/dL triglycerides by maturity. The average serum lipid levels from wildtype (WT) mice were 144 mg/dL and 161 mg/dL respectively, and more than half of adult KO mice were within a normal range. Circulating blood Uric Acid levels did not differ substantially between normal and KO animals of any age (Figure 3-2c).

Blood glucose measurements transitioned rapidly near weaning, from pups with severe and unremitting hypoglycemia, to adults with general normoglycemia (70-200mg/dL) (Figure 3-2d) with only intermittent hypoglycemic events. Mice that became mildly hypoglycemic were usually capable

of rebound, and 50 mg/dL blood-glucose was found to represent a general cut-off for normal activity, behavior and feeding. While transient, survivable episodes of more severe hypoglycemia were intermittent throughout the six-month time course, the longest-lived KO mice were able to maintain serum glucose levels above 50 mg/dL on 92% of days measured (Figure 3-2d).

Liver and Kidney Histology Does Not Appear to Improve with Age

Histological analysis of liver tissue revealed distortion of the hepatic microarchitecture in both young and mature KO mice (Figure 3-3 a,c) compared to WT animals (Figure 3-3 b,d). PAS-stained sections revealed excess glycogen accumulation in GSDIa mice compared to WT, independent of age (Figure 3-3 a,c vs. b,d). Analysis of histology and glycogen content revealed the same phenotypic manifestation in the kidney of KO animals compared to WT (Figure 3-3 e,g vs. f,h). Aperio slide scanning and image analysis software was used to quantify relative staining intensity among mice of both genotypes and age brackets. Data revealed a clear genotype-dependent trend in glycogen storage, with mean staining intensity lowest in G6Pase+/+ mice, slightly higher in G6Pase+/- mice, and highest in KO mice. An age dependent trend was also apparent, with younger mice storing slightly more glycogen than older mice of their same genotype. ANOVA statistical analysis of staining intensity across the 6 groups of animals produced a p-value of .078 with 95% confidence level. Trichrome staining for type 1 collagen did not reveal significant fibrosis of either the liver or kidneys of KO animals (Figure 3-4).

GSDIa Adult Mice Can Breed Naturally and Produce Viable Litters

Successful mating of G6Pase-/- males to heterozygous females produced the expected 1:1 ratio of heterozygous and null pups (Figure 3-5a). Surprisingly, female KO mice were capable of withstanding pregnancy (Figure 3-5b), and a cross between two KO parents produced normal sized litters of 100% KO pups. The G6Pase-α-/- female maintained safe blood-sugar levels throughout the majority of the pregnancy, and had uncomplicated delivery. Approximately 90% of pups were viable at

birth; however, the mother was unable to nurse adequately. Providing a healthy lactating surrogate female will negate this problem, should G6Pase-/- mothers be unable to nurse future litters.

G6Pase-β is Not Upregulated with Age in GSDIa Mice

G6Pase-β regulation was evaluated at the level of transcription, translation, and functional phosphohydrolase activity. Quantitative Real Time rt-PCR showed no significant up-regulation of G6Pase-β transcription in the muscle of GSDIa mice as compared to WT animals (Figure 3-6a). In agreement with this, Western blot analysis of protein extracts from muscle tissue did not eveal differential regulation of the beta isoform between G6Pase-α deficient and wild-type mice (Figure 3-6b). The phosphatase activity assay confirmed the absence of G6Pase activity in the liver of GSDIa mice at each age tested (data not shown). While recent investigations employed an *in vitro* over-expression system to examine G6Pase-β kinetics, (12, 15) this approach was not available for our study. These results suggest that G6Pase-β upregulation is not an adaptive mechanism in post-weaning G6Pase-α KO mice.

Discussion

Since GSDIa was first described in 1929, nutritional protocols for both children and adults with the disorder have been carefully defined for optimal maintenance of health. The meticulous dietary guidelines have been made available in many formats, and with strict adherence and interprandial supplementation, patients with GSDIa have a fairly good prognosis. Even so, the lifestyle required to maintain the health of a child with GSDIa is an incredible challenge. The severity of symptoms is often more pronounced in childhood, and the potential for the next life-threatening hypoglycemic event is ever-present. Careful management and continued dietary adherence is a continuing battle, but meticulous glycemic control can produce striking clinical and metabolic improvement, and is closely linked with overall prognosis.(53,160) Moreover, the severity of symptoms is known to lessen with

age (2002 chou/chen, 2003 Shieh/Chou, 2004 Shieh/Chou). Nonetheless, the threat of acute hypoglycemia, malnutrition, osteoporosis, hyperlipidemia, hepatic malignancy, and liver or kidney disease never subsides throughout the patient's lifetime. To date orthotropic liver transplantation is the only means available to correct the genetic disorder. However, beyond the risks implicit to allogenic transplantation, GSDIa patients also have a heightened chance for complications in long-term management.(59) Therefore an alternative means for eradicating the disease is essential for offering a chance at a normal life.

Over the past ten years or so a number of translational studies have set out to eliminate the manifestations of GSDIa utilizing a gene therapy approach to shuttle exogenous G6Pase-***α*** to KO animals.(52, 57, 61, 74, 77, 159) Throughout the cited literature, untreated G6Pase KO pups were shown to suffer acutely from classical disease-related symptoms including stunted growth, unremitting hypoglycemia, and pronounced elevation in serum cholesterol, triglyceride and uric acid levels. Survival of afflicted pups beyond weaning has been described as a 'rare occurrence' unless some type of intervention was implemented. To the contrary, these studies clearly showed that KO pups treated with adeno- or AAV viral vectors at two weeks of age not only survived weaning, but also showed phenotypic improvement by week 4, and were virtually indistinguishable from WT littermates with respect to each disease parameter measured by maturity.(74)

The high fatality of KO pups had eliminated the chance for comparison between vector-treated and untreated adults. Thus, prior studies relied upon the phenotype of immature KO pups as a baseline for gauging the long-term effects of gene therapy. In this setting, vector administration was concluded to have caused the improved health of treated animals.(74) Notably however, untreated KO pups that survived to the periweaning period in these studies also showed moderate increase in average blood glucose, with values falling between those of afflicted neonates and normal age-matched siblings.

Most remarkably, the few untreated KO mice reported to survive past weaning displayed marked improvements in serum and growth profiles as well.(74) Although these data were noted graphically, its significance has not been addressed.

To our knowledge, no prior study had considered the potential for natural adaptation of KO mice in the evaluation of GSDIa treatment efficacy. In the absence of gene therapy, we observed remarkable improvement in serum lipid profiles, glycemic control, size, appearance, and liveliness. We also observed some relative improvement in hepatomegaly, but little change in nephromegaly, or liver lobule microarchitecture. Not surprisingly, PAS staining of both tissues revealed that glycogen accumulation did not improve with age, however Masson's Trichrome stain for type 1 collagen did not reveal development of pathological fibrosis. Interestingly, while lethal hepatic and renal lesions have been reported in untreated KO pups by 4-5 weeks of age,(159) this result was not reproduced in our study, even in mice as old as 6 months. Though the reason for this discrepancy is unclear, dietary supplementation with Nutragel® likely contributed to the relative health of our animals.

In the clinic, G6Pase-deficiency leaves patients highly susceptible to hyperuricemia from the overproduction and under-excretion of urate. In humans, build up of lactic acid from alternative metabolic breakdown of G6P only worsens the hyperuricemia, as uric acid must compete with lactic acid for excretion by a common renal anion transporter.(47) For yet unknown reasons, the KO mice do not typically manifest lacticacidemia. With normal lactic acid levels, KO mice would have a greater capacity to eliminate circulating uric acid and avoid hyperuricemia, as observed in our study of the model.

The robustness of the adult G6Pase KO mouse was again demonstrated when KO males produced viable litters with both heterozygous and KO females. Notably, female KO mice were able to carry litters of normal size to term, and deliver without complication. In common with some human

patient reports (personal communication with Dr. David Weinstein), KO mice did not successfully nurse, but providing a healthy female surrogate may allow for rearing of 100% KO litters. The capacity for such high-throughput production is particularly useful to this model, as a fraction of KO pups in each litter will die despite the best available care. Also alternative to the standard heterozygote cross, breeding heterozygous females with KO males will still double the efficiency of producing KO pups in numbers suitable for studies of age-matched littermates, without the need for a surrogate mother.

Upregulation of a G6Pase isoform, G6Pase-***β***, has been postulated as a partial explanation behind the phenotypic improvement in humans. With the animal model capable of an even more drastic change, it was important to investigate whether the transition was due to up-regulation of a compensatory enzyme, in addition to modifications in husbandry. Because the largest glycogen reserves are found not in the liver, but throughout the skeletal muscle; it would then be logical to suspect that some analog of G6Pase might also exist in association with this tissue. The initial published characterization explored the enzyme's gluconeogenic potential first *in vitro,* and then *in vivo* using the young GSDIa mouse to eliminate background from G6Pase-***α*** activity.(155, 158, 161) The results showed that G6Pase-***β*** phosphohydrolase has 12% of the activity of hepatic G6Pase-***α***. The *in vivo* study also revealed that G6Pase-***β*** transcript, protein, and enzyme activity levels were roughly equivalent among young WT and G6Pase-***α***-/- mice;(158) thus providing evidence that G6Pase-***β*** is not differentially enhanced in young mice lacking G6Pase-***α***. With the KO mouse now available for analysis throughout the stages of development, our study addressed the possibility that upregulation of this secondary enzyme could be responsible for age-dependent improvement.

Characterization of our G6Pase-***α***-/- mice verified that, per unit mass of tissue, G6Pase-***β*** is not upregulated with age (see Figure 3-6). Quantification of G6Pase-***β*** by Real Time rtPCR and Western blot analysis did not show an increase in G6Pase-***β*** transcript or protein expression, respectively, and

the activity assay did not suggest enhancement of muscle-bound G6Pase-***β*** to nearly the magnitude of the functional hepatic isoform (data not shown). Furthermore, because no other related enzymes were predicted by genomic search,(155) it is unlikely that a third yet-unknown analog is responsible for the improvement. Despite the absence of upregulation, it is still probable that G6Pase-***β*** can play a role in age related improvement. As relative muscle-to-body mass increases from 20% in neonates to 40-45% in adults,(158) a greater percentage of tissue is able to produce the gluconeogenic enzyme. While the clinical importance of muscle-bound G6Pase-beta will have to be explored with human tissue, increased muscle mass very likely contributes to the improved metabolic control that patients experience as they mature. Moreover, the patient's increased response to physiological cues and steady dietary optimization is also likely to have a role in the lessening of their symptoms.

Mice undergo a more pronounced improvement in robustness than humans do because mouse pups manifest the disease much more severely. The reasons for this discrepancy are not exhaustively defined, however the simple increase of bioavailable glucose that occurs with weaning is almost certainly behind the improvement seen in mice. While human infants with GSDIa are fed a lactose-free formula,(53) mouse pups continue to nurse exclusively. In the absence of G6Pase-***α***, KO pups are unable to metabolized lactose efficiently, and much of the consumed nutritive value is inaccessible. Prior to weaning, malnutrition impedes development, and excess sugar is shunted down alternative metabolic pathways, precipitating hyperlipidemia. Replacement of milk with standard adult chow and easily-ingestible high-calorie Nutragel® supplement gives young mice a better balance of more accessible nutrition, at which point their condition improves dramatically. Additionally, the addition of glucose to their water allowed for a quick glycemic boost interprandially, and lead to improved survival rates.

Undoubtedly, the previous reports that employed gene therapy for the treatment of GSDIa have produced marked improvements in histology and glycogen accumulation with restoration of G6Pase-$\boldsymbol{\alpha}$ activity. These changes alone verify the value of gene or cell-based therapies. It is also evident that the viral vectors have assisted afflicted pups through the pre-weaning 'crisis' period, where without any palliative support these animals would likely have died. However, our study reveals that relative glucose homeostasis, normalized serum panels, normal growth and even reproductive ability can be achieved by modifications in husbandry.

Our method for rearing G6Pase-α KO mice into maturity provides a critical tool, which may potentiate many advances within this research field. The adult KO mouse will provide a vital prerequisite for any investigational therapies requiring a larger, healthier pool of animals. Particularly, those procedures employing cell-transplantation and those wishing to investigate gene therapy potential in fully matured animals. With our current knowledge of hepatic cell transplantation, certain procedures must be implemented in order to prime the hepatic niche for engraftment of transplanted cells. With rare exception, this priming requires chemical inhibition of endogenous cells, followed by physical injury to the liver.(162, 163) Sickly KO pups would not likely survive the preconditioning for engraftment, nor withstand the recovery period from surgical cell-infusion. With the resilient adult mouse now available, research on hepatocyte and stem cell transplantation will now be much more feasible.

In summary, this detailed characterization of the mature KO mouse provides a critical standard for evaluating the true efficacy of potential treatments. The capacity to raise untreated GSD mice to maturity also provides the opportunity to test new treatment options; whereas direct stem cell transplantation to the GSD liver would be devastating in neonates, it would be relatively uncomplicated in mature animals. By breeding knockout mice, we have also greatly improved our

capacity to produce litters with knock-out pups in sufficient numbers for study. Finally, our work has shed new light on the parallel in age-related improvements seen in both mice and humans, and furthered our general understanding of this serious disease.

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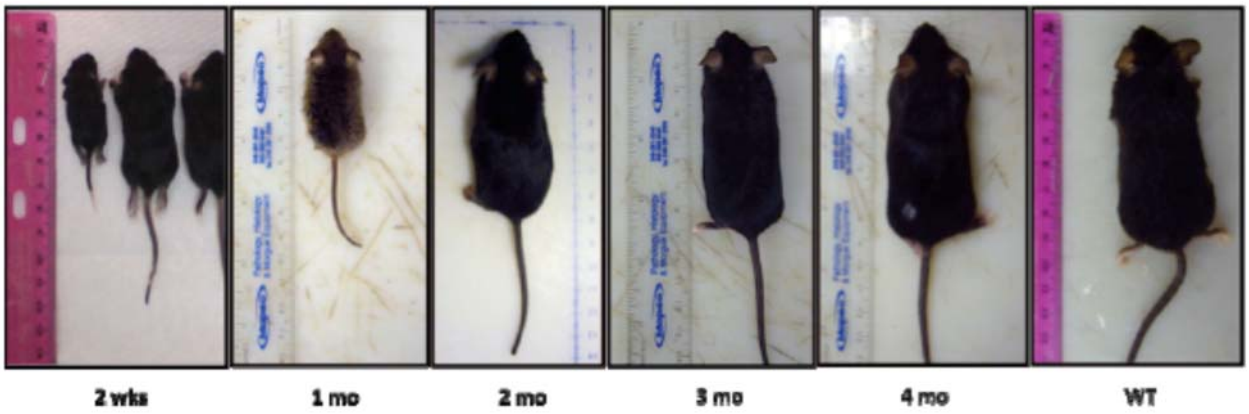
A



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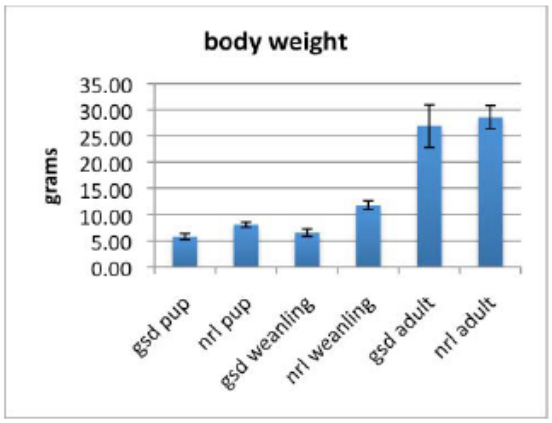

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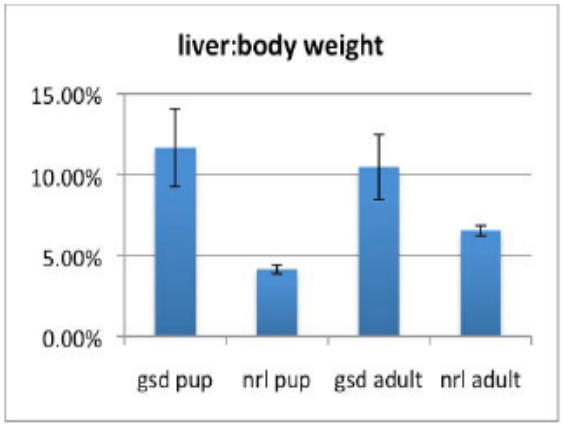

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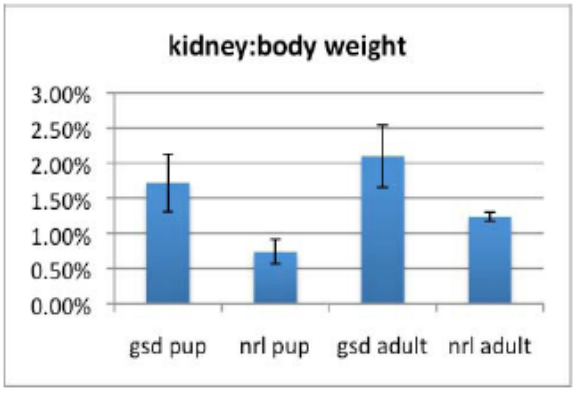
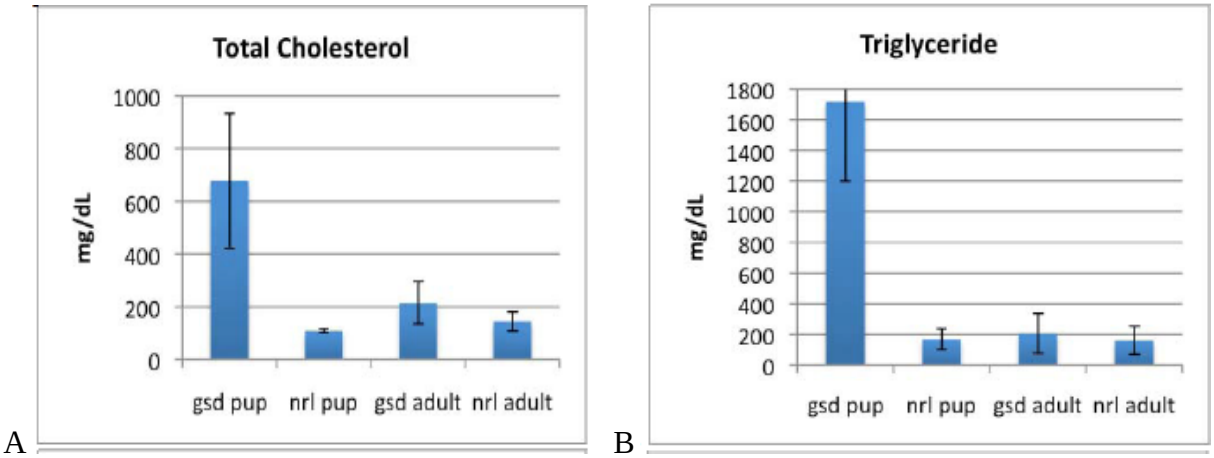
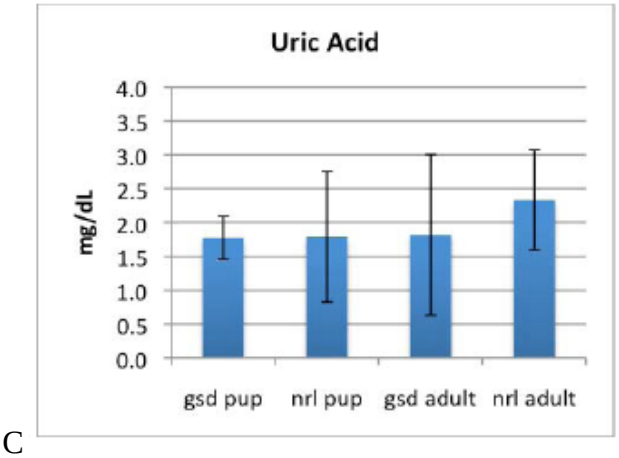


Figure 3-1. G6Pase-/- mice undergo innate morphological change as animals reach maturity. Pups that were markedly undersized were often determined to be homozygous null for G6Pase. A) Pictures of representative KO mice spanning 2 weeks to 4 months old with age-matched WT siblings at each end. Mice are pictured at the same scale. B) Total bodyweight of mice from 6 representative categories. Weights represent pups at 11 days old (n=4), weanlings at 21 days old (n=7), and adults from 3 to 6 months of age (n=6). C,D) Liver:body and kidney:body weight ratios, respectively, of pups (n=4) and adults (n=6).





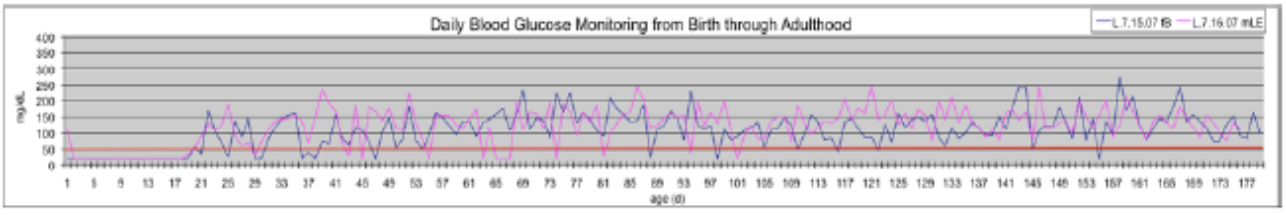


Figure 3-2. G6Pase-/- mice undergo innate change in serum chemistry as animals reach maturity. A,B,C) Serum panels from pups age 11 d (n=4) and adults age 3-6 mo. (n=6) charting total cholesterol, triglycerides and uric acid, respectively. D) Blood-glucose readings from the oldest KO mice, studied over 6 months (n=2). Mice were monitored for a total of 177 days, 155 of which were after the normal weaning point.

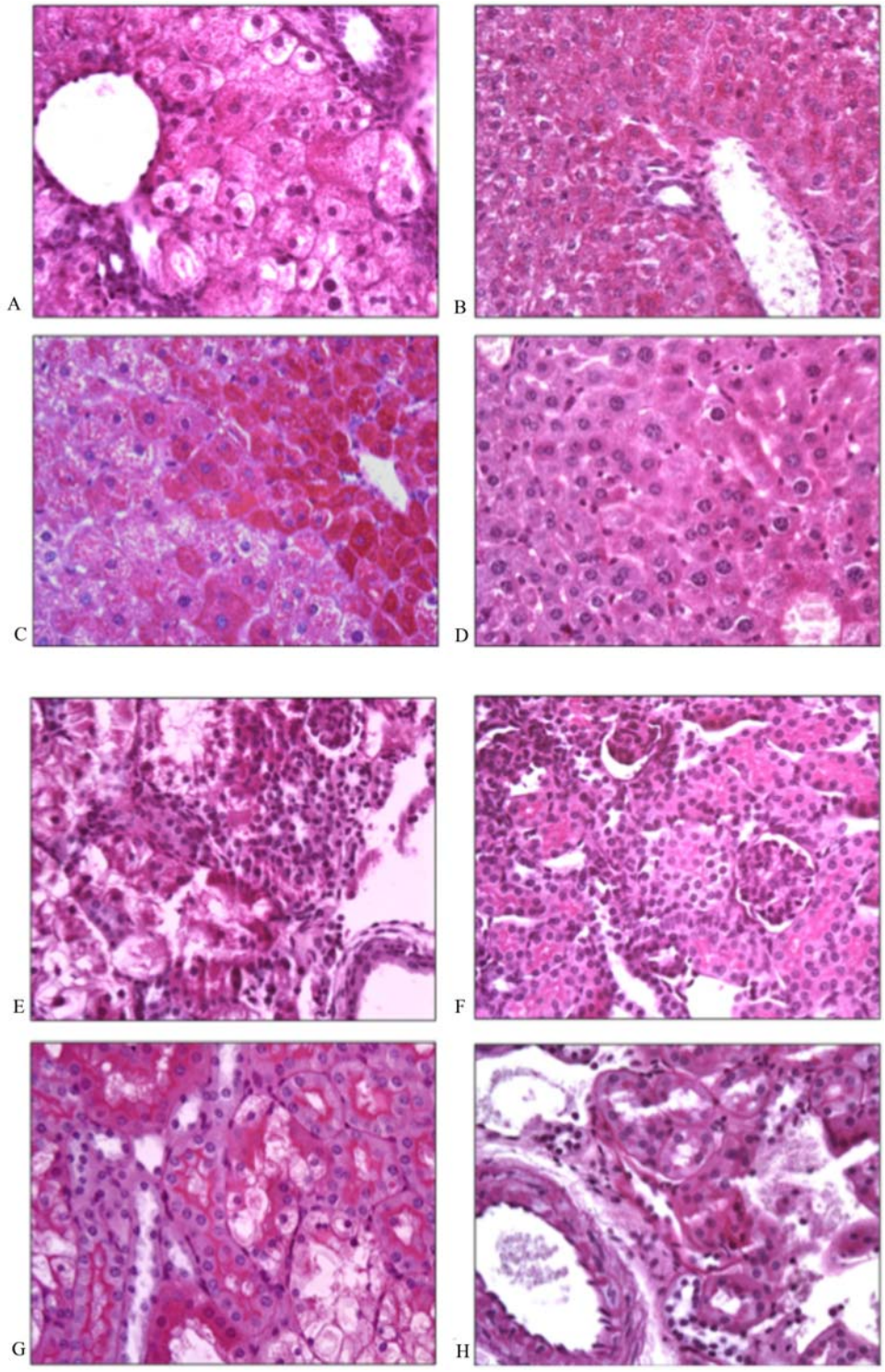


Figure 3-3. Histology and glycogen storage in the liver and kidneys of KO mice does not improve markedly with age. PAS stained liver (a-d), and kidney (e-h), from KO pup, WT pup, KO adult, respectively. Relative glycogen deposition is indicated by accumulation of magenta staining within the cell cytoplasm. All shown at 400x total magnification.

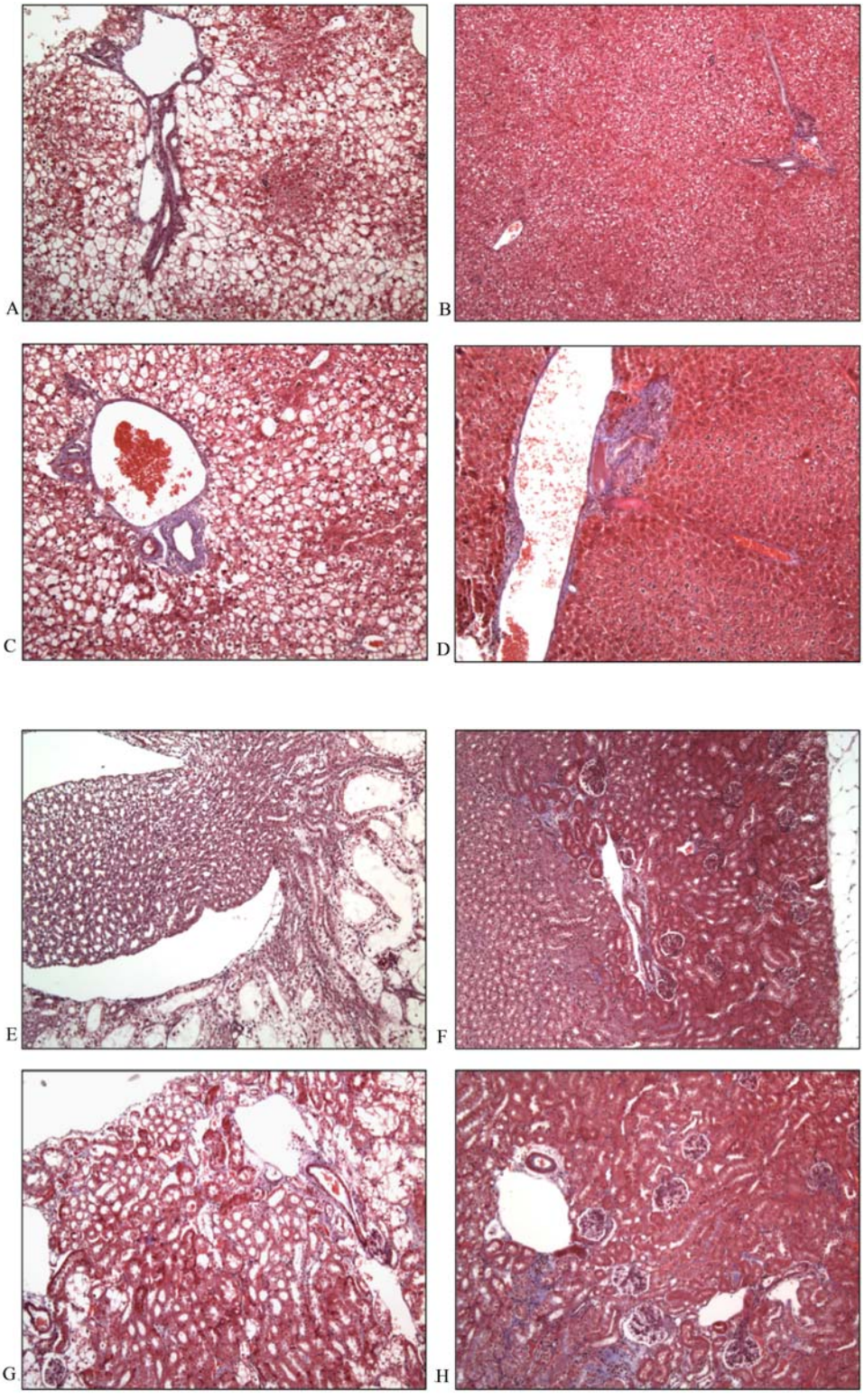


Figure 3-4. Fibrosis in the liver and kidney of KO mice does not worsen markedly with age. Masson's Trichrome stained liver (A-D) and kidney (E-H) from KO pup, WT pup, KO adult, and WT adult, respectively. Relative fibrosis is indicated by blue-stained tissue collagen. All shown at 100x total magnification.

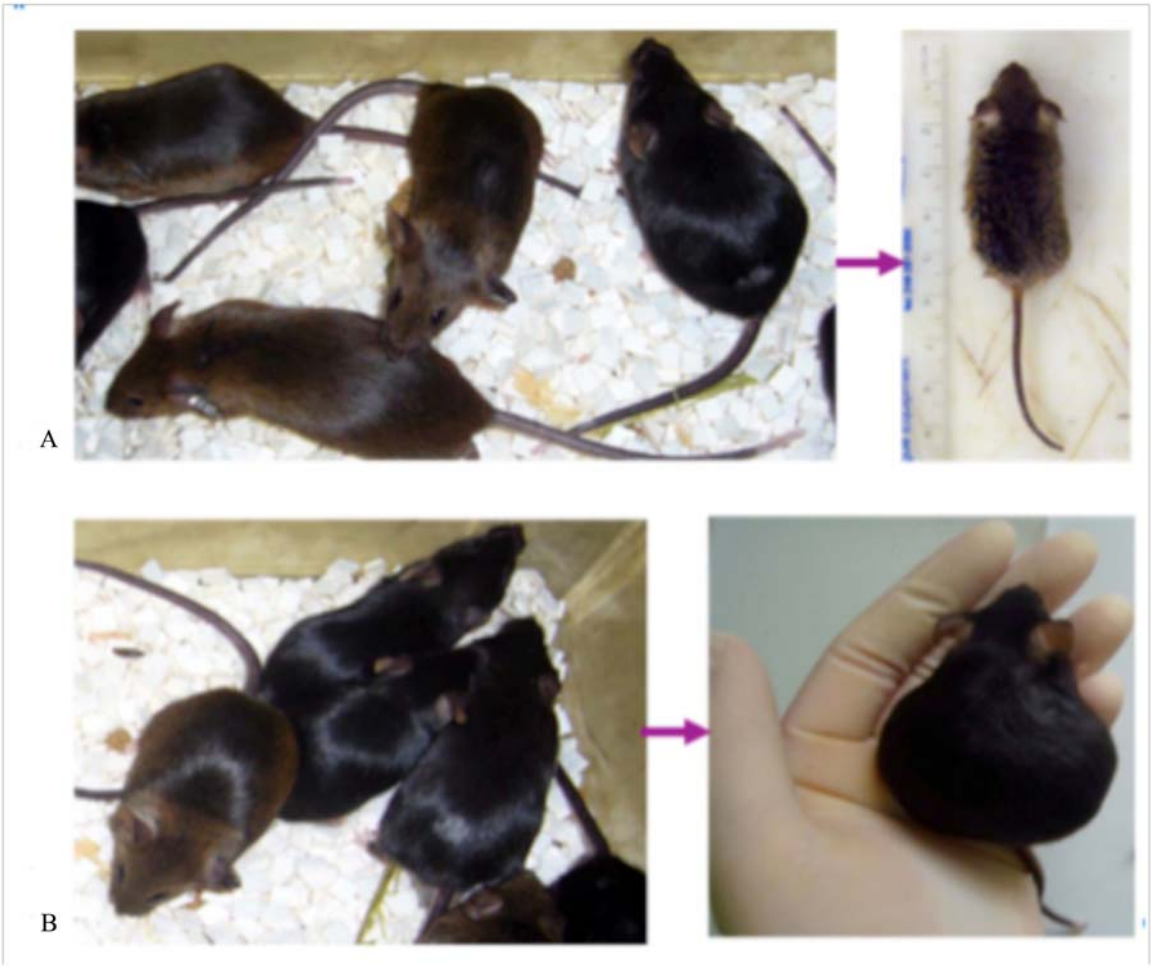


Figure 3-5. G6Pase-/- mice of both genders are able to breed to produce viable litters. A) Male KO mouse (black, top right) x female heterozygote (brown, bottom left) produced first reported litter from a KO parent. Representative KO progeny shown at right. B) Male KO (black, right) x female KO (black, center) produced first reported 100% KO litter. Pregnant KO female shown at right.

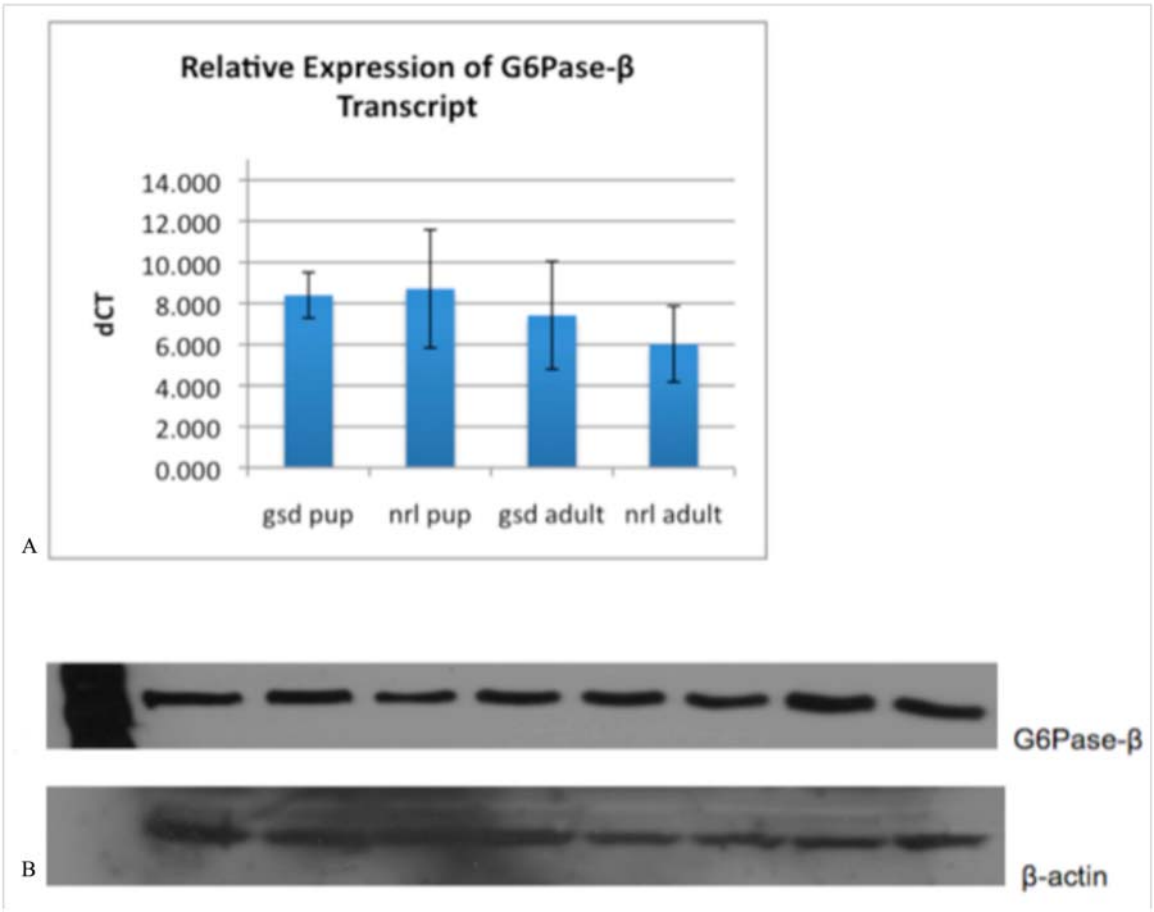


Figure 3-6. G6Pase-β was not differentially expressed in KO (G6Pase-α-/-) animals relative to WT. (a) Quantitative rtPCR of G6Pase-β mRNA in muscle tissue from adults and pups of both genotypes (KO n=5and WT n=4, both age groups). Numbers represent difference in target copy number relative to β-actin, therefore a lower mean-value indicates higher expression (b) Western blot of G6Pase-β protein in muscle tissue from adult KO (right) and WT (left) mice (n=4, both). Membranes were stripped and re-probed for β-actin.

CHAPTER 4
PREVIOUSLY UNPUBLISHED FINDINGS FROM CHARACTERIZATION OF THE MOUSE MODEL OF GLYCOGEN STORAGE DISEASE TYPE 1A

Supplementary Evaluation of GSD Mouse Model Study

Glycogen Quantitation in the Livers of the GSD1a Mouse *vs.* Healthy Littermates

Relative glycogen deposition in liver sections, as evaluated by Aperio image analysis software, displayed clear trends according to genotype and age bracket. Graphical depiction of data revealed that mean staining intensity was lowest in G6Pase+/+ mice, slightly higher in G6Pase+/- mice, and highest in KO mice. It also showed an inverse association with age, likely reflecting the feeding frequency (Figure 4-1). ANOVA statistical analysis across the 6 groups of animals (3 genotypes x 2 age categories) produced a p-value of .078 with 95% confidence level.

We suspect that the difference in glycogen storage across genotypes may actually be more pronounced than this assay suggests. The reasons are twofold: firstly, glycogen storage fluctuates and is influenced by food intake, but fasting was infeasible in this model. The PAS staining thus reflects the liver's glycogen content at sacrifice, but does not indicate the amount compulsorily stored. Secondly, the exact quantitation of staining is dependent on the range of wavelengths chosen for analysis. This eliminates inter-sample variation, but it is possible that different wavelength parameters may be more ideal for quantitating the color developed on our slide.

Analysis of Dietary Impact on Improvements in Health

Addition of glucose directly to the water supply provides a quick glycemic boost to counteract bouts of hypoglycemia, and the longer digestion required by the adult diet likely contributes to the relative maintenance of normoglycemia seen in mature mice.

Therapeutic Implications for G6Pase-β

Although G6Pase-β was not upregulated in the GSD1a mouse model, this widely expressed enzyme may still have therapeutic value; with adequate glycogen reserves, increased

muscle phosphohydrolase activity may prevent dangerous hypoglycemia. In particular, the new generations of lentiviral vectors have many of the qualities sought for gene-transfer, and a recent study by Richard *et al.* (2008) showed correction of GSDII in myocytes by lentiviral vector with muscle-specific tropism.(164) Based on this study and pending similar success *in vitro*, the potential for preventing hypoglycemia in GSD1a could be explored by providing G6Pase-***α***-/- mice with additional G6Pase-***β*** via this muscle-targeted vector.

The Early 'Mock-Sick' Phenotype

This is a brief description of the discovery and phenomenon of an aberrant or 'mock-sick' transient phenotype that has repeatedly been observed during our rearing of the GSD1a mouse colony.

Our Introduction to the G6Pase-α KO Mouse

The mouse model of Glycogen Storage Disease was known to be delicate, and thus a visit to the original founders of this mouse strain was made to obtain hands on training prior to launching a second colony at University of Florida. After this briefing on the model characteristics and handling, we returned and began our own GSD1a mouse colony with 2 breeding pairs of G6Pase+/- mice from the original colony (a kind gift of Dr. Janice Chou, NIH).

To date, all of the literature that we referenced on GSD animal models reports that animals with at least one good copy of the G6Pase gene are phenotypically healthy. To the contrary, all homozygous recessive pups are born small and hypoglycemic; a condition which persists until their death. In our hands, some of these KO pups died within a day or two of birth (complete failure to thrive), while others were able to survive until weaning; throughout which time they were critically hypoglycemic, increasingly growth-stunted, had worsening abdominal

enlargement due to liver enlargement form excessive glycogen stores, and developed a very weak bone structure with forelimbs which bent beneath their own weight.

While it is reasonable to expect some variance of size and strength of newborn mice from a common litter and genotype, a readily visible and testable difference was nonetheless understood to exist between the smaller 'runts' of the litter vs. true KO pups: typical 'runts' presented with slightly low birth weight, but were otherwise unremarkable, whereas true G6Pase-alpha-/- were markedly undersized and severely hypoglycemic.

Excitement over 'The Pellet'

In the early stages of our work, we sought more handler-friendly means of meeting the demands of this very high-maintenance animal model. Accordingly, we began a collaborative effort with Innovative Research of America Inc. on the development of an implantable slow-release glucose pellet to reduce the frequency or demand for frequent glucose injections. Following subcutaneous implantation, the 3mm pellet was designed to release glucose at a rate of 0.5mg/day over 21 days, and be replaced with a fresh pellet as needed. If it worked, our new slow-release glucose pellet would provide a much more amenable approach to managing the blood-glucose levels of the KO mice by eliminating the need for frequent injections.

We had already found that not all of the sick pups are able to survive with the prescribed 3x daily injections of 15% glucose alone, and this was also the case for those implanted with the pellet. However, those pups that did survive after receiving the glucose pellet were often found to improve in growth and glycemic control (Figure 4-2a and b, respectively). More convincingly still, in cases where 'the pellet' seemed to be working, there were clear and documented hypoglycemic events that seemed to correlate with anticipated duration of the implant (Figure 4-2b). The conclusion from such striking improvement was that the slow-release glucose pellet had ameliorated the effects of the enzyme deficiency by providing the reliable energy supply that the

KO pups had struggled for. Furthermore, it was thought that by minimizing physiochemical challenge of elevated stress-hormones from wildly fluctuating blood-sugar, the pellet was assisting in normal development.

The Glitch We Can't Fix

Despite its apparent promise, a lingering problem with the pellet remained in that it's success rate was seemingly low. While a handful of recipient pups would survive and then flourish, many did not benefit and died between implantation and weaning. These cases of 'pellet failure' prompted further exploration of post-implantation events, and surgical exposure of the implants revealed that they had crumbled rather than holding form and slowly dissolving (Figure 4-3a). After much tinkering with different pellet formulations and methods of implantation, we decided to go back and reevaluate the mice that had survived the transplantation and subsequently transitioned from small, frail and hypoglycemic to phenotypically normal.

Detection of the G6Pase gene is accomplished by PCR using two sets of primers. The first set flanks the gene itself: F' = 5' AAG.TCC.CTC.TGG.CCA .TGC.CAT.GGG 3', and R' = 5' CCA.AGC.ATC. CTG.TGA.TGC.TAA.CTC 3' , and together amplify a 264 bp fragment from +/+ and +/- mice. The second set flanks the neo cassette that replaces the G6Pase gene: F' = 5' ATA.CGC.TTG.ATC.CGG.CTA.CCT.GCC 3' and R' = 5' CAT.TTG.CAC.TGC.CGG. TAG.AAC.TCC 3' and together amplify a 667 bp fragment from +/- and -/- mice. Heterozygote mice have both bands, whereas homozygous (-/-) produce only the heavy fragment and homozygous (+/+) produce only the light fragment upon amplification.

Discovering the True Outcome of Pellet Implantation

Re-evaluation of these 'treated' survivors lead to a devastating discovery: Departure from the expected genotype:phenotype correlation in the mouse model of GSD1a resulted in early misjudgment of the pellet's efficacy. Specifically, phenotypic delineation between typical runts,

and true KO pups, was found to be error-prone in the GSD1a mouse model. Rigorously optimized genotyping protocols revealed that heterozygous pups fell into one of two categories: The majority began life with normal development and metabolic control; yet a fraction of heterozygote pups very closely mimicked the true homozygous-nulls. Figure 4-2a (right frame) shows a single litter of pups just under 1 week of age. Though two of these pups were very small and hypoglycemic, it was determined that none of them were true homozygous-null for the G6Pase-α gene; instead, they were merely 'mock-sick' heterozygote runts.

Despite the truly compelling evidence, reevaluation exposed the reality that the pellet did not help with survival of afflicted pups at all. Indeed, the true KO pups did not survive long past surgical implantation, and those pups that did survive were actually a subset of 'mock-sick' pups heterozygous for the functional G6Pase-alpha allele. Further study of the population dynamics in the GSD1a mouse colony revealed that a small percentage of heterozygous pups did bear this illusory phenotype and effectively mimic true KO pups; however, these 'mock-sick' heterozygotes were also seen to improve physically prior to weaning and usually continue on to a normal development without further intervention. This normalization includes their ranking in size and weight, as well as their ability to maintain euglycemia.

Also noteworthy, although 'mock-sick' heterozygous pups develop the ability to overcome or manage their affliction, acute incidents such as temporary food restriction, bearing a litter, or a sometimes-unknown antagonist have caused temporary resurgence of hypoglycemia. Although the young-adult 'mock-sick' heterozygous mice usually recover, there were two individuals which died with co-incident hypoglycemia. To our best approximation, there have been a dozen heterozygotes that have presented this way in the GSD1a mouse colony. We cannot exclude the possibility that a gene with similar or connected function may have been corrupted in a member

of the colony's P-generation; nor can we be certain that the 'mock-sick' phenomenon never occurs in pups homozygous for the functional G6Pase-α gene. However, we also recognize that an epigenetic modifier or supplementary gene may be naturally variant in the mouse population. If this is the case, then weakness in the G6Pase-α dosing (heterozygocity) may precipitate the intermediate phenotype that we describe in heterozygous mice of a one variant, while leaving other heterozygotes relatively unaffected.

Although this peculiar phenotype is very worthy of remark for researchers beginning their use of the GSD1a mouse, there has been no prior report of such 'mock-sick' heterozygous pups in the literature. Whether or not the founders of this particular mouse strain have witnessed this illusory phenomenon within their colony at the NIH is not known. Nonetheless, it is also notable that such a phenomenon of mildly to moderately hypoglycemic and undersized heterozygotes has also been observed in the naturally-occurring dog model, under study at University of Florida and several other institutions (personal communication with Dr. David Weinstein). Moreover, parents of GSD1a children, who are proven heterozygotes, often report a personal tendency towards interprandial hypoglycemia. These heterozygous individuals do not however seem to follow the same pattern of acute presentation in childhood followed by a more moderate adulthood (personal communication with patients/families). The question of whether or not the murine 'mock-sick' phenomenon represents an 'accident' in a relatively small genetic pool, or a clue to a fundamental genetic interchange, offers an interesting subject for future study.

We were fortunate to discover the fluke in the GSD1a mouse model fairly early in our study, and refocusing our efforts on improved means of caring for this fragile mouse strain has lead to other very substantial findings in the field, as detailed in our 2009 research publication: A Detailed Characterization of the Adult Mouse Model of Glycogen Storage Disease 1a.

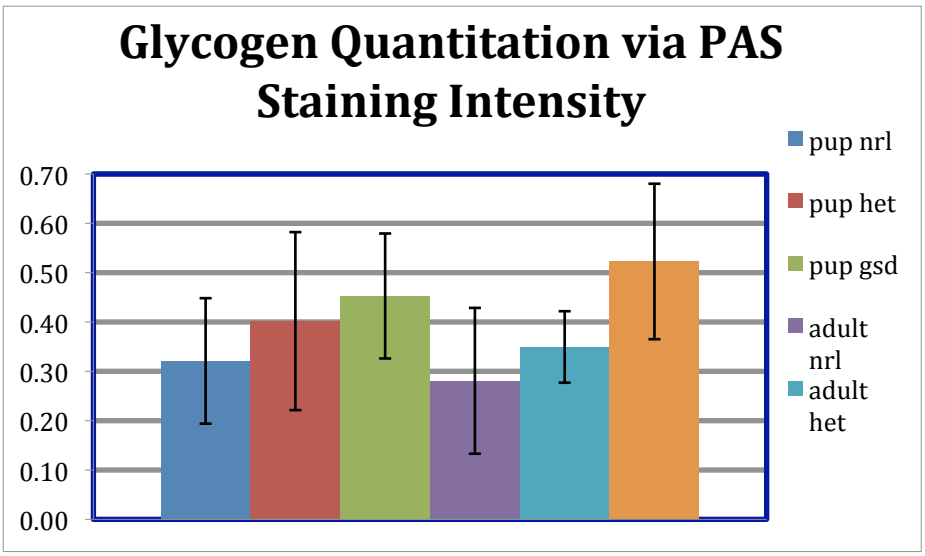


Figure 4-1. Liver histology and glycogen do not improve markedly with age. Quantitation of PAS staining in representative samples by Aperio slide scanning and image analysis program. Statistics were analyzed by ANOVA with 95% confidence level.

A

B

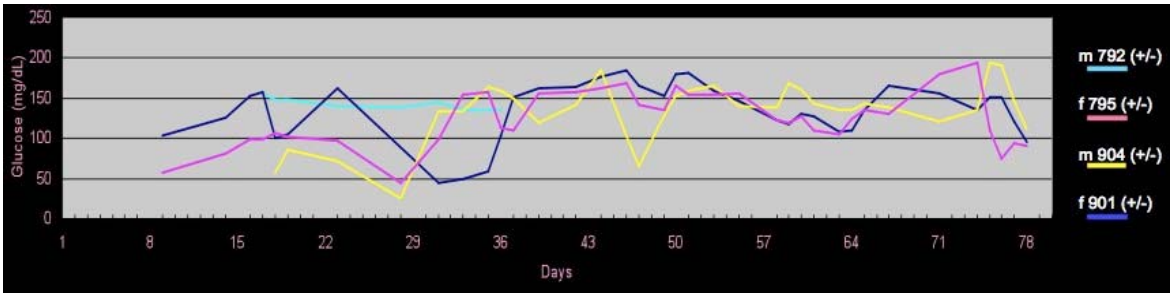


Figure 4-2. The 'Pellet': Early (incorrect) interpretation of the illusory evidence. A) Leftmost frame shows a littermates of various genotypes. The purple bars indicate the size of the small 'mock-sick' pups and the normal siblings. Middle frame shows suspected KO mouse receiving subcutaneous pellet implant. Rightmost frame shows these same littermates in maturity without obvious morphological pathology. B) graphical depiction of blood glucose levels of implanted 'mock-sick' vs. healthy mice from d8 through d78 of life. The turquoise band represents a phenotypically normal heterozygote, whereas the other 3 bands represent 3 mock-sick mice from 2 different litters.

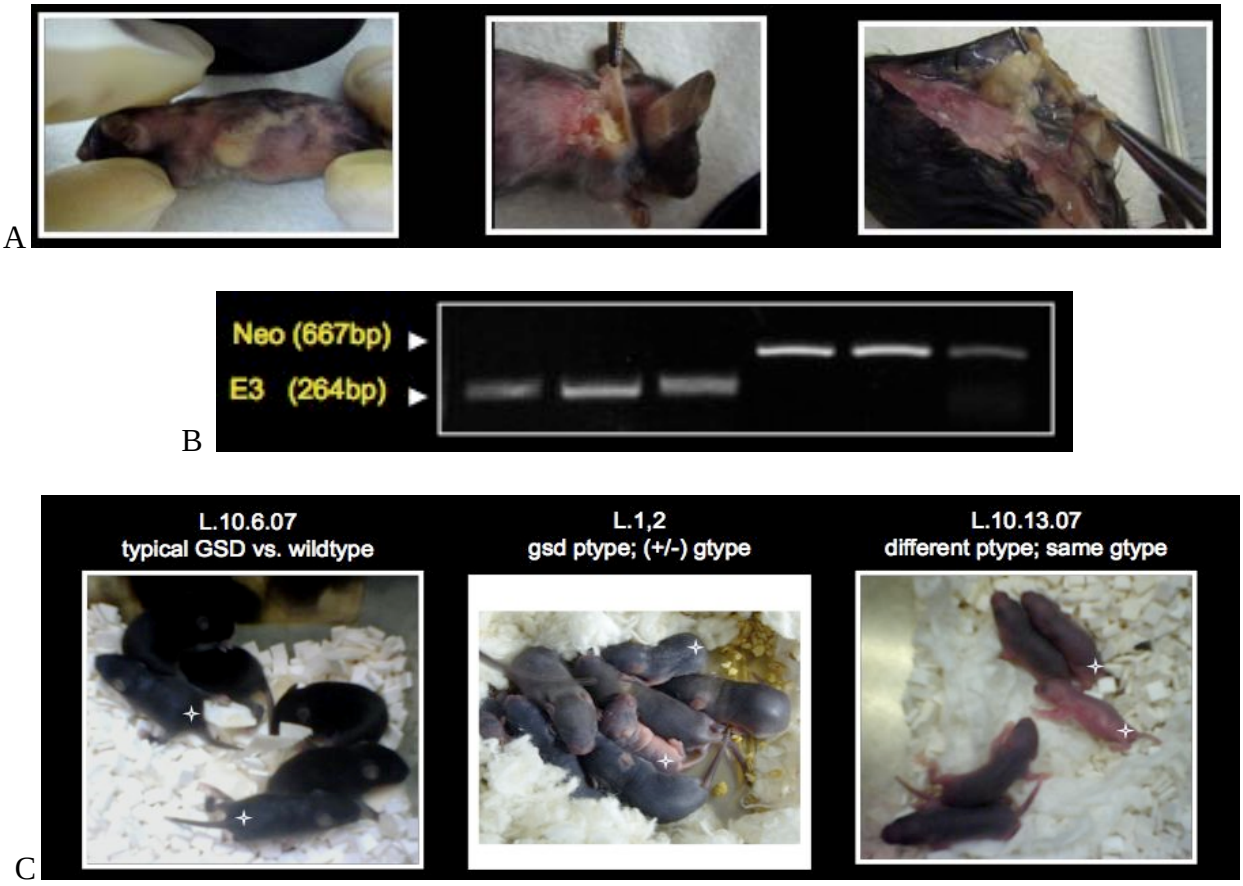


Figure 4-3. Many of the very small hypoglycemic pups did not improve despite pellet implantation. A) 3 week old G6Pase-α KO mouse pup with subdermally placed glucose pellet. Shaving the fur clearly reveals the visible remnants of the degrading pellet (leftmost frame). Placement of the pellet does not alter or improve the outcome (center and rightmost frame). B) Optimizing of genotyping protocols reveals that not all 'very small, hypoglycemic pups' are G6Pase-α null. PCR from 3 such pups confirms that all 3 are heterozygous for the G6Pase-α gene and thus 'mock-sick'. C) further elucidation of the departure from the expected genotype:phenotype correlation in the mouse model of GSD1a . Leftmost frame is a standard visual delineation of KO pups (asterisks*) alongside healthy (+/- and +/+) littermates. Center frame shows two heterozygous 'mock-sick' pups (asterisks*), with a phenotype mimicking that of true KO pups, alongside healthy littermates. Rightmost frame shows two homozygous KO pups (asterisks*) with two different phenotypes alongside littermates.

CHAPTER 5
FORMERLY UNPUBLISHED WORK ON SA1: EMPLOYMENT OF CURRENT PROTOCOLS FOR LIVER REPOPULATION IN THE GUNN RAT MODEL OF CRIGLER NAJJAR SYNDROME

Abstract

Studies in recent years have shown a seemingly limitless capacity for plasticity in adult stem cells. While it does not appear that every stem cell subtype is efficient for repopulation of every tissue, progenitors are known to exist for every mature tissue type. This study was initially designed to test the hypothesis that different hepatic progenitor cells have different capacities for repopulating the recipient liver following transplantation. Specifically, we sought to determine which cell type, mature hepatocytes, hepatic oval cells (HOCs) or bone marrow derived cells (BMDCs), was better suited to rescue the recipient animal from a metabolic liver dysfunction. The Gunn rat model of hyperbilirubinemia was selected because of the straightforward monogenic cause of the disease. It is well documented that hepatic engraftment with donor cells is more substantial when a niche is prepared in the recipient organ. In rodent models, prior to partial hepatectomy (PHx), monocrotaline (MCT) has been used to inhibit endogenous cell proliferation and confer the desired selective advantage to the infused cells. We demonstrate that MCT is not well tolerated by the Gunn rat, and suggest that the original chemical retrorsine be used to prepare the hepatic niche for engraftment.

Introduction

The Gunn rat has offered a useful model of congenital enzyme deficiency with a straightforward monogenic cause. This natural model of hyperbilirubinemia appeared a non-inbred mutant Wistar rat. As in the case of patients with Crigler Najjar Syndrome, the homozygous recessive Gunn rat has a deficiency of the liver enzyme uridine diphosphate glucuronyltransferase (UDPGT), which is responsible for conjugating bilirubin to bilirubin-

diglucuronide. Homozygous knockout rats are jaundiced at birth, and all bilirubin is in unconjugated form.(64)

For our comparative cell transplantation study, female Gunn rats which lacked the functional UGT-1A1 enzyme (Gunn j/j) were pretreated to encourage hepatic engraftment of donor cells, and then infused with either i) total bone marrow ii) oval cells or iii) hepatocytes from healthy male Gunn rats (denoted Gunn n/n or Gunn n/j). Because the donors and recipients share a genetic background, the transplantation was syngenic and mimicked a patient being both donor and recipient of their own cells. However, since the donor cells are from healthy Gunn rats with normal enzyme activity, no transducing viral vector was needed.

Preparing the Hepatic Niche for Engraftment

The concept that cells deriving from the bone marrow are capable of hepatic reconstitution has been shown a number of times through reports of cross-gender bone marrow transplantation. In particular, Theise *et al.* (2000) showed that female mice that were lethally irradiated and then rescued with bone marrow from male donor mice harbored upwards of 2.2% Y-chromosome + hepatocytes through the end of an 8 month study.(28) While Theise's study did not employ hepatic injury *per se*, other studies have shown that transplanted cells must hold a growth advantage relative to the recipient's own parenchymal cells if they are to maintain a useful presence in the recipient liver.(165) To accomplish this, the recipient animal is primed with injections of a pyrrolizidine alkaloid to inhibit endogenous hepatocyte proliferation.(166) Pyrrolizidine alkaloids such as retrorsine and monocrotaline (MCT) which arrest endogenous hepatocyte replication by alkylating proteins and DNA, and producing accumulation of cells in late S and/or G2 phase. This inhibitory effect reportedly lasts for weeks or months, despite the chemical being rapidly metabolized.(165)

After preparative treatment in the month leading up to cell transplant, the recipient receives a 2/3rds partial hepatectomy followed by immediate donor cell infusion.(6) Together, these two preparations halt endogenous cell proliferation in the organ, and open the niche in which transplanted cells can propagate quite freely (Figure 5-1).

Cell Types Selected for Infusion

Bone marrow derived stem cells (BMSCs) have been shown to produce endothelial, mesenchymal and epithelial cell types.(152, 167) While one specific cell type may be capable of full tissue repair, there are suspicions BMSCs are aided by companion cell types for the process of endogenous hepatic reconstitution. The objective was not to define the cell type responsible for producing the hepatocytes, but rather to show further proof of principle that bone marrow derived cells can rescue a damaged liver upon direct transplantation. Therefore, sub-populations of BMDCs were not separated, but rather were cleared of blood-cells and then co-transplanted.

Hepatic oval cells (HOCs) were also evaluated for their capacity for hepatic rescue following cell infusion. Oval cells appear in the liver upon certain types of injury, and are known to differentiate into both hepatic and biliary lineages. We utilized existing protocols for oval cell activation and isolation from donor animals to gather the cells for transplant.

Hepatocytes are already known to be capable of hepatic repair when properly supplied to the injured liver, so this parenchymal cell type served as a baseline for comparing the efficacy of reconstitution of functional liver tissue. Successful engraftment and function was expected to manifest as a marked decline in serum bilirubin.

Evaluation of Outcome

Evaluation of successful reconstitution is approached by a few different analyses. Blood-bilirubin levels are charted as a primary indicator of successful treatment. Conjugated (direct) bilirubin is water-soluble and reacts directly when dyes are added to the blood specimen. The

non-water soluble, free bilirubin does not react to the reagents until alcohol is added to the solution. Therefore, the measurement of this type of bilirubin is 'indirect'.(38, 64) Total and direct bilirubin are measured by spectrophotometry according to this reaction. Tissue will also be evaluated for evidence of successful cell establishment. DNA, RNA and Protein extracted from tissue homogenate can be used for PCR and southern blot, RT-PCR and Northern blot, or Western blot, respectively. Immunohistochemistry for UGT and SRY should indicate the location of donor cells in recipient tissue. Should IHC produce sub-par results or supporting data be desired, in situ hybridization will also be utilized to locate donor cells – in this case by transcripts to the SRY and UGT genes. Gross histology was also noted, both as it applies to disease pathology, and any obvious damage incurred by MCT exposure.

Materials and Methods

Gunn rats were obtained from Harlan Laboratories at 8 weeks of age. Animals were housed within the Animal Care Services facilities at University of Florida College of Medicine. They were given *ad libitum* access to water and standard rodent chow, and treated in accordance with the regulations of the UF Institutional Animal Care and Use Committee.

Animal Sacrifice and Tissue Collection

Upon sacrifice by isofluorane and cervical dislocation, liver tissue was collected and partitioned into cassettes for paraffin embedding, freezing in OCT, and microfuge tubes for storage at -80* c until ready for molecular analysis. Blood collected at the time of sacrifice was analyzed at the core diagnostics labs of Shands hospital for the levels of serum bilirubin.

Experimental and Control Groups

The initial study involved three sets of three female Gunn j/j rats as the enzyme-deficient recipients, and 5 n/j male Gunn rats as healthy, syngenic donors. Recipient animals were initially given 2 injections containing 2.25 mg MCT (150ul of 15mg/mL solution), 14 days apart, to stifle

endogenous liver cell propagation. 2 weeks following the second injection, the females underwent 2/3rds PHx and received transplanted healthy male cells via splenic injection. 3 females received plain hepatocytes from untreated male rats, 3 received BMDCs (mixed population bone marrow extract) from these same untreated males, and the last 3 received hepatic oval cells donated by male rats which had undergone the 2AAF/PHx protocol for oval cell induction.

After this initial set of recipients had been treated, a second, larger study was conducted as follows: Three experimental groups each contained 6 female Gunn j/j rats which had been treated with hepatic inhibitor MCT, and given a 2/3rds PHx prior to infusion with one of the three donor cell populations. Several controls were in place to enable correct interpretation of the usefulness of each cell type transplanted: A control group of 3 Gunn j/j females did not receive any treatment, and thus represented an ongoing baseline for animals with hyperbilirubinemia. Another control group of 3 Gunn j/j females received only a hepatic injury and PHx. This was done to show the effects of the protocols that are used to prepare the liver for cell engraftment, in lieu of treatment. This also served to normalize any beneficial effects seen with the transplanted females – because transplanted cells will not only have to combat disease, but also any damage that the liver accrued during its pretreatment. A third control was 3 Gunn j/j males who were inhibited, injured, and rescued with male hepatocytes. This control was designed to reveal any gender difference, including potential minor-histocompatibility rejection reported in female rats given male SRY+ cells. A final control made use of 3 Gunn n/j (healthy) male rats who are inhibited, injured and receive no treatment. Because their health was not affected by a worsening metabolic condition, these enzyme-competent rats only had the chemical and physical insults to

deal with. This control served as a background to reveal the consequences of pre-transplant conditioning in the healthy animal.

Obtaining Donor Cells for Transplant

Rat oval cell induction

Petersen lab developed a reliable protocol for the production and isolation of large numbers of highly purified oval cells from mouse and rat liver. For rat oval cell induction, a small pellet of 2-acetylaminofluorine (2AAF) is implanted in the lower left intraperitoneal space. The pellet provides slow release of the chemical over a 28 day period. This procedure takes approximately 3-5 minutes, and the animals recover quickly from the anesthetizing agent. Using 2-AAF pellets alleviates undue stress to the animals associated with multiple 2-AAF oral gavage and reduces the amount of human exposure to the 2-AAF. 1-2weeks post implantation, the rat receives PHx as a second means of liver insult, as described by Higgins and Anderson.(6) Oval 'stem' cells appear in the liver in high numbers on day 9-11 post PHx, presumably as a way of seeding it for regeneration. At this time, the liver is perfused (see protocols).

Oval cell harvest/purification:

Rat hepatocyte and non-parenchymal cells (NPC) isolation was performed by a 2-step collagenase digestion of the liver according to the protocol established by Seglen (1976). Small oval cells are separated first from the quickly-pelleting hepatocytes, and then from other NPCs of the 250 x g supernatant fraction. This finer secondary isolation is achieved via MACS with primary anti-body tagging the Thy-1(+) oval cells, and secondary magnetic antibody tagging the FITC end of the primary antibody. Untagged cells are eluted from the magnetic column, while labeled oval cells are retained until the magnetic field is removed. The viability of the isolates is immediately checked using trypan blue exclusion, and all retained sufficient cell number to allow for culture and/or transplant.

Alternatively, more stringent oval cell isolation can be accomplished using flow cytometric cell sorting (FACS) technology. The NPC fraction contains the hepatic oval cell population as described by Yaswen *et al.* (1984).(168) We have also reported that the NPC fraction contains the highest percentage of oval cells. A portion (approximately 30-40% of the total 2x10^8 cells) of the NPC fraction was further purified using flow cytometry. Fluorescein isothiocyanate (FITC)-conjugated anti-rat Thy-1 (1 mg/million cells) is used to label the target oval cells. A FITC rat IgG1 is used as an isotype control. Cells were sorted into two populations Thy-1+ and Thy-1-, performed on a Becton Dickinson FACS Vantage-Plus DIVA flow cytometer with 9 color capabilities. In addition, sorted cells were gated at 10-12 μm based on an approximate size for oval cells. Keeping the gate at such a small size excludes mature hepatocytes from our sample. Viability and purity was determined on all collected cell populations. Typically this procedure will yield anywhere from 5-20 x 10^6 oval cells per sort. Immunohistochemistry was randomly performed on the parenchymal and NPC fractions to ensure that the cells of interest are in the NPC fraction. Rat oval cells can be detected by OV-6 specific antibody. Those animals whose livers' are perfused will have one of the smaller lobes surgically removed via ligation and excised prior to cell isolation procedure. This tissue was used for light microscopy as well as for *in situ* hybridization and IHC. This excised liver tissue was divided in half and either fixed in a 10% buffered formalin solution or placed in O.T.C. compound, frozen in cold 2-Methylbutane (Fisher Scientific, Pittsburgh, PA) and stored at -80^{o}C. Isolation of mouse oval cells from the DDC model was accomplished in a similar manner, with Sca1 antibody in place of Thy1. Also, mouse oval cells can be identified by A6 antibody.

Hepatocyte harvest/purification:

After 2-step liver perfusion, hepatocytes are isolated from the unwanted cell types by speed-dependent centrifugation. Specifically, hepatocytes are pelleted at 50 x g leaving the smaller non-parenchymal cells in the supernatant. One rat will yield roughly 1e8 hepatocytes.

Bone Marrow Derived (Stem) Cell harvest/purification:

BM was obtained from femurs and tibias (long bones) of donor rats sacrificed via lethal dose of pentobarbital (100 mg/kg). The bones are sterilized by immersion in 70% ethanol, and the skin and muscles removed. BM was exposed by cutting the ends of the bones, and extruded by inserting a needle and forcing Iscov medium supplemented with 10% fetal calf serum through the bone shaft; gentle pipetting generates a single-cell suspension. One animal yields about 5-8 x 10^7 total bone marrow cells, of which approximately 0.5-4 x10^6 are stem cells. Cell suspension is depleted of red blood cells (RBC) by incubation for 5 minutes at room temperature with 4 mL of autoclaved RBC lysing buffer (8.32 grams NH4Cl, 0.84 grams NaHCO3, 0.043 grams EDTA per liter of deionized distilled water). Mononucleated cells are then pelleted at 250 x g. Beyond the bone marrow stem cells themselves, we suspect that the surrounding mesenchymal cell population may be needed or at least useful for efficient engraftment and differentiation in the injured liver. With this in mind, the population of cells isolated for transplant is not further screened based on marker repertoire. Should further sifting be desired, an assortment of MSC markers including Sca1, CD29, CD44, and CD106 can be employed for FACS analysis of mouse or rat, using a Becton Dickinson FACSVantage SE flow cytometer. For the GSD model, BMSC from mice were isolated in the same fashion with male mice as donors.

Partial hepatectomy (PHx):

For the PHx procedure, mice are hepatectomized under general anesthesia similarly to the

methods described by Higgins and Anderson (1931).(6) Briefly, the animals were anesthetized by isofluorane inhalation. The abdomen is shaved and sterilized using a betadine/ethanol antiseptic scrub. A small incision along the midline of the abdomen (approximately 0.5 inch in length) just below the xyphoid process is made. Following this, part of the liver is pushed out from the abdominal cavity. Three separate lobes are then ligated using 2-0 black braided silk suture material and excised. Any excessive bleeding is cauterized using a cauterizing pen. The animal is then closed; first by suturing the abdominal muscles together (about 3-4 sutures, 3-0 Coated Vicryl) then stapling the outside epidermis (about 2-3 staples). The wound was scrubbed again with betadine/ethanol antiseptic to clean the area around the sutures. The animals will then be placed in a sterile area and monitored following the operation for any postoperative stress then placed back into their normal caging for the remainder of the experiments. Again, this is a very simple operation and the whole procedure takes only minutes. All animals are expected to recover and gain normal daily activities.

Cell transplantation via splenic injection:

During the PHx procedure oval cells are injected immediately following the removal of the resected liver. The liver is then situated in such a way as to expose the spleen as the point of entry. Intrasplenic injection is performed as shown in Figure 5-2, and described by Genin *et al.* (1999).(169) Intrasplenic injection has been reported as a safe and reliable method to transplant for cell transplant to liver. Between 1.5-2e6 cells were supplied to each female recipient rat in 150-200ul saline solution. Recipient rats will receive no more than 2e6 cells per tx, as higher numbers are reported to provide no added benefit, and may even damage parenchyma.(170)

Blood loss is minimized by a tamponade of the injection site with a cotton tip along with surgical absorbable hemostat (Johnson and Johnson, Arlington, TX). An alternative way to

transplant cells is to use the hepatic vein as the site of entry. The hepatic vein method is also reported as a reliable method of cell transplantation, but is more difficult to perform successfully.

Detection of Donor Cells from Recipient Tissue

Analysis of the procedure outcome was approached at the molecular and functional levels. DNA was extracted from the livers of transplant recipients, and PCR was used to amplify any UGT gene present from donor cells. Primers to the SRY gene were also used to indicate sustained contribution of the liver parenchyma by male donor cell engraftment. Blood samples were measured for bilirubin content (conjugated, and total) from before treatment through their participation in the study, at approximately 2 wk intervals. Liver tissue from all transplanted female rats was eventually collected for paraffin embedding, frozen sectioning, and protein and DNA extraction.

PCR

A segment of tissue from the recipient liver was set aside for DNA extraction using Promega's 'DNA Wizard' kit, as per manufacturer's instructions. Briefly, a tissue-degrading solution of EDTA, Nuclei Lysis Solution (provided), proteinase K, and the tissue sample is added to a screw-cap microfuge (1.5ml preferred). The sample is dissolved overnight at 55^{o}C in a shaking incubator. To separate the DNA from the proteinaceous component of solution, the suspended proteins are precipitated and pelleted (solution included in kit). The DNA-contianing supernatant is transferred to a new microfuge tube, and DNA is precipitated with isopropanol, washed with 70% ethanol, dried, and re-eluted in 1x TE. Primers to the desired donor gene are then used to amplify a fragment from the DNA sample via PCR.

SRY is first detected using simple PCR. The 1st set of primers to the SRY gene are 5'-GGA.GAG.AGG.CAC.AAG.TTG.GC-3' and 5'-CTT.CAG.TCT.CTG.CGC.CTC.CT-3', which together amplify a ~ 135 bp product. The 2nd set is 5'-CAT.CGA.AGG.GTT.AAA.GTG.CCA-

3’ and 5’-ATA.GTG.TGT.AGG.TTG.TTG.TCC-3’. Both sets have high specificity for the Y chromosome with no reactivity to female DNA. UGT PCR Detection: UGT is initially checked by simple PCR with primers nestled within the gene’s coding region. Primers are 5’-GTC.ATC. CAA.AGG.CTC.GGG.C-3’, and reverse, 5’-AGG.GCG.TTT.TCC.AAT.CAT.CG-3’, Digestion of the PCR fragment with BstNI indicates homozygous Gunn rats by the double loss of a BstNI restriction site.

Western blot analysis

Microsomes are isolated by homogenizing 100mg of tissue in homogenization buffer (250 mmol/L sucrose, 1 mmol/L EDTA, 50 mmol/L Tris, pH 7.5), supplemented with protease inhibitor cocktail (Roche Diagnostics GmbH). The homogenate is centrifuged for 10 min at 16,000g to remove nuclei and debris and subsequently the supernatant was centrifuged at 170,000g. The microsome pellet is resuspended in homogenization buffer and protein concentration was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Microsomal protein (20 Ag/lane) was resolved on a 12% SDS–polyacrylamide gel and electroblotted to a nitrocellulose membrane (Protran; Schleicher and Schuell, Dassel, Germany). The membrane is preincubated in blocking buffer (5% nonfat milk powder, 0.1% (w/v) Tween 20 in PBS) and incubated with an appropriate diution of antibody. Subsequently, the membrane is incubated with a 1:1000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG antiserum (DakoCytomation). Immunoreactive protein bands are visualized on X-ray film using a bioluminescence assay kit (ECL Plus; Amersham Biosciences). For reference, a commercial preparation of pooled human liver microsomes can be used (BD Biosciences, San Jose, CA).

Immunohistochemistry

DAKO, citrate and High pH antigen retrieval methods were tested for suitability with each protein detected. The detection kit used is ABC vector elite, with vector avidin/biotin blocking

kit. Paraffin embedded tissue slides are buffered in TBS-T. Slide pretreatment from paraffin embedded sections involves deparafinization with xylene, rehydration through serial ethanols to water, followed by 10minute incubation in 3% hydrogen peroxide. Chosen retrieval method is employed. For DAKO antigen retrieval, slides are incubated in DAKO target reagent for 20' @ 95^{o}C then for 20' @ RT. For high pH retrieval, slides are incubated DAKO high pH retrieval solution for 20' @ 95^{o}C followed by 10' @ RT. 20' incubation in rabbit serum containing 4 drops/mL avidin solution serves as the serum block. Biotin block 15' is followed by a wash in TBS-T and 1* antibody incubation O/N @ 4^{o}C. The next day, slides are washed in TBS-T and secondary antibody and ABC solutions are made according to kit instructions. Slides are incubated in 2* antibody for 30' @ RT, then washed in TBS-T and incubated for 30' @ RT with the ABC reagent. Slides are counterstained with hematoxylin and run back to xylene. Coverslips are applied with xylaseal, and allowed to dry @ RT overnight. Immunofluorescence detection may also be used on an as-needed basis.

UGT is was also detected by IHC in attempts to support the molecular data. The 1* antibody goat anti-rat UGT was applied at 1:50, followed by the secondary rabbit anti-goat and rabbit serum background block. Difficulty in staining is often encountered because of the protein's microsomal localization, and although there was a noticeable difference in staining intensity between UGT KO and WT liver tissue, the staining was not so discrete as to enable identification of individual donor-derived cells. As with SRY gene product, UGT transgene may also be determined by *in situ* hybridization using the molecular probe to UGT on tissue slides. The probe is synthesized according to manufacturers specifications (PCR DIG Probe Syndissertation Kit, Roche, #1636090)

***In situ* hybridization**

DIG-labeled probe is made according to manufacturers specifications (Roche, #1636090), then purified with the High Pure PCR Product Purification Kit (Roche #1732668) as per manufacturers recommendations. To assess the size of the probe as well as approximation of quantity, it was run on a 1% agarose gel. Hybridization may be done on frozen or paraffin embedded tissue sections. Frozen Sections: OCT embedded tissue was cut into 5μm sections and stored at -80°C until use. The slide was fixed in 4% Paraformaldahyde. The slides will then be dehydrated in EtOH washes. Paraffin embedded sections: 4μm sections were cut and floated onto Superfrost+ slides. Sections were dried by baking for 1hr and allowed to cool to RT. Paraffin was removed by two 10 min submersion in Xylene at RT. The slide was dehydrated as described for frozen sections, air dried, aged in 2X SSC for 10 min at 75°C, and again dehydrated. Slides (frozen or paraffin embedded) are then incubated for 10 min in pepsin solution (Zymed #BC0011; 0.25mg/mL in H2O stored frozen, immediately prior to use solution is diluted to 50,000X with 0.01N HCl). After rinsing the slide in H2O to deactivate the pepsin, the slide will again be serially dehydrated. At this time the probe was added to the hybridization solution (5mL Formamide, 2mL Dextran Sulfate, 0.2mL 50X Denhardts, and 0.75mL fish sperm DNA [Roche#1467140]) and warmed to 37°C. 12-35μl hybridization solution was added to each slide, which was covered with a plastic coverslip. Heating the slide to 95°C for 5min will denature the probe and target DNA. Hybridization was performed at 37°C overnight (16hr) in a humid chamber.

After hybridization, DIG-and DNP-labeled probes are detected by incubation with either alkaline phosphatase-conjugated polyclonal rabbit anti-DIG (Roche Diagnostics) or rabbit anti-DNP antiserum (Molecular Probes, Eugene, OR, USA) followed by an alkaline phosphatase-

conjugated secondary antibody (goat anti-rabbit IgG; DakoCytomation, Glostrup, Denmark). Alkaline phosphatase activity is visualized using NBT/BCIP staining (Roche Diagnostics). Briefly, the slide was soaked for 5min in Buffer #1 (8.76g NaCl, 1.34 Tris-OH, 14,04g Tris-HCl, qs to 100mL and pH to 7.5). The slide is then blocked for 30min at 37°C with Buffer #2 (1g Roche #1096176 blocking agent in 100mL1X buffer, dissolved by heating to 37°). The slide will then be incubated for 2hrs at 37°C in a humid chamber with diluted Anti-digoxigen AP conjugate (Roche# 1093274; 1:500 in Buffer 2). After 2 washes with Buffer #1 for 15 min each, the slide was equilibrated in Buffer #3 (10mL 1M Tris, pH 9.5; 10mL 1M NaCl, and 90mL H2O) for 5 min at RT. The slide will then be incubated for 2hrs in the dark with 10-35μL of detection agent (NBT/BCIP [Roche#1681451], 1:50 in Buffer #3). The reaction is stopped by incubation with TE buffer for 5min. The slide will then be counterstained with neutral red and a coverslip mounted with aquamount (Lerner Labs #13800).

In situ hybridization for SRY is often invaluable when regular IHC detection methods are unsuccessful. The following DIG-labeled SRY-specific sequences are from the 3' untranslated region of the mRNA: SRY1, 5' AGA.TCT.TGA.TTT.TTA.GTG.TTC-3'; SRY2, 5'-TGC.AGC. TCT.CCA.GTC.TTG-3'. *In situ* hybridization for UGT can also be done on liver cryosections. DIG-labeled human UGT1A1-specific sequences are from the 3' untranslated region of the mRNA, including the WPRE (from the netherlands plasmid construct), to avoid interaction with rat UGT1A sequences: UGT1, 5'-TTG.GAA.ATG.ACT.AGG.GAA.TGG.TTC.AAA.ATT. TTA.CCT.TAT.TTC.CCA.CCC.AC-3'; UGT2, 5'-TGC.AAA.GTA.TTT.CCT.TAA.TAA.GAA. TAA.AAT.GAA.TTT.AAC.ACT.GAT.TCT.G -3'; UGT3, 5'-CCA.CAT.AGQ.CGT.AAA.AGG .AGC.AAC.ATA.GTT.AAG.AAT.ACC.AGT.CAA.TCT.TTC-3'. Control probes specific to rat albumin mRNA are as follows: ALB1, 5'-GGG.CTC.TTG.TTT.TGC.ACA.GCA.GTC.AGC.CA

G.TTC.ACC.GTA.GTT.GTC.ACG.AA-3'; ALB2, 5'-GGC.TGC.TTT.GTC.AGA.CTC.TGT.GC A.GCA.CTG.GGT.CAG.AAC.CTC.ATT.GTA.TT-3'; ALB3, 5'-TGT.TGC.CAA.TTT.GGQ.TC A.TTT.CTG.CGA.ACT.CAG.CAT.TGG.GGA.ATC.TCT.GGG-3'.

Results

Tissue of female rats that had received an infusion of male cells was tested for the presence of the SRY gene, indicating successful donor cell engraftment. PCR amplification of the SRY gene clearly showed banding from the male control sample, as well as the spleen and liver of rat 3-0, and the liver of rat 5-0 (Figure 5-3 lanes 1,2,3 and 5 respectively). There was also evidence of some SRY+ cells from lane 4 and 5 (rat 4-1's liver and 5-1's spleen), but the banding was barely detectable. The negative control showed no representative band.

Five of the nine transplanted rats survived throughout the first 2.5 month study, with bilirubin levels that seemed to decline (Figure 5-4). While these results may suggest some therapeutic value offered by the cell transplantation protocol we employed, a larger study was necessary to address any ambiguity. The four rats that died before the studies termination all showed substantial hemorrhaging of the lungs, and some of the intestines upon exploratory necropsy and tissue harvesting. Adipose tissue had a strong yellow tinge, indicative of trapped bilirubin. Blood obtained from deceased animals indicated a sharp spike in bilirubin (known as a "crisis") both leading up to and shortly after their death. From each untreated donor animal, a sample of the bone marrow cells and hepatocytes were collected for expansion in culture and storage. 2AAF/Phx treated animals likewise provided oval cells for *in vitro* work. During this early culturing, cell-maintenance techniques were fine tuned, and both bone marrow mesenchymal cells, and hepatic oval cells can be maintained to satisfaction in culture. Hepatocytes have provided a far greater challenge for long-term maintenance, and in the absence

of a more refined long-term culture system, these cells were cultured only long enough to interpret transduction efficiencies via GFP-bearing vector.

Discussion

While an obvious decrease in bilirubin over time has fairly straight-forward interpretations, lack of disease correction can be traced to an assortment of causes, by just as many methods. A frequent misconception is that rising conjugated-bilirubin is indicative of successful correction. While Total levels are expected to decrease, direct levels are also expected to stay low. This is because conjugated bilirubin is in water-soluble form, and is flushed from the body accordingly. UGT-1A1 DNA, RNA and protein should all increase as transplanted cells take hold. If immunohistochemical staining for the UGT-1A1 protein is found to be diminished relative to gene or transcript, it is possible that staining is not working sufficiently; this problem has been reported in the past, and *in situ* hybridization is often a compensatory approach.

If unpleasing results are accurate depictions of the transplant, the first suspicion would be that a problem arose during isolation (low cell survival), transplantation (insufficient numbers, or failure to localize following splenic injection), or establishment (cells reach the liver, but fail to function as hepatocytes).

Use of Monocrotaline

The results of both cell transplantation studies were not as promising as we had hoped, but the primary reason was most likely an unexpected intolerance to MCT among Gunn rats. This chemical inhibitor of resident hepatocytes was investigated for use in rodent cell transplantation studies as an alternative to retrorsine. Retrorsine has been used successfully for hepatocyte transplantation in rodents,(165) but was at times very difficult or expensive to obtain, and MCT's structural and molecular similarities made it a likely substitute among the pyrrolizidine alkaloid.(166) When administered according to dosing protocol, MCT followed by PHx was

found to greatly enhance liver repopulation by donor hepatocytes contributing to upwards of 20% of the liver within the first 6 weeks post-transplantation.(166)

The primary paper reporting the successful application of MCT to enhance donor hepatocyte engraftment used the F344 rat strain. While no significant growth of transplanted hepatocytes was observed in rats receiving only PHx, addition of 30 mg/kg MCT allowed donor cell engraftment contributing to nearly 20% of the re-grown liver on average.(166) Complications from MCT toxicity including acute pulmonary hypertension and intestinal damage were not reported to occur in the F344 strain until higher doses had been administered. (166) Given the study results, the therapeutic index of MCT was large enough in the F344 strain to be useful for improving donor cell engraftment without prohibitive side-effects.

Monocrotaline is Contraindicated for Use in the Gunn Rat

The Gunn rat is not an inbred Fisher strain but rather is a mutant variant of Wistar rat. Although the rats in our study received only a third of the dose administered to F344 rats in the earlier study , our Gunn rats experienced acute symptoms of MCT toxicity; the most prominent of which was massive pulmonary hemorrhaging resultant of pulmonary hypertension. The difference in genetic background of the animal, as well as the stress of elevated bilirubin levels may account for the sensitivity to MCT at dosage that would be well tolerated by other strains.

In addition to excluding the use of MCT in the Gunn rat strain, the severe reaction that our rats had to the chemical also prohibited analysis of any calculable therapeutic effect that might have been conferred by the donor cell transplantation. Both the rat model and human patients often experience what is known as a ‘bilirubin crisis’ when faced with other environmental insults. During these events, the body decompensates and bilirubin levels in the blood elevate dramatically.(39) Because of this negative response to external stress, it was not possible to determine whether the transplanted enzyme-proficient cells had actually had a positive effect on

health, or if the relatively steady bilirubin levels were simply resultant of rats that did not experience full decompensation.

The mixed results of the experiment beg the question of why some animals did quite well post cell Tx, and others did not. It was clear from necropsy that the animals which died early suffered notable morbidity associated with monocrotaline administration, particularly with hemorrhage of the lungs and, to a lesser extent, the intestine. While all rats were of approximately the same weight and received the same dose, the exact site of injection, strength of the animal in light of its disease, and simple individual variation could have left certain animals more susceptible to this sort of toxicity. Secondarily, it is entirely possible that transplanted cells had low survival at the time of transplantation relative to the 80-90% desired. As a first attempt at this sort of isolation and transplant, a lengthy and technically challenging procedure which must be completed in a single day, there is most likely room for improving the speed and elegance of the procedure. A second colony is now in study, repeating the initial experiment with a much larger sample size, and multiple controls. Particular care was taken to avoid cell death prior to transplantation, and should enable clearer interpretation of the potential of these various cell types.

Alternatives to MCT

An alternative to direct transplantation of bone marrow cells to monocrotaline treated animals would be indirect repopulation. In this procedure, bone marrow cells from male rats are transplanted through the tail vain into lethally irradiated female recipients. After bone marrow reconstitution, (monitored by screening for SRY), rats are started on 2AAF/PHx protocol to activate oval cell compartment and possibly bring bone marrow cells to rescue. After 4 weeks animals can be placed back on normal rodent chow to allow hepatocyte growth. Described technique could be used if problems with direct transplantation are encountered.

It is possible, but unlikely, that none of the cell populations we tested was able to duplicate the results obtained with bone marrow cells in previous literature.(8) Such an outcome may simply result from skewed bilirubin results due to monocrotaline intolerance. Alternatively however, it may also suggest that whole bone marrow transplant to liver is not ideal for regeneration. We would then test pre-identified progenitors isolated by sorting with HSC and MSC- specific markers. Another complication is that existing liver tissue has to be eliminated for new hepatocytes to take hold. Although this can be accomplished through toxicity protocols, they may create an environment not conducive for the newly transplanted cells, as toxicity and inflammatory responses could make establishment unlikely. Gross organ histology should indicate any heightened MCT-related damage. Simple DAB staining of tissue sections can also be used to visualize unusual infiltration of inflammatory cells.

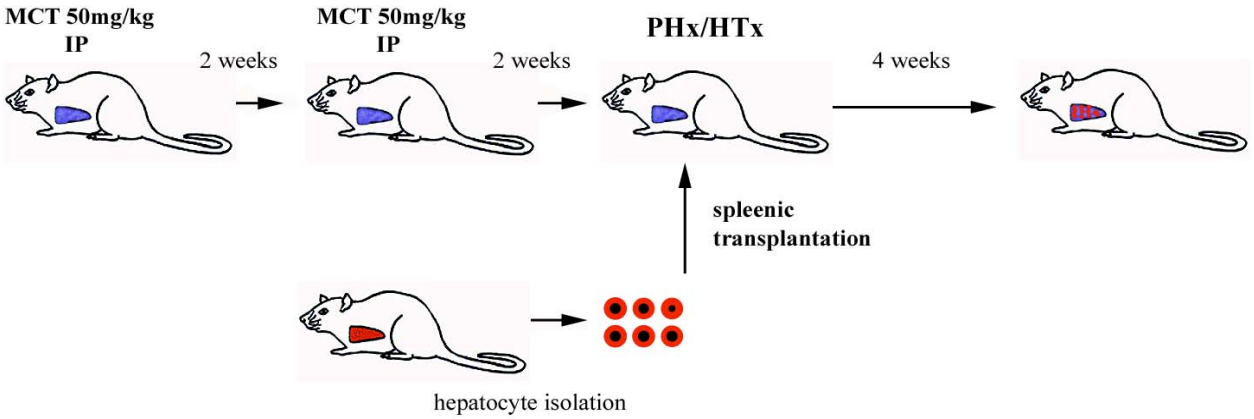


Figure 5-1. Cell transplantation from healthy donor Gunn j/j recipient. For hepatocyte and BMDC transplantation, cells are simply collected from the donor, enriched and transferred to the recipient via spleenic injection. For oval cell transplantation, the donor rat is induced to produce oval cells by 2AAF/PHx hepatic injury, prior to cell collection (Diagram by Rafal Witek).

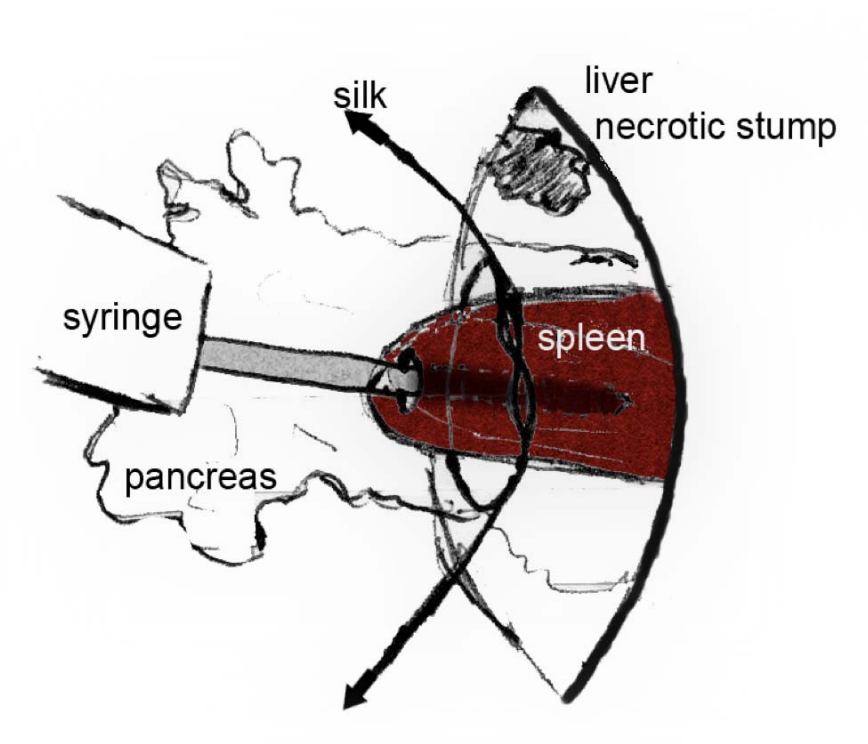


Figure 5-2. Cell transplantation via splenic injection. Transplanted cells are shuttled directly to the liver via the natural circulation.

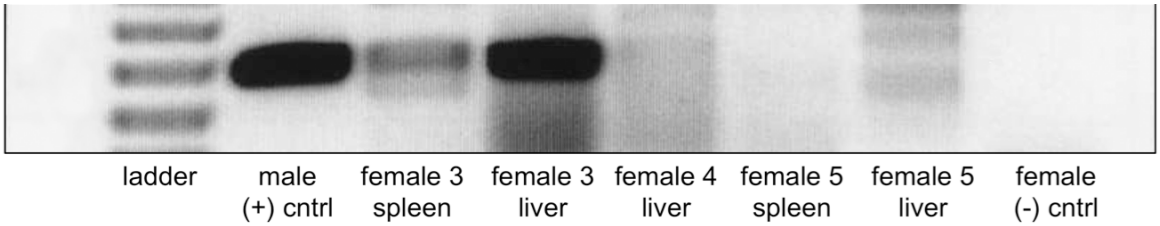


Figure 5-3. PCR products of SRY gene amplification from recipient rat tissue. SRY Banding position (300bp) is indicated by middle rung of ladder, with bands present for lanes 1, 2, 3 and 6 (representing positive control, rat 3's spleen, rat 3's liver, and rat 5's liver, respectively). There is little evidence of SRY+ cells from lane 4 and 5 (rat 4's liver and 5's spleen) as the banding not clearly detectable.

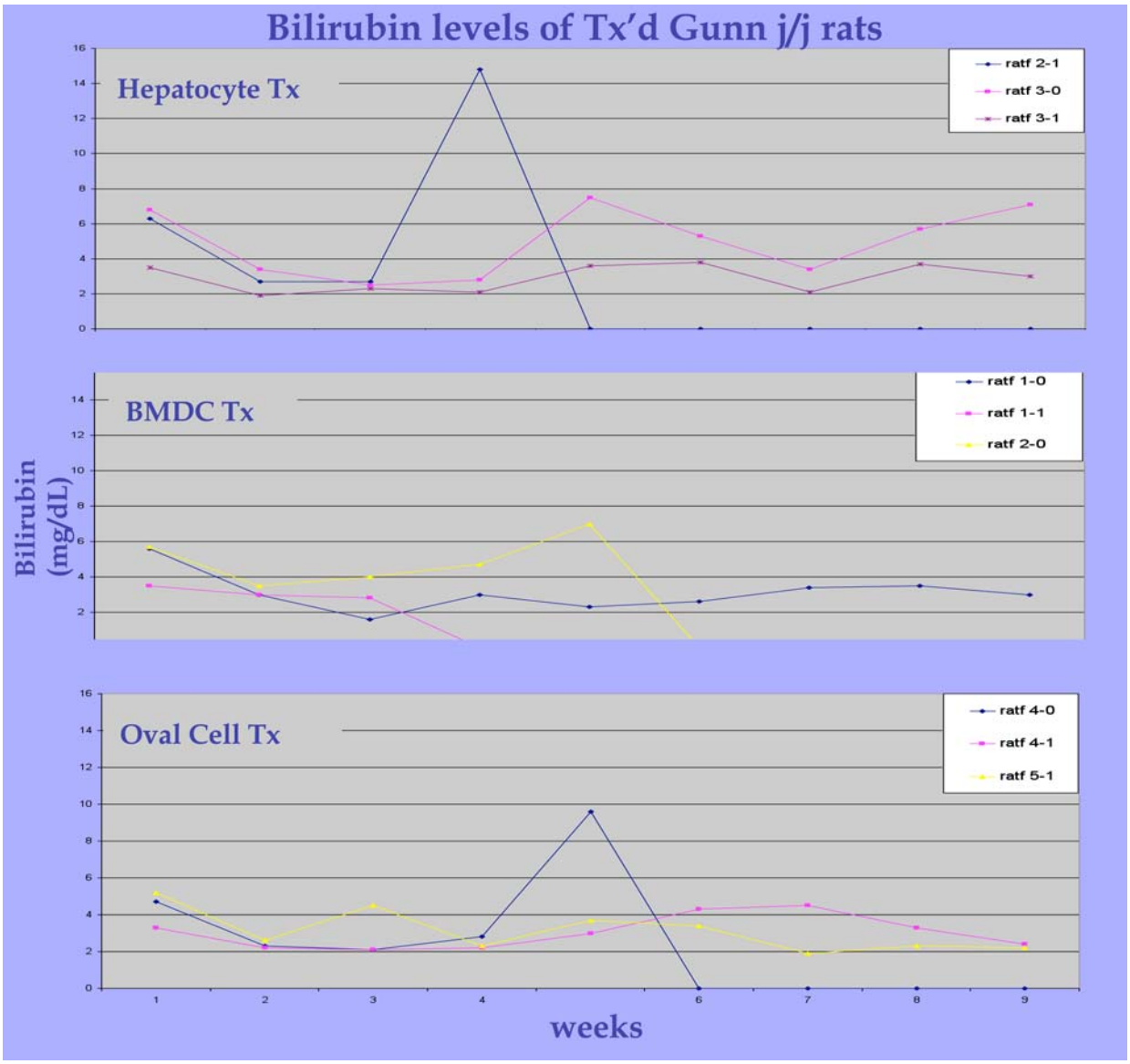


Figure 5-4. Total Bilirubin levels (in mg/dL) from Gunn j/j rats post cell Tx; Top graph depicts recipients of donor hepatocytes; middle depicts recipients of BMDCs; bottom depicts recipients of hepatic oval cells.

CHAPTER 6
FORMERLY UNPUBLISHED WORK ON SA2: TO EXPLORE THE POTENTIAL FOR *EX VIVO* TRANSDUCTION WITH VECTOR BEARING THE UGT1A1 OR G6PASE-ALPHA GENE CASSETTE

Abstract

SV40 and Lentivirus both have a number of assets which make them potentially valuable in the gene therapy arena. As a prerequisite step to *ex vivo* transduction and reimplantation, transduction efficiencies of the vectors should be determined for the various cell types used in the engineered cell infusion studies, namely hepatocytes (as baseline for cell Tx), bone marrow derived cells (BMDCs), and hepatic oval (stem) cells (HOCs). Although this comparative study was truncated to accommodate other ongoing investigations, good efficiency of lentiviral transduction was observed in the cell types tested *in vitro*.

Introduction

Crigler Najjar Syndrome (CNS) and Glycogen Storage Disease (GSD) are both liver-based metabolic disorders arising from a single mutated gene of comparable size. Because of these similarities, we suspected that we could employ a single viral vector to treat both respective animal models, by simply swapping the gene expression cassettes prior to vector production.

In order to select the best vector:cell combination for subsequent *ex vivo* transduction/ reimplantation experiments, we first designed an experiment to cross-test the transduction efficiency of Lentivirus, as well as SV40, separately in cultures of hepatocytes, HOCs and BMDCs; each of which has appeal for liver-based applications.

A recent paper from the Netherlands-based group of by Dr. Bob Scholte documented a fairly successful amelioration of CNS in the Gunn rat model with systemic infusion of lentiviral vector.(171) In order to tackle both CNS and GSD with a single systematic approach, we arranged to team our own expertise in stem cell and liver-directed therapies with the proficiency

in viral vector production offered by the Scholte lab group. Pending our own assembly of the SV40 constructs, we also arranged for collaboration with Dr. David Strayer of Jefferson Medical College for production of SV40 vector stock.

Simian Virus 40

Building the SV40 Construct

Prerequisite to making the full engineered SV40, the G6Pase cassette was excised from pGEM, and spliced into pT7[RSV-LTR] (provided by Dr. David Strayer, Thomas Jefferson University, PA). DH5alpha bacteria were transformed with pGEM11z-G6Pase (carreir construct) and separately with pT7[RSV-LTR] SV40 plasmid to increase the stock of constructs. Cultures were pelleted, both plasmids were purified (Qiagen kit), and both were cut with matching (but non-homologous) restriction enzymes. For plasmid ligation, XL10 bacteria were co-transformed on selective LB-neo plates. Only colonies bearing the pT7 plasmid were able to grow, and of these, PCR identified which ones bore a ligated, G6Pase-containing construct. The ligated **construct** was grown up in 500ml culture, and plasmid purified by CsCl ultracentrifugation. The ligated construct was sequenced with 2 separate primer sets to reconfirm presence of gene cassette, and its orientation checked by PCR and restriction enzyme digest. Ligation and proper orientation of the G6Pase gene cassette within the SV40 construct was verified by gene sequencing, PCR (Figure 6-1), and restriction enzyme digest with not1 and xho1 (Figure 6-2).

Verifying the Construct

Successful production of the SV40 vector construct was further tested by transfecting 3T3 cells with pT7[RSV-LTR]-G6Pase. With rtPCR of 3T3 lysates verifying G6Pase transcription (Figure 6-3), samples of the construct were sent to Dr. Strayer for production of transgene-bearing SV40 vector. This lab is specializes in SV40 gene therapy vectors and agreed to provide us with stocks of transduction-ready, fully-assembled vector. The transduction efficiency of this

vector is said to approach 100% for MSCs, and will be examined in the other cell types of interest to our investigation. We await receipt of vector from the Strayer group before continuing on to cell transduction assays.

Lentiviral Vector

Building the Lentiviral Constructs

Lentivirus was produced in-house according to manufacturer's protocols, as outlined in Invitrogen's Virapower manual.(172) Production of the lentiviral particles involved the use of a three-vector system. The first step in lentiviral vector production was generation of the expression construct containing the gene of interest. Briefly, the gene-of-interest was spliced into the pLenti6/V5 expression plasmid (Invitrogen's Virapower lentivirus production kit). Design of the vector calls for specific ends on the insert; the cassette could not simply be excised with restriction enzymes, but rather had to be amplified and primer extended to provide appropriate ends for ligation; one a specific overhang, the other a blunt end. Ligation of a blunt-ended cassette, requires a proof-reading polymerase that does not add "A's" to the ends of the polymerized sequence, as ordinary Taq would. Thus, Pfu polymerase was used for amplification of the G6Pase cassette.

After cassette amplification, plasmid ligation was achieved by cotransforming DH5alpha with G6Pase cassette and pLenti6/V5 plasmid. Equation 6-1 is used for calculating the molar ratio of gene:pLenti to use in the transformation/ligation:

$$\frac{\text{ng vector x kb gene}}{\text{kb vector}} \times \frac{\text{X } \mu\text{L gene}}{\text{ratio}} = \text{ng gene} \qquad (6\text{-}1)$$

According to this equation, given that pLenti6V5 = 20ng/μL, so 1ul = 20ng pLenti, we deeded to determine how many μL contained 20ng gene. This calculation determined that 7ul G6Pase gene stock is 20ng, while just 1ul pLenti stock is 20ng. Gene:pLenti ligation ratios of

1,2, and 3:1 (20ng gene = 7ul; 40ng gene = 14ul etc.) were tested to determine maximal efficiency. TOPO isomerase enzyme is coded for by the pLenti6/V5 plasmid, so additional ligase was not needed upon plating bacterial cells with ligation mix.

Bacterial colonies were selected, grown in 5ml culture and miniprepped for analysis of their plasmid content. Transformed bacterial colonies were verified to contain ligated construct by restriction enzyme digestion with spe1 and xho1 (Figure 6-4). Culture was expanded from these verified colonies, and the plasmid DNA was extracted from culture pellet.

Testing the Lentiviral Constructs

Lipofectamine 2000 was used to transfect 3T3 cells so we could test the construct's ability to confer the transgene. After incubation, RNA was extracted from transfected 3T3s Qiagen's RNAeasy kit. RT-PCR of the transfected 3T3 cells was used to verify presence of G6Pase transcript, and a lenti-GFP construct was used as a control for transfection (Figure 6-5 a,b).

HEK293-FT cells were then transfected with the expression cassette for G6Pase. Western blot confirmed that transfected cells were able to produce G6Pase protein as a fusion product with the vector's V5-epitope. The V5-epitope served as the standard target for antibody-detection, and the V5-G6Pase fusion protein was visible as a ~ 40kDa band (36kDa for G6Pase; 5 for V5). Beta-actin served as a protein loading control (Figure 6-6).

Lentiviral Production

With the construct confirmed, HEK293 producer cells were plated in antibiotic-free media so that they would be at ~90% confluence at the time of transfection. The following day Lipofectamine 2000 was used to co-transfect HEK293FT producer cells with expression construct, and an optimized 'packaging mix' of two vectors bearing trans viral enzymatic genes and envelope genes; the necessary components for assembling the virus, under the control of non-retroviral promoter. The expression construct contains gene of interest and its promoter,

flanked by cis regions necessary for targeting to the viral capsid and eventual chromosomal integration. This construct allows production of viral RNA encapsulated by transgene-encoded proteins, resulting in viral particle production. During incubation, this cell line assembles the plasmids into full therapeutic lentiviral vector particles.

After overnight incubation, media is replaced and cells are again returned to incubation. By 48-72 hrs post-transfection, virions are off-gassed into the culture medium, and this viral supernatant is collected into aliquots for determination of titer, transduction efficiency, and storage in liquid nitrogen.

Lentiviral Titer Determination

At least one 6-well plate was used for every lentiviral stock to be tittered (one mock well plus five dilutions). The day before transduction (Day 1), cells were counted and plated in a 6-well plate such that they would be 30-50% confluent at the time of transduction. They were incubated 37°C overnight in a humidified 5% CO2 incubator. On the day of transduction (Day 2), lentiviral stock was thawed and 10-fold serial dilutions prepared ranging from 1:100-1,000,000. For each dilution, lentiviral stock is diluted into complete culture medium to a final volume of 1 mL. Culture medium is removed from the cells, and replaced with a mL of viral stock. For increased transduction efficiency, Polybrene® was added to each well to a final concentration of 6 μg/mL. Plate was gently agitated to distribute the solution and then incubated at 37°C overnight in a humidified 5% CO2 incubator. The following day (Day 3), media containing virus was replaced with 2 mL of complete culture medium. Cells are again incubated overnight at 37*c. Starting on Day 4, cells were subjected to blasticidin selection. The blasticidin selection marker within the viral expression construct enabled stable propagation of exclusively transduced cells in the presence of blasticidin antibiotic as described on page 12-14 of the

Virapower user manual.(172) Cells transduced by the packaged virus gained blasticidin resistance and thus survived in the presence of this media additive, while those that were not transduced did not survive. To achieve this selection, culture medium was replaced with fresh medium containing antibiotic every 3-4 days. After 10-12 days of selection (day 14-16) no live cells were visible in the mock well (well that had not received viral titer), but discrete antibiotic-resistant colonies were present from at some wells that had received a dilution of viral stock.

Crystal Violet staining enabled identification of surviving and non-surviving cells in wells containing various media concentrations of blasticidin, as well as various dilutions of viral stock. To achieve this, media was removed and cells were washed 2x with PBS. Crystal violet solution was added to each well (1 mL for 6-well dish; 5 mL for 10 cm plate) and the dish was left to incubate for 10 minutes at RT. After incubation, excess stain was removed by two washes with PBS. At this time, a count of blue stained (surviving) colonies provided an estimate of the number of viable virions per volume of viral stock. Colonies were counted from the well that contained visible colonies after exposure to the highest vector dilution. The well with the next highest dilution was also counted. The titer was calculated as the average of the cell counts x their respective dilution. An example calculation would appear as follows: at 10^6th there are 5 colonies visible, and at 10^5th there are 46 visible. The average of 5 x 10e6 and 46 x 10e5 = 4.8e6 TU/mL = the viral titer produced by the HEK293FT culture. Figure 6-7 illustrates an estimated viral stock titer of ~ 4.8e6TU/mL as indicated by the degree of cell viability.

Although notable cell death was observed amongst the original HEK293FT producer cells in our preliminary assay, our viral titers were calculated at 3e5 TU/mL. While this first production round produced a relatively low viral titer, the viral supernatant may be further concentrated by ultracentrifugation.(173, 174)

Lentiviral Transduction Efficiency

The Virapower user manual indicates that 10 mL of lentivirus at > 1e5 TU/mL is generally sufficient to transduce at least 1 million cells at a multiplicity of infection (MOI) = 1. In our hands, the viral transduction efficiency was tested by exposing various types of cultured cells to virus bearing the GFP transgene cassette. Viral supernatant was removed after 12 hours and replaced with fresh culture media to prevent cell toxicity. Transduction efficiency was reflected by the fraction of cells expressing GFP 3 days after exposure to viral titer.

With desirable results from the initial GFP experiment, plasmid expression constructs were made for each gene of interest (G6Pase-α for GSD1a or UGT-1A1 for CNS) by swapping out the GFP cassette. Following the same procedure for cell transduction, we had further planned to quantitate the production of each exogenously provided enzyme product by antibody mediated targeting (immunohistochemistry and/or Western Blot).

Discussion

A growing body of scientists have recognized the value of combining stem cell and gene therapy approaches for the correction of disease. With regard to hepatic disorders originating from an enzyme defect, tremendous improvement in health outcomes may be made by harvesting one's own cells for genetic correction *ex vivo,* and then returning them to the patient where their function can reverse the course of disease.

Bone marrow derived stem cells (BMDCs), hepatic oval cells (HOCs) and regular hepatocytes are each suspected or known to hold promise for use in interventional liver repair. While some statistics are known for BMDC and hepatocyte transduction efficiencies, little is known about oval cell receptiveness to transduction, and even less their ability to express a transgene product once they mature and establish residence in the liver. This specific aim was

designed to reveal which cell:vector combination was best at conferring transgene activity to the enzyme-deficient rodent liver.

Although this aim was a prerequisite step en route to the planned *in vivo* investigations, multiple pitfalls highlighted the complexity of culture-based optimization. Microbial contamination, low viral titers, variable transduction efficiency and unsustainable cell lines were just a few of the speed bumps to determining which cell types, in what culture conditions, with which virus administered at what m.o.i., might be most advantageous for correcting a metabolic disease in a living animal. While some cells are highly robust, others are very particular in regards to their plating density, media replacement rate, and growth matrix. Meticulous attention to sterile technique greatly reduced the risk of culture contamination, yet the use of antibiotics was absolutely necessary to prevent microbial takeover. Penn/Strep was the first line of defense, but kanamycin, fungizone and others were also useful for combating infections.

Problems inherent to the viral stocks were fundamentally more difficult to troubleshoot. If culture-related factors are ruled-out, retitering was indicated, as viability is known to decline with shelf age and/or repeated freeze/thaw cycles. Titers that come back low required determination of whether the stock had lost viability, or was low in titer to begin with, which would necessitate further optimization of the production and harvesting protocols.

Safety precautions were also crucial at all stages of work with the viral vectors. Invitrogen responsibly designed their pLenti6/V5 vectors to be self-inactivating (a “SIN” vector).(172) This was achieved by having the packaging signals, necessary for production of new virions, provided exclusively by the HEK293-FT producer cell line. Once virions assembled and diffused into the culture medium, they were capable of a single infection, but unable to replicate once they have infected a target cell. Essentially, the infection is ‘contained’ relative to

the initial viral titer that the body was exposed to. Nonetheless, because the loss of skin integrity could still allow for primary infection by SIN vectors, exposure still could result in a oncogene activation in a given transduced cell, or a positive preliminary HIV test, which would need to be dispelled by further molecular testing. The primary concern over handling SV40 vector stock was in relation to its natural tumorigenicity. This undesirable tendency was addressed by the production of modified versions of the virus which no longer harbor the gene encoding the insidious large T-antigen (Tag).

Several constructs bearing the EF1a, CB, or CMV promoter are still in storage for future investigations, however, the investigator should note that improvements in vector design are always being made, and that our archives are best used for their individual cassettes, rather than as stand alone constructs. The albumin promoter may be another one worthy of investigation, however its function *in vitro*, particularly in non-hepatic cell types may be insufficient. CMV is unlikely for further employment as it is often silenced in the liver.

The pending collaboration with the Netherlands-based Scholte group offers another testable lentiviral system, with different promoters that have proven to yield high transgene product. These promoters include the CAG (aka. CB) as a baseline, and SFFV, which has far outperformed other promoters in all cell lines this group has tested (up to 100x better expression than CB). Efficacy of liver-specific hAAT (presentation - GSD conference) offers another promoter option to weigh against the others. Additionally, the increasingly popular WPRE element, shown to increase stability of the lenti-conferred transgene, is among the cassettes used in this vector to increase its appeal. Demonstrated improvements in transduction efficiency with this vector system may negate the need for in-house production of pLenti6/V5-UGT.

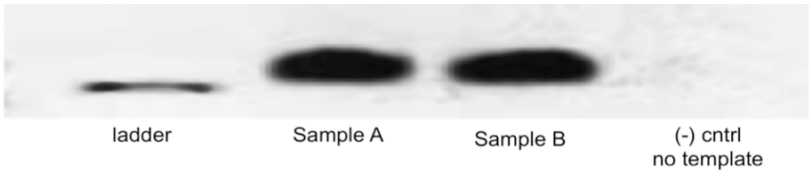


Figure 6-1. PCR of G6Pase gene insert from ligated pT7-SV40 plasmid. pT7[RSV-LTR]-G6Pase plasmid was tested to confirm presence of gene cassette. Ligation was carried out in bacteria grown on selective plates so that pT7 uptake was needed to survive. Strong bands at 1kb verify presence of G6Pase cassette as well.

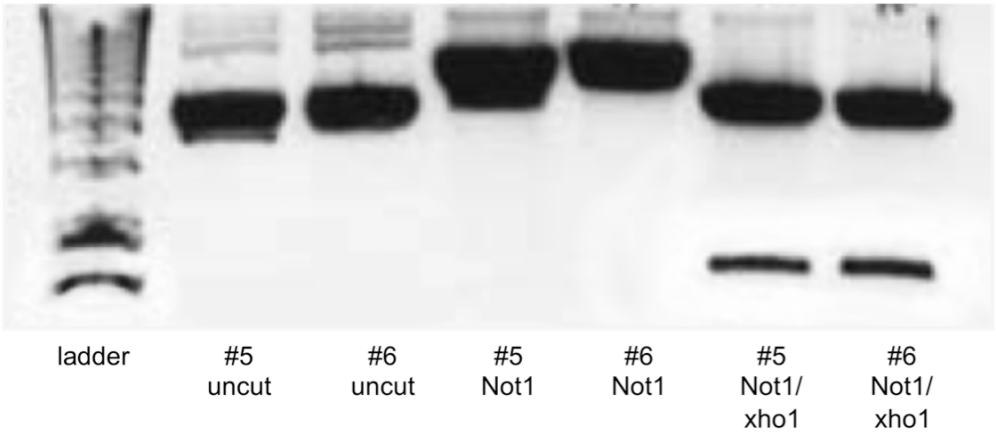


Figure 6-2. pT7[RSV-LTR]-G6Pase RE Digest. From large culture of prescreened colonies. Single and double digestion of ligated plasmid stock indicate presence of cassette. Lanes 1 and 2 show samples of uncut plasmid. Lanes 3 and 4 show single-cut plasmid, and lanes 5 and 6 show excised G6Pase cassette at 1kb, with an equivalent loss of size to the pT7 band.

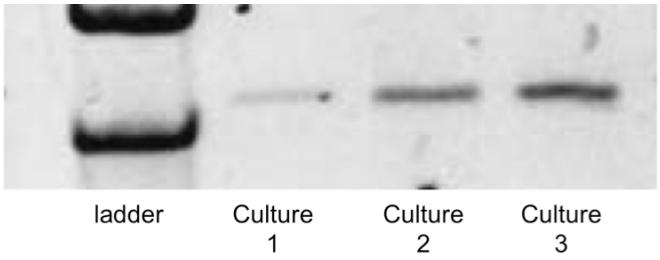


Figure 6-3. pT7-G6Pase RT-PCR from transfected 3T3 cells. RT-PCR was performed on RNA samples from three wells of 3T3 cells transfected with the ligated SV40 plasmid. Transcription of the plasmid's G6Pase cassette is clearly depicted by the banding at 1kb.

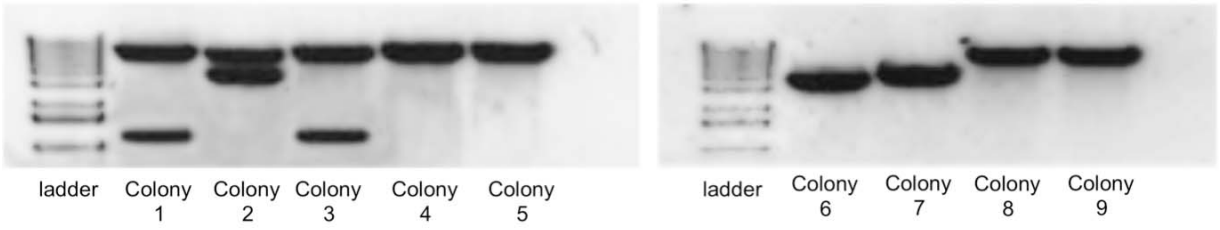


Figure 6-4. pLenti6V5-G6Pase RE Digest. Screening colonies for growing up in large culture. Double digestion of ligated plasmid stock indicates presence of plasmid. Lanes 1 and 3 show excised 1kb G6Pase cassette, with equivalent loss of size to the pLenti band.

A G6Pase-α

β-actin

B

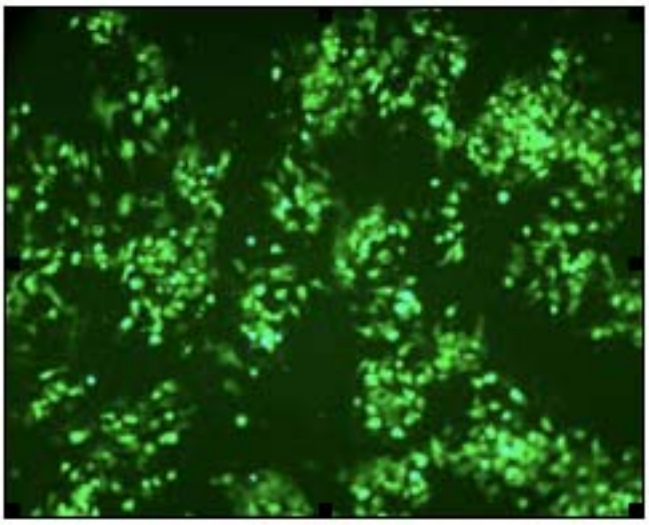


Figure 6-5. pLenti6/V5-G6Pase transfected 3T3 cells. A) RT-PCR of RNA samples from 3 wells of 3T3s transfected with SV40 construct. Transcription of the plasmid's G6Pase cassette is clearly depicted by the banding at 1kb. β-actin served as a sample loading control. B) pLenti transfection control – pTYF-UGT-pIRES-eGFP from transfected 3T3 culture. Control well was transfected with GFP-plasmid for visual confirmation.

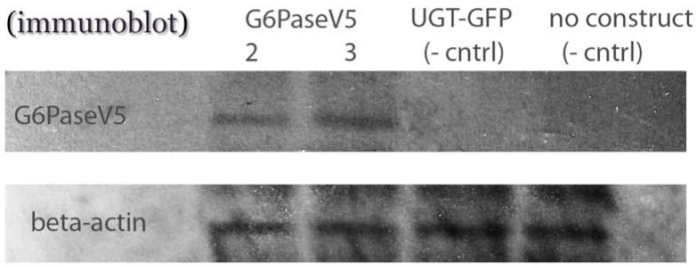


Figure 6-6. pLenti6/V5-G6Pase western blot from transfected HEK293FT lysate. 41kDa band represents antibody recognition of the G6Pase + V5 fusion protein. β-actin served as a sample loading control

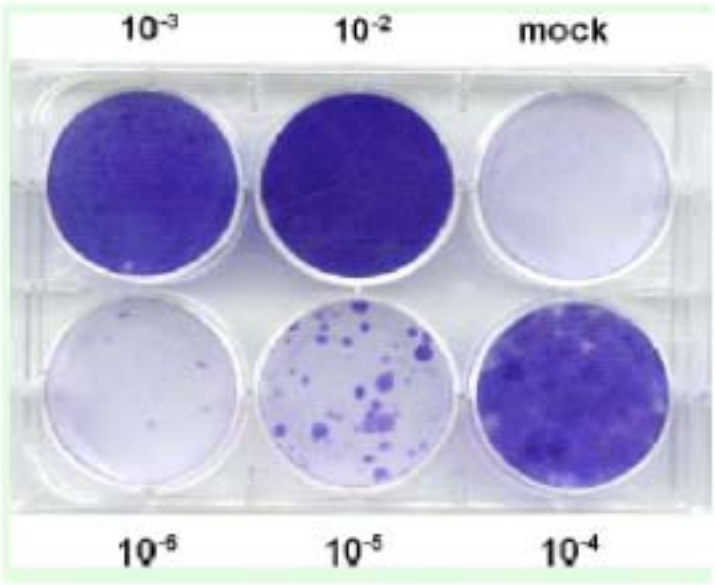


Figure 6-7. Example plate from crystal violet titering assay. Live cells stain blue. Cells are plated at the same density, and given various dilutions of vector. Only transduced colonies survive in selective media.

CHAPTER 7
FORMERLY UNPUBLISHED WORK ON SA3: TO EXPLORE THE THERAPEUTIC POTENTIAL FOR TREATING INHERITED METABOLIC DISORDERS *IN UTERO* BY INFUSION OF STEM CELLS OR LENTIVIRAL VECTOR

Abstract

Today's burgeoning technology offers many promising strategies for curing patients with inherited metabolic disease. Indeed, our capacities for treating disease have transcended the traditional stages of independent life, and now offer hope to patients that are still in the womb. Our investigation sought to determine whether we could reliably provide intrahepatic transgene activity to mice *in utero* via direct infusion of lentiviral vector or bone marrow derived cells into the developing mouse liver. Both of these modalities employed GFP as a visible marker of both location and penetrance of the therapeutic vehicle. While neither approach seemed to yield hepatic GFP expression in levels sufficient for therapeutic value, both methods produced expression patterns with potential value to other investigations.

Introduction

When one thinks of repairing a genetic disorder, the strategies which most often come to mind involve infusion of genetically engineered cells or therapeutic viral vectors to the individual who has presented with the disease. In turn, the individual is typically envisioned as an adult, child or even infant who has come to the clinic with an evident dysfunction. In reality however, our limitations have ever less to do with size or robustness of the individuals in need of treatment, but are instead merely dependent on our ability to accurately diagnose them. With the modern day availability of amniocentesis and high-precision robotics, we now have the technical capacity to deliver treatments directly to a developing fetus while still in the womb.

By intervening at this very early stage, patients who are otherwise destined to enter the world with very challenging physical limitations could be spared a great deal of hardship and

instead only know a 'normal' life. This is certainly the case patients identified *in utero* to have an inherited metabolic disorder caused by a single wayward gene.

While we were in the midst of developing our protocols for rearing a full colony of robust GSD1a adult mice, our investigation of *in utero* treatment strategies sought to bypass the difficulties of working with the KO mouse in its young and excessively frail form. In common with the future studies planned for the adult KO animal, our *in utero* study aimed to reliably provide intrahepatic transgene activity via direct infusion of lentiviral vector or bone marrow derived cells (BMDCs) into the developing mouse liver. Both of these modalities employed GFP as a visible marker of both location and penetrance of the therapeutic vehicle. Neither the stem cell transplantation nor the lentiviral infusion seemed to confer useful amounts of transgene expression to the liver, the GFP signal that was detected within post-partum mouse tissue may hold valuable clues for investigations with alternative objectives.

Materials and Methods

Animal Housing and Husbandry

Our mouse colony began with 2 breeding pairs of G6Pase-alpha $^{+/-}$ mice on the Black6 background strain (a kind gift of Dr. Janice Chou, NIH). Mice were given *ad libitum* access to standard rodent chow and housed within the Mouse Pathology Core at the University of Florida College of Medicine.

Bone Marrow Derived Cell Isolation and Preparation for Transplant

First, we isolated BMDCs from a GFP-expressing mouse (kind gift of the Ed Scott lab). Briefly, long bones of hind limbs were stripped, one head of the bone clipped off, and a 25 gauge needle was used to flush 200ul of BMSC culture media through the bone marrow shaft and into a collection tube. Slurry was concentrated by 5' centrifugation at 1000xg. The top 150ul supernatant was decanted from the sample and the pellet plus the remaining 50ul supernatant was

resuspended. This concentrated cell slurry was placed on ice briefly prior to use, and extra slurry was combined with DMSO (10% final solution) and stored at -80^{o}C.

Preparation of Viral Vector for Transplant

Lentivirus was provided by the Steve Ghivizzani lab at a practical infectivity of 5ul to 100,000 plated cells. The viral stock was stored at -80^{o}C until use, and did not need further preparation prior to infusion.

Infusion of Cells or Vector *In Utero*

Timed matings between mice were verified by the presence of a post-coital vaginal plug. On day 14.5 of pregnancy and embryonic development (e14.5) female mice were anesthetized by intraperitoneal administration of 300ul Avertin, made by combining 2.5 gm 2,2,2 Tribromoethanol, 5 mL 2-methyl-2-butanol (amylene hydrate, tertiary amyl alcohol, and 200 mL distilled water of neutral pH.(175) Laparotomy was preformed to reveal the two uterine horns containing the developing embryos.

A hand-crafted glass needle was carefully inserted through the uterine wall and embryonic sac directly into the peritoneal cavity of fetal mice, targeting the liver. 5ul of solution containing either the lentiviral vector stock or the prepared GFP-expressing BMDCs cells was pneumatically delivered directly to the hepatic region of each e14.5 fetal recipient. This procedure was done on 3 separate occasions: the first time with untreated BMDCs injected into 3 embryos at e14.5, the second time with RBC lysis buffer treated BMDCs injected into 5 embryos at e13.5, and the third time with lentiviral stock injected into 4 embryos at e14.5 of development.

After the infusion, the pregnant female mouse was sutured, received topical antibiotic and anesthetic, and was allowed to come out of general anesthesia. Under cage rest, females carried the litter for the remainder of gestation and had uncomplicated deliveries.

External observation of 1 day old pups under ‘GFP goggles’ revealed some noticeable green around the lower abdomen and in some cases laterally. We bore in mind that milk in the stomach/intestine could appear as a GFP signal with only naked-eye examination, and proceeded to collect tissue for more detailed evaluation.

Animal Sacrifice and Tissue Collection

Pups were sacrificed by overdose of isofluorane on day 1, or on day 9 of life. One day old pups were then sectioned through the midline. The right and left halves were placed in separate cassettes. Because of their size, 9 day old pups were sectioned through the midline, and then the left or right halves of the cranium, thorax and limbs were placed in separate cassettes. Paraffin embedding was not appropriate because the alcohol dehydration steps prior to staining denature the protein and destroy the GFP signal. Nonetheless, the natural vibration of the GFP protein necessitated its fixation to prevent gradual deterioration. To address this, samples were fixed O/N in 4% paraformaldehyde (PFA), and then embedded in OCT and frozen at -80 degrees Celsius. Fixation of GFP+ tissue was important to prevent degradation of the fluorescent signal.

Evaluation of Samples Post-Harvest

OCT embedded fixed-frozen tissue was sectioned at 6um thickness. Representative sections were cut and HandE stained in order to help identify anatomical structures (Figure 7-1). The remaining sections were not stained, but examined directly under a dual GFP filter comprised of a turquoise light, and a yellow light more specific to GFP wavelength emission. Presence of GFP signified successful engraftment GFP BMDCs or transduction by the GFP-lentiviral vector.

Results

In Utero Bone Marrow Cell Transplantation

For the first round of transplantation, 3 pups were infused on e14.5 of development with 5ul of GFP+ BMDCs. All 3 were born healthy, and sacrificed on day 1. All 3 pups had obvious clusters of GFP-expressing cells (Figure 7-2). The transplanted cells were not however localized to the liver, but rather were clustered in the mesenteric tissue, the intestine, and scattered throughout the parenchyma.

The second round of transplantation was instead carried out at e13.5 (one day earlier), and utilized BMDCs that had been exposed to RBC lysis buffer (eBiosciences, San Diego, CA). Although the cell isolation and injection steps seemed to be uneventful, only 4 out of 5 infused embryos were alive at full-term birth. Pup #4 from this second round did not appear to contain any GFP-expressing cells upon histological examination (data not shown).

In Utero Lentiviral Vector Infusion

The third round of transplantation utilized eGFP-expressing lentiviral vector. Four e14.5 embryos were injected IP as close as possible to the liver without complication. The results of this vector infusion were generally less promising than the BMDC infusion. Little transduction was noted in pups sacrificed at either day one (pups 1 and 2) or day nine (pups 3 and 4). Both one day old pups had very few GFP+ cells in what appeared to be the intestinal epithelium (Figure 7-3) The nine day old pups had a spattering of GFP+ cells in the intestinal epithelium (approximately 11 GFP+ cells in a single 200x frame). As seen from the BMDC infusion, no GFP signal was detected in the liver of any of the pups examined.

Discussion

It is unclear why pups from the second round of BMDC infusion did not appear to harbor any GFP signal. It is our suspicion that the viability of transplanted cells was compromised

following treatment with the RBC lysis buffer. Another possibility is that the donor animal was not truly GFP(+); the genotype of the donor animal was ensured by the prior handler, however the 'GFP goggles' were not available and thus expression was not personally confirmed.

A general limitation to using GFP+ cells as a gauge of engraftment efficacy is that only an estimated 30% of cells from so-called GFP+ mice individually express GFP. With 70% of donor cells not expressing the fluorescent surface marker, it is difficult to fairly estimate the number of donor cells which successfully engrafted into the fetal recipient. Moreover, in reality the fraction of cells expressing GFP varies by individual cell population, and certain cell types may or may not express it at all. A separate mouse strain with enhanced-GFP expression (upwards of 80% of cells GFP+) was also known to represent a possible alternative, but the excessive cost, likely due to its poor breeding capacity, prevented utilization for our study.

One possible remedy to the problem of spotty GFP expression within a cell pool is to FACS sort the cell slurry taken from the long-bones of the GFP+ donor prior to infusion, and inject only those cells which actively fluoresce. If a pure mesenchymal stem cell population was desired, cells could also be sorted for markers relevant to this progenitor type (ie. Negative sort for CD-34, CD-45, CD-11b and c-kit, with a positive sort for CD-105, CD-90, CD-44 and Sca-1). This does not however guarantee that these same transplanted stem cells will continue to express GFP once they have differentiated and engrafted *in vivo*.

One additional concern in relation to our particular investigation is that the donor mice we received produced GFP under control of the CMV promoter. This promoter is known to be silenced in the liver when applied to viral vector transgene expression, and we could not be certain that this did not also occur in the context of cell transplantation.

The reason for the low signal observed in pups that had received lentiviral infusion *in utero* is also not defined. While the literature mentions the rapid activation of the type-1 IFN response in the liver of adult animals exposed to lentivirus,(82) this same response was not necessarily anticipated to occur from the immature immune system of the developing embryo. It is also possible that the lentiviral stock lost considerable viability prior to use, but exact determination of this factor will require another repeat investigation with vector from a single stock divided for concurrent use in embryos as well as in test culture.

A major overarching consideration for *in utero* infusion of G6Pase (or any corrective gene) via cells or vector is the issue of being able to accurately genotype the pup once it is born. Genotyping could be confounded because it may be difficult to ensure that all cells from the tissue sampled were free of exogenous gene. If for example a stem cell from that transplanted bone marrow slurry made its way out to the tail tip and propagated, it is possible that genotyping would reveal a false positive for the G6Pase gene from a pup that would be a total G6Pase-KO in the absence of earlier intervention. Possible way around this problem is to take a sample of the amniotic fluid at the time of *in utero* infusion, but this could come with risks to the tiny mouse fetus, as well as leave the sample vulnerable to contamination by maternal cells. Because we knew that none of the fetuses had any endogenous GFP expression, this was fortunately not a complication for our preliminary cell tracking study.

In terms of therapeutic value however, neither GFP-expressing stem cells nor lentiviral vector maintained a substantial presence in the post-partum murine liver. While it is not possible to ensure a direct translation in meaning between GFP and G6Pase signal localization, it is likely that G6Pase that localized in this way would not be therapeutically useful. Still there are questions of what the true implications of localization are. For example, wild-type stem cells that

are transplanted into a KO fetus would not likely express G6Pase unless they differentiate into hepatocytes in vivo; however, this may not be the case for transduced cells that express G6Pase under the control of a constitutive promoter. Furthermore, the outcome of a G6Pase-α expressing stem cell which differentiates into working muscle is not necessarily simple to predict, as the muscle of GSD1a mice naturally contains the slower-acting G6Pase-β analog, and speedy utilization of muscle glycogen stores could have unpredictable results (rapid depletion, or the capacity for euglycemia). Even in the latter scenario, it is likely that hepatic enlargement would continue as liver glycogen stores remained untapped.

Given the results of our *in utero* infusion studies, another worthy consideration is that the transgene localization resulting from either the cell or vector infusion approach may hold therapeutic value for entirely different treatment objectives. While it was clear that neither approach produced substantial transgene expression in the liver, the mesenteric, and more importantly the intestinal tissue did contain transgene expressing in levels that may be of use for certain applications. Successful transduction or engraftment of mesenteric tissue could, for example be useful for continued systemic release of hormones or other biochemicals. Alternatively, the capacity to effectively incorporate exogenous gene activity into intestinal tissue may hold value for treating gastroenterological disease, such as that seen in Crones disease or certain manifestations of Irritable Bowel Syndrome.

Moreover, the promise for correcting liver IMDs specifically may be evident in larger animals, even though hepatic contribution in mice was minimal. There is a very real possibility that stem cell and or viral vector infusion is capable of bringing transgene function to the liver stably, and that this was not readily achievable with the tools and the tiny animal model that we had to work with. The mere size of the human fetus would provide tremendous advantage for

highly specific targeting of infusion into the liver parenchyma; especially with the employment of more high-tech robotics. While our pilot studies did not reveal a shot-gun approach to curing the GSD1a mouse prior to birth, it is clear that the results still hold clues worthy of consideration for the planning of future studies.

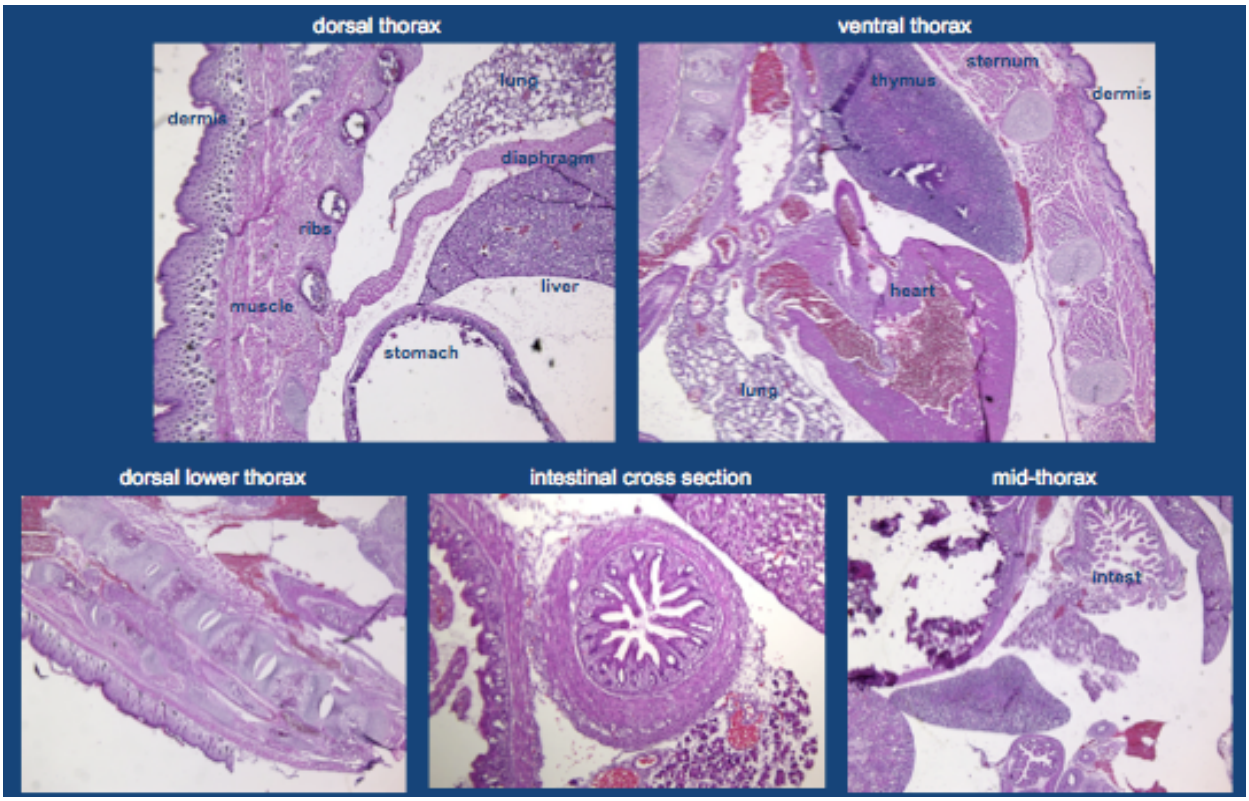


Figure 7-1. H&E staining of tissue sections from a 9 day old mouse pup. Anatomical structures are labeled for ease of orientation.

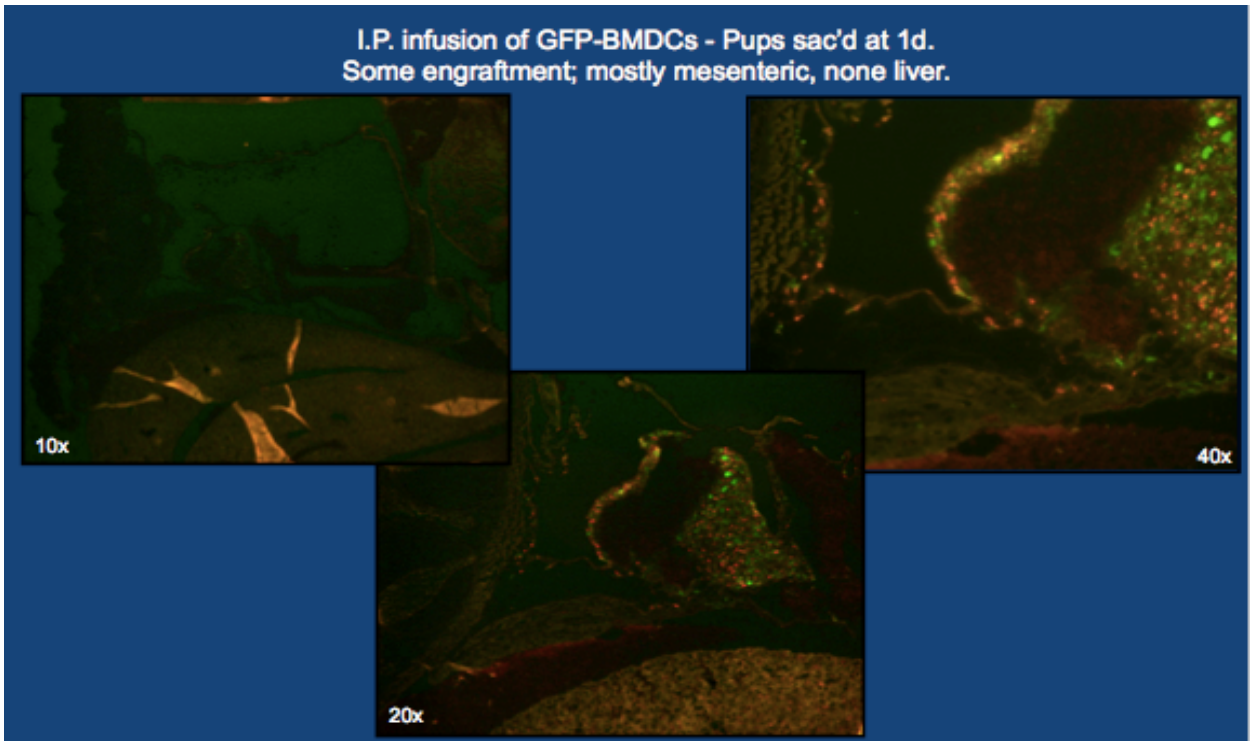


Figure 7-2. Cross sections from 1 day old pup that had received IP infusion of GFP-BMDCs at e14.5 of embryonic development. From left to right, shown under 10x, 20x and 40x objective (equivalent to 100, 200 and 400x magnification, respectively).

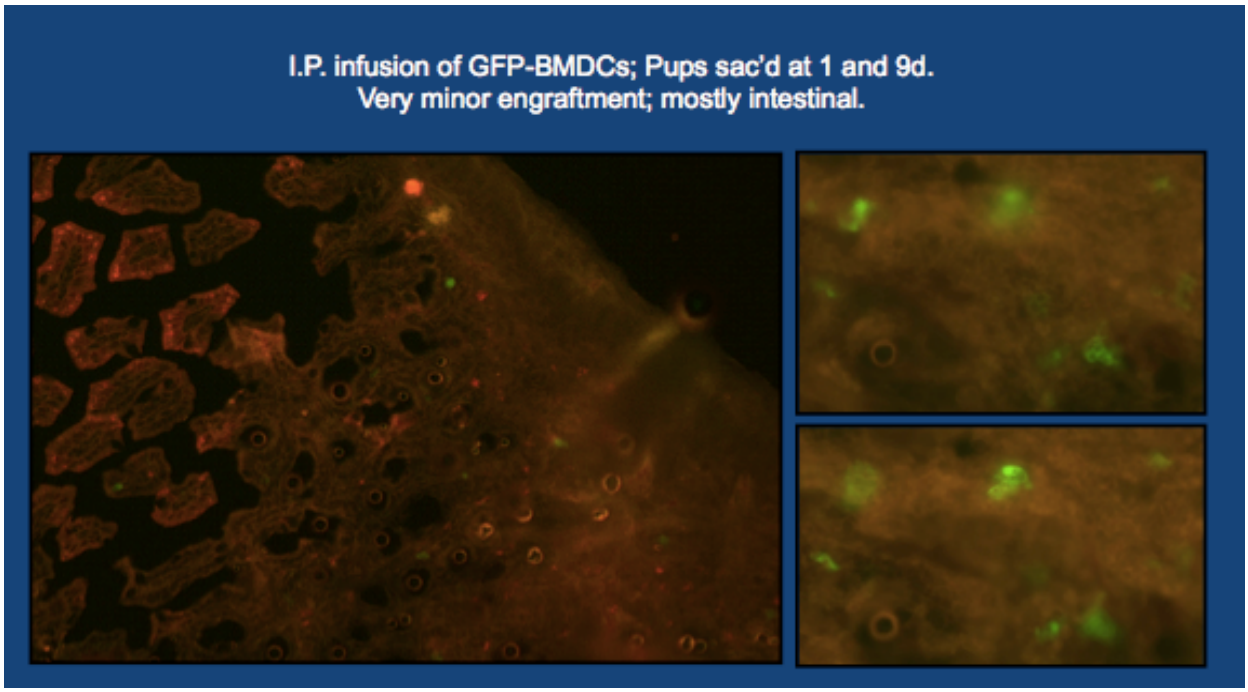


Figure 7-3. Intestinal cross section from 9 day old pup that had received IP infusion of eGFP-Lentiviral vector at e14.5 of embryonic development. Clockwise from left, shown under 40x and 100x objective (equivalent to 400 and 1000x magnification, respectively).

CHAPTER 8
CONCLUSION

Valuable Lessons for Young Scientists, and Reflections for the 'Experienced'

The many experimental avenues that we explored in the course of our research produced a wealth of both positive and data that, with acute interpretation, can hold value for upcoming investigations in a number of different fields. While not all of our primary assumptions were initially correct (ex. the efficacy of the glucose pellet) reevaluation of our findings produced clarity, as well as some valuable overarching lessons of research; namely that:

- things are not always as they seem
- you should not always believe what you think
- peculiarities are not always outliers, but are sometimes clues

Such reevaluation should be frequent, conscientious and objective in any branch of scientific inquiry, and the researcher must avoid personal attachment to the immediately desired outcome; for even unsought outcomes often uncover new purpose for study.

Meaning to the scientific community

Successful completion of a repaired stem cell approach to therapy could springboard a health-preserving therapy to patients with severe metabolic liver disorders. While many industrious teams pursue ameliorative treatments by way of viral vector or cell transplantation alone, the unity of these approaches may turn out to be a strategy that is hard earned, but well suited. We are certainly not alone with our inkling that combinatorial approaches hold promise in future biomedicine; indeed, groups worldwide, including our Netherlands collaborators are carving a path into this expanded territory.

The complications we faced in the use of our animal models ultimately rerouted our efforts towards filling in the critical gaps that had been abandoned for the more technological advancement-oriented investigations. As our investigation has shown, such obstacles are not

only guaranteed in the process of research, but are also needed for an occasional regrounding of the scientific community in terms of where it truly stands in the course of its progress. Despite the unavoidable pitfalls of highly ambitious research, the survey of these new approaches to medicine has been promising, and careful and reflective pursuit will unveil information that will hold value to research across the biomedical fields.

Future Directions for Related Research

Questions that will need to be answered prior to serious consideration in the clinic include the feasibility of harvesting various cell types, and safely expanding them *ex vivo* when necessary. Future investigators will be faced with a mountain of required proofs, showing vector purity, interactive tendency in the genome, and minimal immunological disturbance. The need for transducing the recipients own cells *ex vivo* will demand extreme meticulousness for preventing any sort of contamination from the external environment, and also reassurance that the cells you pull out retain their obliging multipotency at the time of reintroduction. Long-term study of transgene stability, both alone and in the context of cell survival, will need careful evaluation. Also, the means of handling an unusual patient response with swiftness and confidence will need time-tested demonstration before any such genetic therapy goes mainstream.

Equally as important is a more thorough understanding of the pathologies associated with these disorders, especially with regard to their origin, degree, and potential for turn-around. Volumes have been learned about the clinical implications of GSD and CNS within just the past few years. Beyond the ever-growing literature, international meetings bring both afflicted families and working scientists to the apex of new knowledge; indeed, the communications there can save months research on what would otherwise be a fatally-flawed experiment. Still many details - the molecular interactions of these wayward proteins, the reasons that tumors develop

in some patients but not in others, the complete explanation to age-related improvement, and so many more, await full understanding.

We recognize that a direct and accelerated association exists between the stage at which a medical intervention is employed, and the ultimate level of health that the patient still has the potential to attain. It is our hope that the publication of our investigative findings, in full disclosure, will nudged curative therapies for GSD1a a significant step closer to the clinic. With conscientious dedication, a greater number of patients will be able to receive effective corrective treatment before the more intractable stages of their disease can take hold.

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BIOGRAPHICAL SKETCH

Susan V. Ellor was born in 1982, in the city of Binghamton, New York. She spent her teenage years in Southern New Jersey where she attended Lenape High School and volunteered as an EMT for the Tabernacle Rescue squad. Starting in the fall of 2000 she entered Cook College (since renamed *SEBS*) at Rutgers, The State University of New Jersey. During her time at Rutgers, Susan participated in and helped start a number of initiatives, including (but not limited to) *A Posteriori*: The Rutgers University undergraduate research journal, as well as The Rutgers Astronomers. Naturally adept in the life sciences, Susan majored in biotechnology pursued undergraduate research on marine microbiology in the laboratories of Dr. Costantino Vetriani and Dr. Lee J. Kerkhoff. Her research background and developing interest in medicine led Susan to pursue a doctoral degree in biomedical sciences at University of Florida's College of Medicine. She entered the Ph.D. program in September 2004, and developed her research in the laboratory of Dr. Bryon Petersen through the Department of Pathology, Immunology, and Laboratory Medicine. Near the end of her graduate studies, Susan co-founded the *Collaboration of Scientists for Critical Research in Biomedicine* (CSCRB, Inc.), which was legally incorporated in January 2009, and has since flourished. Susan received her Doctorate in Biomedical Sciences from the University of Florida in the spring of 2009, and was selected to join the Charter Class of University of Central Florida's Medical School, where she has since pursued her M.D.