INVESTIGATION OF GENE AND CELLULAR THERAPIES TO CURE MAPLE SYRUP URINE DISEASE (MSUD) IN A GENETICALLY ENGINEERED MOUSE MODEL

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MSUD is a serious liver-based metabolic disorder caused by a deficiency in the branched-chain alpha-ketoacid dehydrogenase (BCKDH) complex. Resulting branched-chain amino acid (BCAA) accretion in the body mainly affects the brain, which in most cases results in permanent neurological dysfunction or death without life-long attentive care. Recently it was shown liver transplantation alone restored BCKDH to a level sufficient to correct MSUD. To test novel therapies, a mouse model of intermediate MSUD (iMSUD) was created (Homanics et al., 2006), which mimicked human iMSUD. Therefore, this dissertation focused on the investigation of liver-directed therapeutic approaches to correct MSUD.

In the first aim, iMSUD mice were further characterized and determined to closely mirror the human disease phenotype. iMSUD mice suffered from developmental delay, seizures, and altered brain amino acid and neurotransmitter concentration. iMSUD brains also displayed histological abnormalities while liver morphology was normal.

In the second aim, adeno-associated viral (AAV) vectors were used to deliver E2 to the liver. However, no significant improvement was determined in AAV-treated iMSUD mice compared to controls. The most likely reasons this study was unsuccessful were low treatment dose, a weak albumin promoter, and possible competition and interaction between AAV-derived and iMSUD transgene-derived E2.

The third aim focused on hepatocyte transplantation (HTx). iMSUD-HTx mice had a 75% reduction in BCAA/alanine levels compared to iMSUD controls. BCKDH activity was increased, and Real Time qPCR detected donor-derived E2 in the liver. Dopamine and serotonin, along with several related metabolites, were corrected to control levels. Body weight at weaning and survival were also significantly improved in iMSUD-HTx mice.

The fourth aim focused on differentiated embryonic stem cell (ESC) transplantation. Differentiated ESCs expressed liver-specific markers after 3 days in culture and BCKDH activity was significantly increased over undifferentiated ESC populations. Liver-like ESC engraftment was verified up to 1 month following transplantation into wildtype mouse liver.

In summary, iMSUD mice were determined to be a superior model to test novel liver-directed therapies. Our findings of partial metabolic correction of iMSUD in a mouse model by HTx were very encouraging. Therefore, liver-directed therapeutic intervention for human MSUD should be investigated further.

FORWARD

Never, never, never, never

give up.

- Winston Churchill

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LIST OF ABBREVIATIONS

3-MT	3-methoxytyramine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptophan, serotonin
AAT	alpha-1-antitrypsin
AAV	adeno-associated virus
AFP	alpha-fetoprotein
ANOVA	analysis of variance
BCAA	branched-chain amino acid
BCATc	branched-chain amino transferase, cytosolic
BCATm	BCAT, mitochondrial
BCKA	branched-chain keto acid
BCKDH	branched-chain alpha-ketoacid dehydrogenase
BDK	BCKDH kinase
BDP	BCKDH phosphatase
BH_4	tetrahydrobiopterin
BMSC	bone marrow stem cells
cAMP	cyclic adenosine monophosphate
CCl_4	carbon tetrachloride
cDNA	complementary DNA
cMSUD	classic maple syrup urine disease
CNS	central nervous system
CTx	cellular transplantation
DA	dopamine
DAB	3,3-diamino-benzidine tetrahydrochloride
DBT	BCKDH dihydrolipoamide branched-chain transacylase
DLD	BCKDH dihydrolipoamide dehydrogenase
DNA	deoxyribonucleic acid
DOPAC	dihydroxyphenylacetic acid
E1α, β	BCKDH E1α, β
E2	see DBT
E3	see DLD
EB	embryoid body
ENU	N-ethyl-N-nitrosourea
ESC	embryonic stem cell
FGF	fibroblast growth factor
FVII, VIII, IX	Factor VII, VIII, IX

GABA	Gamma-aminobutyric acid
GFP	green fluorescent protein
H&E	Hematoxylin and Eosin
HIV-1	human immunodeficiency virus
HPLC	high performance liquid chromatography
HSV	herpes simplex virus
HTx	hepatocyte transplantation
HVA	homovanillic acid
IEC	ion exchange chromatography
IHC	immunohistochemistry
iMSUD	intermediate maple syrup urine disease
IP	interperitoneal
iPS cells	induced pluripotent stem cells
ITR	inverted terminal repeat
KIC	ketoisocaproate
KIV	ketoisovalarate
KMV	ketomethylvalarate
KO	knockout
LAD	lipoamide dehydrogenase
LAP	liver-enriched activator protein
LDL	low density lipoprotein
LIF	leukemia inhibitory factor
MEF	mouse embryonic fibroblast
mRNA	messenger RNA
MS-MS	tandem mass spectrometry
MSUD	maple syrup urine disease
NDS	normal donkey serum
NGS	normal goat serum
OCT	optimum cutting temperature
Oct4	octamer 4
OLT	orthotopic liver transplantation
PBS	phosphate buffered saline
PBSC	peripheral blood stem cells
PCR	polymerase chain reaction
PEP	putative endodermal precursor
PVDF	polyvinylidene difluoride
qRTPCR	quantitative Real Time PCR, also Real Time qPCR
rAAV	recombinant AAV
RNA	ribonucleic acid
ROSA	reverse orientation splice acceptor
scAAV	self complementary AAV
SCNT	somatic cell nuclear transfer
SEM	standard error of the mean
TRE	tetracycline transactivator response element
tTA	tetracycline transactivator
WT	wildtype

1.0 INTRODUCTION

This dissertation describes the characterization of a novel genetically engineered mouse model of intermediate maple syrup urine disease (iMSUD), and focuses primarily upon potential therapeutic approaches to treat or correct MSUD in this mouse model. The ultimate goal of this animal-based research is to develop an effective human therapy or cure. Since MSUD is predominantly a liver-based metabolic disorder, and it has been shown through orthotopic liver transplantation (OLT) that restoration of branched-chain α-ketoacid dehydrogenase (BCKDH) in the liver alone is sufficient to correct MSUD (Bodner-Leidecker et al., 2000; Netter et al., 1994; Strauss et al., 2006; Wendel et al., 1999), two liver-directed approaches (cellular and gene therapy) were investigated. The following section will give a brief introduction for each of the main subjects addressed.

1.1 MAPLE SYRUP URINE DISEASE

MSUD is regarded as one of the most significant and serious genetic disorders of amino acid metabolism (Chuang and Shih, 2001). MSUD is a pan-ethnic disorder with an incidence in the general population of 1:185,000 with a slightly higher frequency in distinct populations, such as a Georgia population (1:84,000), the Ashkenazi Jewish population (1:51,000), and countries

where consanguineous marriage is commonplace (Turkey, India, Spain, Saudi Arabia) (Chuang and Shih, 2001; Edelmann et al., 2001). In one such distinct population, the Mennonites of Pennsylvania, a staggeringly high frequency is observed in which MSUD affects 1:176 newborns (Marshall and DiGeorge, 1981). Because the incidence rate is 1000-fold above that of the general population, 37 states including Pennsylvania require mandatory MSUD screening of newborns (Williams, 2004). As more states and countries require screening, increases in MSUD reported cases are expected to demand attention. Although treatments do exist, which are discussed below, there is no universally satisfactory treatment to combat this disorder for all cases.

MSUD is an autosomal recessive metabolic disorder resulting from a deficiency in the BCKDH complex (Dancis et al., 1960; Menkes et al., 1954). This deficiency results in an inability to correctly process branched-chain α-ketoacids (BCKA) (Chuang and Shih, 2001; Harris et al., 2004; Yeaman, 1989). BCKAs [ketoisocaproate (KIC), ketomethylvalarate (KMV), and ketoisovalarate (KIV)] are derived from the branched-chain amino acids (BCAAs) leucine, isoleucine, and valine, respectively (Chuang and Shih, 2001; Harris et al., 2004; Yeaman, 1989). Deficient levels of the BCKDH complex cause toxic accretion of BCAAs and their related metabolites in the cerebrospinal fluid, blood, and tissues, thereby resulting in numerous and serious effects including neurological dysfunction, seizures, and infant death without treatment or constant attentive care (Chuang and Shih, 2001; Cox and Chuang, 1993). Although some BCAA turnover is observed via renal clearance (resulting in a sweet, maple syrup smell of the urine, hence the disease name), it is not sufficient to provide relief from the toxic amino acid levels in the body (Schadewaldt and Wendel, 1997).

1.1.1 The BCKDH complex molecular genetics

The BCKDH complex is a mitochondrial multisubunit enzyme consisting of three catalytic components: E1, E2, and E3 (Harris et al., 2004; Yeaman, 1989). All BCKDH complex genes are nuclear in location and chaperonins are used to escort transcribed precursor proteins into the mitochondria; a mitochondrial leader sequence is incorporated at the N-terminus of all subunits, which is later cleaved once it reaches its final destination (Chuang and Shih, 2001; Danner and Doering, 1998; Mueller et al., 1995; Pieneman and Danner, 1994). Rather than a specific amino acid sequence, the common feature of mitochondrial leader sequences, which direct proteins to the inner matrix, is an inclination to fold into an amphiphilic α-helix with a net positive charge clustered at one end of the helix and uncharged hydrophobic residues clustered at the other (Alberts et al., 2002).

E1, or branched-chain ketoacid dehydrogenase E1, exists as a heterotetramer of α and β subunits in the BCKDH complex and functions as a BCKA dehydrogenase/decarboxylase (Chuang and Shih, 2001). E1α is located at chromosomal position 19q13.1-13.2 and consists of 9 exons which encode a 1.6kb mRNA transcript, which translates to 378aa with a 45aa leader sequence (Zhang et al., 1988). E1β is located at chromosomal position 6q14 and consists of 11 exons which encode a 1.4kb mRNA transcript, which translates to 342aa with a 50aa leader sequence (Chuang et al., 1996; Nobukuni et al., 1990). E2, or dihydrolipoamide branched-chain transacylase (DBT), is a dihydrolipoyl transacylase, exists as a homo-24mer with octagonal symmetry in the BCKDH complex, and is the functional "core" of the enzyme complex due to the presence of lipoyl bearing domains which act as long swinging arms to shuttle intermediates among the other active sites/subunits of the complex (Chuang and Shih, 2001). E2 is located at

chromosomal position 1p31 and consists of 11 exons which encode a 1.46kb mRNA transcript, which translates to 421aa with a 56aa leader sequence (Danner et al., 1989; Nobukuni et al., 1989). E3, or dihydrolipoamide dehydrogenase (DLD), exists as a homodimer in the BCKDH complex and functions as a dihydrolipoyl dehydrogenase located at chromosomal position 7q31-32. E3 consists of 14 exons which encode 2.2 and 2.4kb mRNA transcripts which translates to 509aa with a leader sequence of undetermined length (Pons et al., 1988; Scherer et al., 1991). E1 and E2 are unique to the BCKDH complex while E3 is also present in two additional mitochondrial enzymes (the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes important in the function of the Krebs cycle) and is also known as the glycine cleavage system L protein (Chuang and Shih, 2001; Johnson et al., 1997; Yeaman, 1989).

1.1.2 BCAA metabolism

BCAAs are three of nine essential amino acids which cannot be synthesized by animals and must be acquired through diet (Harper et al., 1984). BCAAs are important for growth and overall good body health in addition to protein, neurotransmitter, and fatty acid synthesis. During normal BCAA metabolism, BCAAs are ingested, delivered to the liver via portal circulation, and transported into liver cells by the L-transporter, a leucine-sensitive transport system (**Figure 1**) (Harper et al., 1984; Le Cam and Freychet, 1977). Once inside a liver cell, BCAAs undergo reversible transamination via branched-chain aminotransferase proteins (BCATc and BCATm, which are present in the cytosol or mitochondria, respectively), which yield corresponding BCKAs, 80% of which are recycled into new proteins (**Figure 1**) (Harper et al., 1984; Schadewaldt and Wendel, 1997; Wu et al., 2004). The BCKDH complex in the

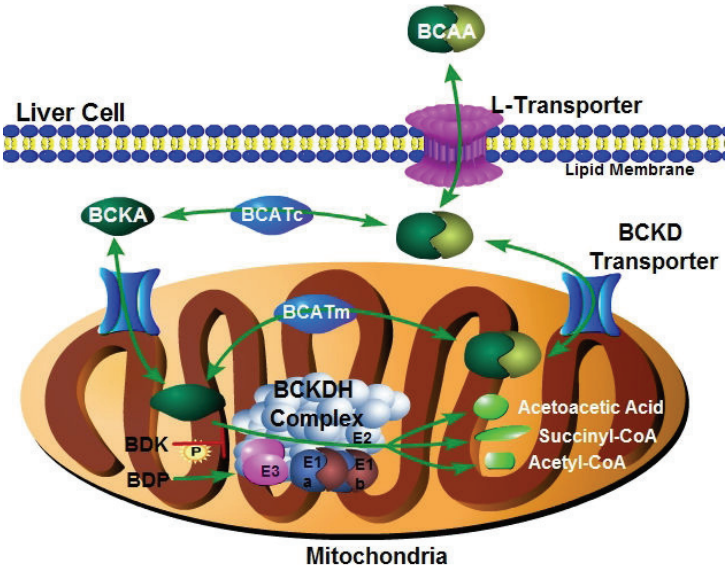

Figure 1. **Overview of BCAA metabolism in a liver cell**.
BCAA are transported inside the liver cell by the L-transporter and converted to BCKA via the BCAT proteins, a reversible reaction. Inside the mitochondria BCKAs are permanently broken down via the BCKDH complex, which is comprised of E1α, E1β, E2, and E3 subunits. The enzyme is under post-translational control: a phosphatase (BDP) activates the enzyme complex while a kinase (BDK) inactivates it. Figure made using Pathway Builder 2.0.

mitochondria is responsible for the irreversible oxidative decarboxylation reaction of the remaining 20% of BCKAs (**Figure 2**, step marked by *), which bind each of these compounds to their own discrete degradation pathway to produce acetyl-CoA, succinyl-CoA, and acetoacetic acid (**Figure 1**) (Chuang and Shih, 2001; Harris et al., 2004; Yeaman, 1989). **Figure 2**, which was adapted from a review article by Wellner and Meister, does not show the final steps of valine and isoleucine metabolism, which results in the production of succinyl-CoA (Wellner and Meister, 1981). In isoleucine metabolism, propionyl-CoA is converted to methylmalonyl-CoA by propionyl-CoA carboxylase; in isoleucine and valine metabolism methylmalonyl-CoA is then converted to succinyl-CoA by methylmalonyl-CoA mutase.

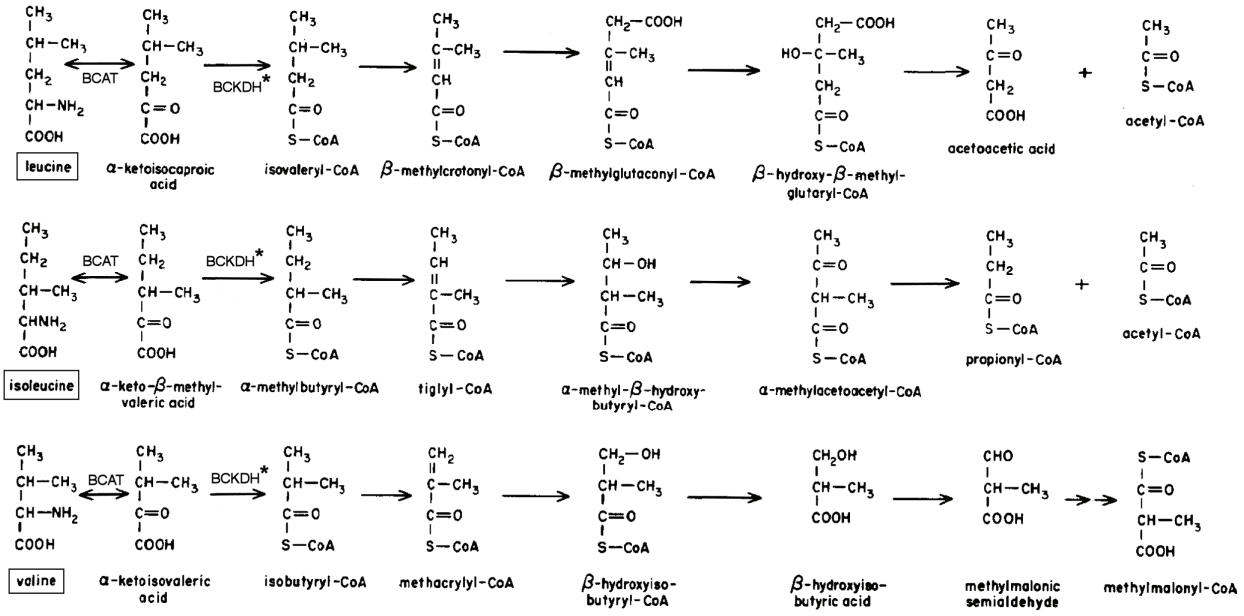

Figure 2. Mechanism of branched-chain amino acid metabolism.
Step by step metabolism of the three BCAAs, leucine, isoleucine, and valine. The first step is catalized by the BCAT proteins reversibly converting BCAAs to BCKAs. BKCDH, marked by an *, permanently breaks down BCKAs and directs them to their own unique degradation pathway to produce succinyl-CoA (produced from further degradation of methylmalonyl-CoA and propionyl-CoA), acetyl-CoA, and acetoacetic acid. Figure adapted from (Wellner and Meister, 1981)

1.1.3 Regulation of the BCKDH complex

The BCKDH complex is associated with a specific set of post-translational regulatory proteins: BCKDH kinase (BDK) and BCKDH phosphatase (BDP) (Damuni et al., 1984; Harris et al., 2004; Paxton and Harris, 1982; Paxton et al., 1986; Popov et al., 1992; Shimomura et al., 1990). When BCAAs are needed for protein synthesis, BDK covalently modifies the complex by phosphorylating specific sites in E1α causing inactivation of the BCKDH complex (Harris et al., 2004; Paxton and Harris, 1982; Paxton et al., 1986; Popov et al., 1992; Shimomura et al., 1990). Conversely, dephosphorylation of E1α by BDP occurs when excess BCAAs are present (Damuni et al., 1984; Harris et al., 2004). In MSUD patients, BCKDH complex activity typically ranges from 0-40% of normal levels, and although 80% of catabolized BCAAs are still

recycled into new proteins, the remaining 20% cannot be processed and instead become trapped and build up to toxic levels (Thompson et al., 1990a; Thompson et al., 1990b).

More recently, it was discovered that the BCKDH complex was susceptible to transcriptional regulation via hormones. Stimulation by a cyclic adenosine monophosphate (cAMP) analog, acidification, dexamethasone, and glucocorticoids increased BCKDH enzyme activity in cultured cells (Chicco et al., 1994; Wang et al., 2001); these stimuli were also independently able to increase BCKDH mRNA levels in hepatocytes. cAMP and dexamethasone have been reported to mediate expression of amino acid metabolism enzymes in hepatocytes, such as the tyrosine aminotransferase enzyme complex (Schmid et al., 1987), and urea cycle enzymes (Nebes and Morris, 1988). BCKDH enzyme activity was also significantly increased after subjection to a high protein diet (Harris et al., 1985; Soemitro et al., 1989). This phenomenon was hypothesized to be linked to increased glucagon in the portal circulation following a high protein diet given that glucagon action is mediated by cAMP (Chicco et al., 1994; Eisenstein et al., 1979). cAMP is an important second messenger responsible for the mediation of hormonal effects on many cellular processes. In many of these studies, E2 was determined to have a major role in enzyme flux. When stimulated with cAMP and dexamethasone, the E2 subunit, which exists as a homo-24mer in the final enzyme complex (Chuang and Shih, 2001), displayed the smallest increase in transcription and translation when compared to E1α, E1β, and E3 suggesting E2 is the limiting subunit for enzyme activity (Chicco et al., 1994). In addition, a pH-responsive element was mapped to a region upstream of the E2 transcriptional start site while dexamethasone could also stimulate E2 promoter activity while concurrently reducing nuclear factor kappa B binding in the E2 promoter (Wang et al., 2001). In

light of these findings, it is likely many more hormonal and protein interactions are able to mediate BCKDH at a pre- and post-transcriptional level.

1.1.4 Many genetic mutations produce disease

All BCKDH complex subunits are essential for enzymatic activity, and each are encoded at different locations in the nuclear genome (Chuang and Shih, 2001; Danner and Doering, 1998; Mueller et al., 1995; Pieneman and Danner, 1994). Therefore, since mutations at any of these loci can produce the disorder, MSUD is a complex disease due to genetic heterogeneity (Chuang and Shih, 2001; Danner and Doering, 1998). There are at least 100 known mutations in BCKDH complex genes that result in the MSUD phenotype; single-base substitutions, and small or large deletions or insertions have all been discovered (Chuang et al., 2006; Chuang and Shih, 2001; Copeland et al., 2004; Danner and Doering, 1998; Pieneman and Danner, 1994). Since the E3 subunit functions in multiple enzyme systems, mutations in E3 affect numerous processes and usually result in prenatal lethality; liveborn E3 mutants are rarely observed (Hong et al., 2003; Johnson et al., 1997). In the Mennonite population, an Y393N point mutation in E1α is most common (Morton et al., 2002). However, in the general population, E2 mutations are the most prevalent (Chi et al., 2003; Chuang et al., 1995; Chuang et al., 2004; Chuang et al., 1997; Schadewaldt and Wendel, 1997; Silao et al., 2004). For this reason classic and intermediate MSUD mouse models, described below, were created by disrupting the E2 subunit of BCKDH (Homanics et al., 2006).

1.1.5 Clinical manifestations

Patients suffering from MSUD show a heterogeneous clinical phenotype due to the disorder's genetic heterogeneity. Four disease classifications exist and all display overlap of gene expression and symptoms (**Table 1**); to further complicate diagnosis, differences in expression and severity of symptoms may be present in individuals with identical mutations (Chuang and Shih, 2001). To aid in classification, disease type is commonly based upon indirect parameters such as increased blood leucine concentration (normal leucine concentration is <0.4mg/mL), age of onset, and residual BCKDH complex activity in cells (Chuang and Shih, 1995; Schadewaldt and Wendel, 1997).

Table 1. MSUD disease variants.

MSUD Variant	Residual BCKDH Enzyme Activity	Age of Onset	Phenotype
Classic (accounts for 75% of all cases)	0-2%	Birth (1-7 days)	neurological dysfunction, brain damage and edema, blindness, profound BCKA acidosis, myelinization failure, hyperammonemia, coma and infant death
Intermediate	3-30%	Late onset (juvenile-adult)	developmental delay, neurological impairment, failure to thrive, ketoacidosis, seizures
Intermittent	5-20%	5-24 months; up to 5th decade of life	Episodic acute behavioral change, ataxia, ketoacidosis, seizures, coma. Episodes occur following increased protein consumption, stress, or illness
Thiamine-responsive	2-40%	Late onset (juvenile)	Similar to intermediate MSUD; responds to thiamine therapy; acts on E2 subunit
E3 Deficient	8-20%	Birth-juvenile	developmental delay, neurodegeneration, hypotonia, ketoacidosis, lactic acidosis, recurrent myoglobinuria and liver failure, reduced ATP synthesis, Leigh's disease

Patients with the most severe or "classic" form of MSUD (cMSUD), usually resulting from a null mutation in the BCKDH complex, have residual enzyme activity between 0-2% of normal levels and exhibit neonatal onset of the disease and many serious clinical manifestations including severe mental retardation stemming from a variety of factors, seizure, coma, and postnatal death (Chuang and Shih, 1995; Chuang and Shih, 2001; Cox and Chuang, 1993; Ogier de Baulny and Saudubray, 2002). Mental retardation frequently observed in cMSUD infants likely occurred as the result of delays in diagnosis and/or treatment at birth (Hilliges et al., 1993; Kaplan et al., 1991; Snyderman, 1988).

Less severe forms of MSUD vary considerably in enzyme activity, disease onset, and symptoms. "Intermediate" MSUD (iMSUD) patients typically have in the range of 3-30% residual BCKDH complex activity and "intermittent" patients have enzyme activity around 5-20%, both with a disease onset ranging from juvenile to late adult (Chuang and Shih, 2001). Individuals with the "thiamine-responsive" form of MSUD present with juvenile disease onset and BCKDH complex activity within 2-40% of normal enzyme levels (Chuang and Shih, 2001). Mutations in the E2 subunit have been associated with thiamine-responsive MSUD (Fisher et al., 1991). E3 deficient MSUD, also known as lipoamide dehydrogenase (LAD) deficiency, is compounded in severity by the fact that E3 is active in two other enzyme complexes both involved in the Krebs cycle and the glycine cleavage system. Most mutations are embryonic lethal, but surviving patients with this disease variant have been reported to contain compound heterozygosity for two mutations (Cerna et al., 2001; Grafakou et al., 2003; Sakaguchi et al., 1986; Shaag et al., 1999). Some LAD patients have developed phenotypic similarities consistent with Leigh's Syndrome (Grafakou et al., 2003). In E3 deficient MSUD, residual enzyme activity

has been reported to be between 8 and 20% and onset is typically between birth and juvenile (Elpeleg et al., 1997).

In general, severity of MSUD clinical manifestations relies mainly on the amount of residual enzyme activity present in patients; increased BCKDH complex activity translates to a milder phenotype with occasional symptomatic episodes (Chuang and Shih, 2001). Therefore, an increase in BCKDH enzyme activity could result in the elimination of disease state.

1.1.6 Treatments and their associated problems

Although there is one widely accepted treatment for MSUD, it is not satisfactory for long-term management of the disease. This method relies on a BCAA-restricted diet, which was first introduced in 1964 as the first successful treatment for MSUD (Snyderman et al., 1964). However, since BCAAs comprise three of the nine essential amino acids, complete restriction retards normal growth and development. BCAAs must be acquired through diet and are necessary not only for growth and overall good body health, but also for protein, neurotransmitter, and branched-chain fatty acid synthesis (Harris et al., 2004). During times of metabolic stress, BCAAs can also serve as energy sources for skeletal muscle, promote protein synthesis, suppress catabolism, and products of BCAA transamination can serve as substrates for gluconeogenesis (Anthony et al., 2001; Gietzen and Magrum, 2001; Layman, 2003; Patti et al., 1998). Dietary management is also complicated by factors causing fluctuation of the body chemistry, such as increased dietary protein, sickness, or stress (Chuang and Shih, 2001). For this reason, cMSUD in particular does not perform well with dietary restriction alone, and high mortality rates and mental retardation is a factor even with attentive care (Cox and Chuang, 1993). The less severe variants of MSUD, although better managed through a BCAA-low diet

due to some residual enzyme activity, can still result in serious side effects if not properly monitored. Also, lack of compliance by patients to stay within the strict dietary guidelines can have major consequences for any variant of MSUD; compliance can be very difficult to accomplish since MSUD is primarily a childhood disease.

Although dietary restriction is still very widely used, new therapies have recently become an option in an effort to find a more satisfactory treatment. Aggressive hemodialysis in conjunction with a BCAA-restricted diet at the time of diagnosis has produced favorable results in MSUD neonates, which significantly reduced toxic accumulation of BCAA by 75% within three hours and reduced the risk for permanent neurological impairment (Puliyanda et al., 2002). Other procedures such as continuous hemofiltration, peritoneal dialysis, and exchange transfusions, which are costly and time consuming, have been done with varying levels of success. These are best used to bring down high BCAA levels very quickly, but are not meant for long term treatment. These treatments are mainly used upon initial diagnosis at birth or when individuals go into crisis and must be hospitalized.

OLT has also been investigated as a possible treatment and cure for MSUD (Bodner-Leidecker et al., 2000; Netter et al., 1994; Strauss et al., 2006; Wendel et al., 1999). Patients receiving OLT have met with great success in treatment of MSUD; they no longer require a BCAA-restricted diet and BCKDH enzyme activity was restored to at least the level of very mild MSUD patients. However, these patients are now required to take immunosuppressant drugs for the rest of their lives, and results seen in OLT treatment are comparable to results seen in the long-established and non-invasive BCAA-restricted diet. Clearly, there is a need for alternative therapies to combat this disorder, but OLT has revealed an important fact. Although BCKDH does function in other tissues, restoration of BCKDH enzyme activity in the liver alone was

sufficient to cure MSUD, thus providing significant rationale for focusing on liver-directed therapies.

1.1.7 Animal models of disease

Current unsatisfactory treatment options require improved therapies to combat MSUD. However, novel treatment development was delayed by a lack of a suitable animal model. Animal models provide an exceptionally useful system to explore the mechanisms of disease as well as test potential therapies that cannot justifiably be explored in humans without established experimental rationale.

1.1.7.1 Polled Shorthorn and Polled Hereford bovine model

A naturally occurring bovine model of MSUD was identified in Australian Polled Shorthorn and Polled Hereford calves, which was characterized by a rapid onset of central nervous system (CNS) neurological impairment, increased BCAA levels and their related metabolites, reduced BCKDH enzyme activity, and death within a few days of birth (Harper et al., 1989). Bovine MSUD, like the human disease, can be caused by multiple mutations in the genes encoding the BCKDH enzyme (Healy and Dennis, 1994) and is inherited in an autosomal recessive manner (Harper et al., 1986). The most common mutation responsible for the bovine MSUD phenotype corresponded to a premature stop codon in exon 2 of the E1α subunit due to a single base pair change at residue 248 in Polled Hereford cattle, and most likely a similar mutation in E1α of Polled Shorthorns (Dennis and Healy, 1999; Zhang et al., 1990). However, brain pathology from a stillborn fetus suggested that disease onset occured prenatally, something aberrant to the human disease, and that a delayed form may also occur in cattle (Harper et al.,

1989). Variations to the human disease also existed in the pathological course of progressive deterioration of the CNS from birth to death. Therefore, in addition to the bovine model being undesirable for use in the laboratory due to expense, its difficulty to handle and manipulate, and the long generation time, the bovine model does not accurately mimic the human disease. Therefore it is not ideal to test proposed therapies to treat and cure MSUD that would later be applied to the human disease.

1.1.7.2 ENU-treated mouse model

In 2004, a mouse resembling a MSUD phenotype was identified after random mutagenesis by N-ethyl-N-nitrosourea (ENU) treatment (Wu et al., 2004). This mouse was found to have severely increased blood BCAA levels, a failure to thrive, muscle weakness, and a decreased lifespan, all characteristics associated with human MSUD. However, when the BCKDH enzyme was analyzed it was found to have normal activity, and sequencing of all subunits revealed no mutations in these genes (Wu et al., 2004). Further investigation revealed a mutation in the exon 2/intron 2 splice site of BCATm (Wu et al., 2004), the mitochondrial protein which catalyzes the reversible transamination reaction to convert BCAAs to BCKAs (**Figure 1**). The mutation resulted in the deletion of exon 2 in BCATm mRNA and a complete loss of BCATm protein and thus enzyme activity in mutant mice (Wu et al., 2004). BCATm was previously identified as a bifunctional protein involved in both BCAA transamination and BCKA transport to the BCKDH complex (Hutson and Hall, 1993). Although ENU-treated mutant mice displayed a similar disease phenotype and responded to a low BCAA diet as seen in MSUD affected individuals, it was not an accurate model of MSUD. Therefore, it is not ideal for testing novel therapies to treat or cure the disease.

1.1.7.3 Dihydrolipoamide dehydrogenase (E3) deficiency in a mouse

In 1997, Johnson et al. created a targeted gene knockout mouse of the DLD (E3) subunit (Johnson et al., 1997), which is active in the BCKDH complex, the α-ketoglutarate dehydrogenase complex, the pyruvate dehydrogenase complex, and takes part in the glycine cleavage system (Chuang and Shih, 2001). The E3 homozygous knockout was embryonic lethal at the early grastrulation stage, but heterozygous mice developed normally with ~50% activity for all four mitochondrial multienzyme complexes. These mice showed increased sensitivity to the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which has been used to model Parkinson's disease (Beal, 2001; Klivenyi et al., 2004), and to malonate and 3-nitropropionic acid, which has been used to model Huntington's disease (Beal et al., 1993; Klivenyi et al., 2004). These data provided increased rationale that mitochondrial dysfunctions, particularly of the KGDH complex which has reduced activity in the brains of Alzheimers, Parkinson's and Huntington's disease patients, may contribute to the pathogenesis of neurodegenerative disease (Klivenyi et al., 2004; Mizuno et al., 1990). It was later determined that mice heterozygous for E3 had altered neural progenitor cells in the hippocampus of adult mice (Calingasana et al., 2008), further supporting the role of mitochondrial dysfunction in neurodegeneration.

The E3 subunit, since it functions as part of the BCKDH complex, is occasionally determined to be the cause of MSUD in some patients (Sakaguchi et al., 1986; Shaag et al., 1999). However, to avoid the embryonic lethal phenotype associated with the homozygous E3 knockout mouse, heterozygotes were used for analysis resulting in ~50% BCKDH activity, which is typically not associated with an MSUD disease phenotype (Chuang and Shih, 2001; Homanics et al., 2006; Johnson et al., 1997). In addition, the role of E3 in other mitochondrial

multienzyme complexes, which manifested as neurological dysfunction and degeneration in this model, makes the heterozygous E3 knockout a poor model of MSUD to test novel therapies (Calingasana et al., 2008; Klivenyi et al., 2004).

1.1.7.4 Classic MSUD (cMSUD) knockout mouse model

A cMSUD knockout model was created by targeted deletion of exon 5 and part of exon 4 of the E2 subunit of BCKDH through standard gene targeting methods (**Figure 3**) (Homanics et al., 2006); E2 was chosen since mutations causing MSUD are the most prevalent in the general population, as previously stated (Chi et al., 2003; Chuang et al., 1995; Chuang et al., 2004; Chuang et al., 1997; Schadewaldt and Wendel, 1997; Silao et al., 2004).

Knockout mouse pups were born in the expected frequency and were indistinguishable from littermates, confirming that BCKDH was not required for normal embryonic development (Homanics et al., 2006). To characterize cMSUD mice, liver BCKDH enzyme activity, BCAA concentration in the blood, and survival was assessed (**Figure 5**, compared to intermediate MSUD mouse models discussed below). Heterozygous knockout mice had approximately 50% of the enzyme activity of wildtype while homozygote knockouts had a complete lack of BCKDH enzyme activity confirming a null allele (Homanics et al., 2006).

BCAA levels expressed as a ratio to alanine, a much more sensitive indicator of MSUD phenotype than BCAA levels alone (Morton et al., 2002), were equal in the blood of heterozygous and wildtype animals, while homozygote levels were elevated as much as 6-fold above wildtype (**Figure 5B**) (Homanics et al., 2006). Knockout E2 mice accurately mimicked phenotypes observed in patients with the classic form of MSUD and, like diseased humans who fail to receive treatment, died within a few days following birth (**Figure 5C**) (Homanics et al., 2006) greatly limiting treatment options regarding this model.

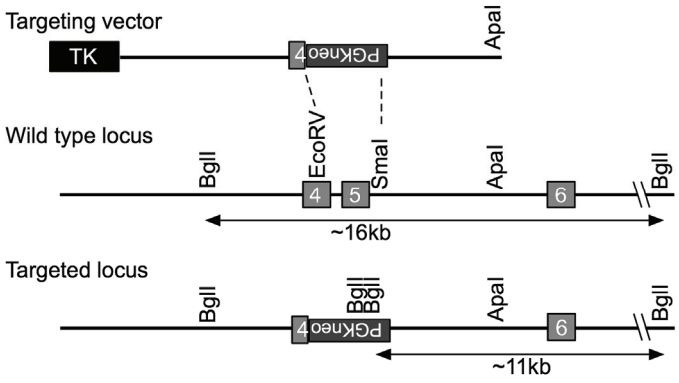

Figure 3. Gene targeting to create the E2 knockout (cMSUD) mouse line.
Exons 5 and part of exon 4 in the E2 subunit of the BCKDH complex (1.67kB) were deleted via standard gene targeting procedures to create a gene targeted knockout. Reproduced with permission from (Homanics et al., 2006).

Dr. Susan Hutson (Wake Forest University, Winston-Salem, NC) is currently attempting to engineer a mouse model of classic MSUD that contains the single point mutation, Y393N, in the E1α subunit, the genetic aberration most commonly found in Mennonite patients (personal communication, Dr. S. Hutson).

1.1.7.5 Intermediate MSUD (iMSUD) transgenic mouse model

An iMSUD model was created by breeding two transgenes onto the E2 knockout background (Homanics et al., 2006). The LAP-tTA transgene contained a liver specific promoter [Liver-enhanced Activator Protein, LAP; (Talbot et al., 1994)] driving the tetracycline transactivator (Kistner et al., 1996). TRE-E2 contained a tetracycline transactivator-regulated human E2 subunit modified to include a c-myc epitope tag at the protein C-terminus (**Figure 4**). The presence of both transgenes directed transgenic human E2 expression to the liver. Animals were designed in this way to allow for tetracycline regulation of gene expression, termed the TetOFF system, which was used previously with great success (Kistner et al., 1996); however this model was not tet-regulatable due to reasons not understood (unpublished observations, G.E.

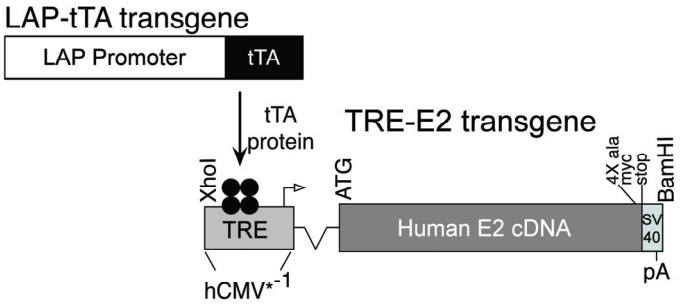

Figure 4. Transgenes expressed on an E2 knockout background.
Transgenes were introduced onto the cMSUD (E2 knockout) background to create iMSUD mice. The LAP-tTA transgene contained a liver specific promoter driving the expression of the tetracycline transactivator (tTA). The TRE-E2 transgene, containing a tetracycline transactivator-regulated human E2 subunit, elicited strong expression when stimulated by tTA. Transgenic human E2 was modified to include a c-myc epitope tag at the protein C-terminus. Reproduced with permission from (Homanics et al., 2006)

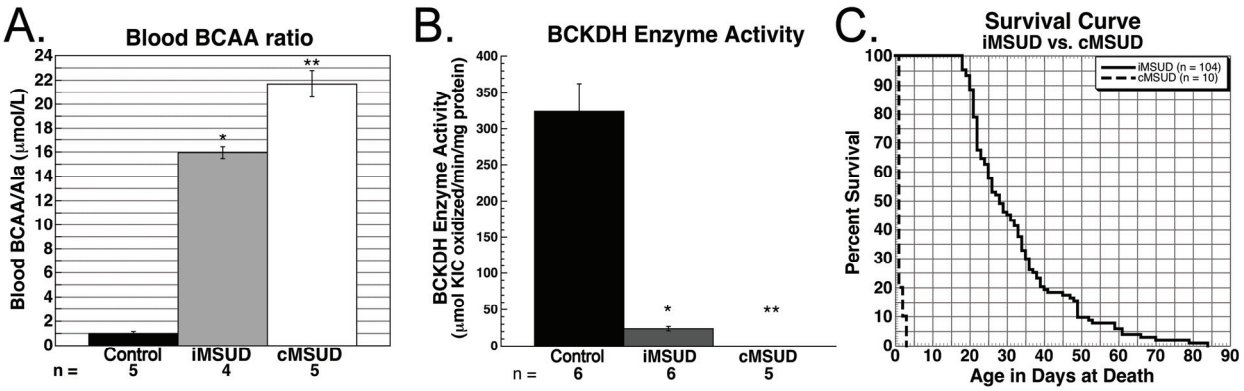

Figure 5. iMSUD mouse BCAA, BCKDH, and survival compared to cMSUD.
A. Blood BCAA/Ala levels were increased ~22-fold in cMSUD animals above Controls (WT). However, levels in iMSUD animals were increased to a value intermediate between cMSUD and Controls. **B.** BCKDH enzyme activity was between 5-6% of Control activity in iMSUD animals. Enzyme activity was not detectable in cMSUD E2 KOs. C. iMSUD survival compared to cMSUD was greatly improved with most animals living past weaning and into early adulthood; * = significantly different from Control and cMSUD ($p<0.0001$), ** = significantly different from Control and iMSUD ($p<0.0001$)

Homanics). Animals in which both transgenes were present transcribed human E2 in the liver, which was sufficient to rescue E2 knockout neonatal lethality increasing survival rate so that most survived beyond weaning and into early adulthood (**Figure 5C**) (Homanics et al., 2006).

Though early characterization revealed that the TetOff system was not functioning in the iMSUD mouse lines, it was discovered that blood BCAA levels expressed as a ratio to alanine were elevated approximately 16-fold above littermate controls (**Figure 5A**) (Homanics et al., 2006). This averaged value was intermediate between Controls (WT) and cMSUD levels (~22-fold above Controls), though values varied substantially between individuals. This was a fortunate surprise; initial rationale for using the TetOff system was so the model would be physiologically normal (i.e., identical to WT) and develop until such time it was given tetracycline to cease E2 expression creating a mature E2 knockout, or cMSUD model. This discovery made tetracycline regulation of the transgenes unnecessary. In contrast to the increase in BCAA levels, alanine, glutamate, and glutamine in the blood were all significantly reduced in iMSUD mice compared to wildtype controls (Homanics et al., 2006). BCKDH enzyme activity in the livers of iMSUD mice was also examined and found to be about 5-6% of normal, which was a significant improvement from the undetectable BCKDH activity levels associated with the cMSUD model (**Figure 5B**) (Homanics et al., 2006). Human iMSUD has been characterized as having a residual BCKDH enzyme activity in the range of 3-30% of normal (Chuang and Shih, 2001). iMSUD mice also suffered from a reduced life span compared to controls though survival was significantly increased over cMSUD, which did not typically survive more than 72 hours (**Figure 5C**) (Homanics et al., 2006). However, it is important to mention iMSUD mice surviving to early adulthood were able to breed and produce normal offspring.

Taken together, the generation of MSUD mouse models has provided excellent animal resources for the testing of novel therapies for MSUD due to their strikingly similar phenotype to human disease patients. Heterozygous cMSUD animals, similar to human disease carriers, had 50% BCKDH activity while maintaining normal BCAA levels, growth, and longevity, which supplied rationale for pursuing therapies to restore BCKDH complex activity since 100% of wildtype activity was not required to be healthy. iMSUD animals displayed intermediate levels of blood BCAA compared to WT and cMSUD mice, had increased BCKDH activity, and were able to survive beyond weaning and into early adulthood in many cases. Therefore, it was determined to be a superior model for testing proposed therapies delineated in this dissertation. Additional characterization of the iMSUD mouse model will be presented and discussed in the upcoming Section 2.0.

1.2 GENE THERAPY

Gene therapy can be defined as the delivery of genetic material, either DNA or RNA, to the cell nucleus in order to manipulate the endogenous nuclear information for the purpose of treating or preventing an inherited or acquired disease (Crystal, 1995; Miller, 1992; Mulligan, 1993). The concept of gene therapy was first introduced in the early 1970's after the discovery of recombinant DNA technology (Friedmann and Roblin, 1972). Though clinical trials were underway very early to test this novel therapy, Friedmann and Roblin called for the development of ethical guidelines, clinical protocols, and opposed any further human application until knowledge of basic genetic processes, such as gene regulation and the relationship between genetics and disease, were expanded upon. Such a vast undertaking would optimize the general

model of gene therapy, increase long-term success, and eliminate a possible misuse by premature application (Friedmann and Roblin, 1972).

1.2.1 Methods of administration: non-viral and viral

There are two basic methods for delivering a therapeutic gene to a cell: viral and non-viral. Viruses naturally evolved to effectively deliver genetic material to a specific cell's nucleus while bypassing host immunosurveillance, and are therefore very attractive for use as a vector system for delivering therapeutic genes (Robbins and Ghivizzani, 1998; Smith and Enquist, 2002; Walther and Stein, 2000). Approximately 70% of clinical trials worldwide utilize gene therapy vectors derived from viruses, but non-viral approaches are gaining popularity due to reduced limitations (e.g., viral packaging restrictions) and less chance of immunologic and mutagenic problems (Eliyahu et al., 2005; Walther and Stein, 2000).

1.2.1.1 Nonviral gene therapy

Non-viral gene therapies (summarized in **Table 2**) include direct injection of naked DNA into cells by a "gene gun" (Albertini et al., 1996; Rakhmilevich et al., 1997), oral gene therapy (Rothman et al., 2005), conjugation of DNA to various proteins (e.g., antibodies, polycations) to efficiently direct the complex to specific cell surface proteins or receptors (Feero et al., 1997; Shimizu et al., 1996), and liposomes. Of the non-viral methods currently available, cationic liposomes, or spontaneously assembled lipids surrounding negatively charged therapeutic DNA, are used most often in human gene therapies (Eliyahu et al., 2005; Gao and Huang, 1995; Liu et al., 1996; Robbins and Ghivizzani, 1998). Liposomes are not pathogenic and can be used for multiple administrations, but transfection efficiency is very low compared to viral gene delivery

– a common problem with non-viral delivery. In order to increase DNA transport into the nucleus, non-viral vectors are manufactured to be more virus-like by conjugation to defective viral particles, proteins, or peptides (Nguyen et al., 1997; Robbins and Ghivizzani, 1998).

Table 2. Non-viral gene therapy advantages and disadvantages.

Non-viral vector	Advantages	Disadvantages
Gene "gun"	Can transfer gene to quiescent cells, cheap and easy to prepare, gold beads are not cleared from skin – may be useful in wound healing, unlimited packaging capacity	Variable transfection efficiency, transient gene expression
Oral	Easy administration that can be done at home, easily altered dose, nonpathogenic, unlimited packaging capacity	Low transfection efficiency; must protect gene from harsh stomach environment, very transient expression – must be readministered very frequently (i.e., daily), expensive
Liposomes (cationic, anionic)	Nonpathogenic, multiple administrations possible, cheap and easy to produce, unlimited packaging capacity	Low transfection efficiency, transient gene expression
DNA conjugation	Efficient direction of a gene or complex to specific cells through cell surface proteins/receptors, unlimited packaging capacity	Low transfection efficiency, depending on the conjugate: risk of immune response
Non-viral/viral hybrids	Higher transfection efficiency, unlimited packaging capacity	Possible immune response to viral proteins

1.2.1.2 Viral gene therapy

There are also several viruses which have been modified for use as efficient gene therapy vectors such as retrovirus, adenovirus, adeno-associated virus (AAV), herpes-simplex virus (HSV), and pox virus, among others. Each viral system, briefly described below, has its own specific advantages, disadvantages, and limitations (summarized in **Table 3**); therefore, each has specific applications for which they are best suited. Each modified gene therapy vector has also

been used in human clinical trials or animal models targeting disease, some of which have been summarized in **Table 4**.

Retroviral vectors, which include lentiviruses and the murine leukemia virus, are derived from small viruses that reverse-transcribe their single stranded RNA viral genome into a double stranded DNA provirus which can integrate into the host genome (Robbins and Ghivizzani, 1998; Walther and Stein, 2000). Although a large packaging capacity and the ability to integrate allowing for long term and stable expression of a transgene make retroviruses an attractive vector for gene therapy, nonspecific or random integration can have dire consequences such as in the recent retroviral X-linked severe combined immunodeficiency (X-SCID) clinical trial (Hacein-Bey-Abina, 2003; Woods et al., 2006). Therefore, until retroviral vectors can perhaps be directed to a specific site in the genome that is known to be harmless, risk of cancer or other genetic disruptions due to random vector integration will be a major concern for their use in gene therapy to treat human disease.

Adenoviruses, discovered in 1953, are non-enveloped, linear, double stranded DNA viruses that can accommodate approximately 7-8 kb of foreign DNA (Enders et al., 1956; Graham and Prevec, 1995). The major disadvantage of adenoviruses as viral vectors is their associated pathogenicity to humans, not only producing a cell-mediated immune response but also a humoral response (Walther and Stein, 2000). Therefore, repeat administration may not be possible. Many attempts have been taken to create an immunologically safer adenovirual vector for gene therapy. However, many viruses with deleted genes still produced viral proteins and stimulated a host immune response (Graham and Prevec, 1995) that resulted in cell death and a loss of therapeutic gene expression (Yang et al., 1995; Yang et al., 1994). However, “gutted” adenoviral vectors devoid of all viral genes leaving only the inverted terminal repeats eliminated

all viral gene expression and thus any host immune response, and increased packaging capacity (Kochanek et al., 1996; Parks et al., 1996). Despite adenovirus disadvantages (**Table 3**), replication-defective vectors have been involved in greater than 60 human trials with varying

Table 3. Gene therapy viral vector advantages and disadvantages.
Table adapted from (Walther and Stein, 2000)

Viral Vector	Advantages	Disadvantages
Retroviral	Large packaging capacity (7-8kb), stable integration into host DNA, broad cell tropism, ease of viral genome manipulation	Random integration into host genome, vector instability, difficult specific cell targeting, infects dividing cells only
Lentiviral	Infect dividing and quiescent cells, stable gene expression, large packaging capacity (10kb)	Presence of viral protein sequences in the constructs, potential insertional mutagenesis
Adenovirus	High gene expression, large packaging capacity (7-8kb), infects dividing and quiescent cells, broad cell tropism	Transient gene expression, host immune response, no integration into host genome
Adeno-associated virus	infect dividing and quiescent cells, broad cell tropism, potential of targeted integration, low to no immune response, nonpathogenic	limited packaging capacity (4kb), typically no integration into host genome, neutralizing antibodies, possible cellular immune response
Herpes-simplex virus	Infects wide variety of cell types, large packaging capacity (50kb), natural tropism for neuronal cells, ability to infect quiescent cells	No integration into host genome, possible toxicity, recombination risk, very short expression except in neuronal cells
Pox virus (vaccinia)	Large packaging capacity (25kB), high cytoplasmic expression, ability to produce humoral response makes it promising for vaccines, wide cell tropism	Produces humoral and cytotoxic immune responses, no integration into the host genome

results (some in **Table 4**) mostly producing a high level, though transient, of gene expression. For this reason, adenovirus may not be appropriate for inherited disorders which require a high level of gene expression for the lifetime of the recipient, but may be more useful for diseases requiring only transient expression such as cancers (Robbins and Ghivizzani, 1998).

HSV is a large linear, double stranded DNA virus with a 50kB packaging capacity (Robbins and Ghivizzani, 1998; Walther and Stein, 2000). While HSV does elicit an immune response (Wu et al., 1996), the virus can also establish latency in neuronal cells lasting for years. That characteristic makes HSV especially appealing for the treatment of neurological diseases, such as Parkinson's and Alzheimers (Robbins and Ghivizzani, 1998), though the virus has a fairly wide tropism for other cell types, specifically muscle and liver. However, a recurring problem with HSV is transient gene expression in non-neuronal cell types; transgene expression is shut down typically within one week post-infection and only the latency promoter of neuronal cells escapes this inactivation (Robbins and Ghivizzani, 1998). This defines a major limitation of HSV driven gene therapy; the reason behind this serious issue must be elucidated and resolved prior to application of HSV for the correction of human disease.

Pox viruses, which are members of the Poxviridae family, such as vaccinia virus, have a high packaging capacity (25kB), can accommodate multiple foreign genes, and are widely used for their high level of cytoplasmic expression of transgenes (Walther and Stein, 2000). They are of great interest for vaccination strategies since Pox viruses are known to induce cell mediated and humoral immune responses. Thus far, Pox viruses have been utilized in clinical trials to treat human papillomavirus, human immunodeficiency virus type 1 (HIV-1), and various cancers (Emery et al., 2005; Kaufmann et al., 2002; McAneny et al., 1996). Despite these successes, due to host immune response associated with Pox viruses, they are not ideal gene therapy vectors to use for correction of metabolic disease.

Table 4. Examples of gene therapy viral vectors targeting disease.

Viral Vector	Animal	Disease and Reference
Retrovirus	Human	MSUD (in fibroblasts) (Koyata et al., 1993; Mueller et al., 1995); X-linked granulomatous disease (Ott et al., 2006); Gaucher disease, arthritis, cancers (Robbins et al., 1994); X-SCID (Aiuti et al., 2002; Gaspar et al., 2004; Hacein-Bey-Abina et al., 2002)
Retrovirus	Mouse	Tyrosenemia type1 (Overturf et al., 1996); melanoma (Abad et al., 2008); Factor IX (FIX) (Chen et al., 2006); type 1 diabetes (Xu et al., 2007); α-thalassemia (Han et al., 2007)
Adenovirus	Human	Ornithine transcarbamylase deficiency (Batshaw et al., 1999; Raper et al., 2003; Raper et al., 2002); cystic fibrosis (Bellon et al., 1997; Boucher et al., 1994); vascular disease (Isner et al., 1995); prostate cancer (Shirakawa et al., 2007); solid tumor cancers (Nemunaitis et al., 2007); squamous head and neck cancer (Kirn et al., 1998)
Adenovirus	Mouse	Hyperoxaluria type 1 (Salido et al., 2006), muscular dystrophy (Deconinck et al., 1996)
HSV	Human	Glioblastoma (cultured cells) (Aghi et al., 2006; Hoshi et al., 2000)
HSV	Rat	Malignant glioma (Boviatsis et al., 1994); FIX, hepatitis B (Miyanohara et al., 1992); Parkinson's disease (neurons) (During et al., 1994); mucopolysaccharidosis VII (Wolfe et al., 1992); chronic root nerve pain (Liu et al., 2004; Zhou et al., 2008)
Pox Virus	Human	HIV-1 (Dorrell et al., 2006; Emery et al., 2005; Ondondo et al., 2006); HPV (Albarran et al., 2007; Kaufmann et al., 2002); prostate cancer (DiPaola et al., 2006); colorectal cancer (McAneny et al., 1996)
Pox Virus	Mouse	Type I diabetes (Dénes et al., 2006); HIV-1 (Bolesta et al., 2006); ovarian cancer (Hung et al., 2007); breast cancer (Zhang et al., 2007)
AAV	Human	Parkinson's disease (Kaplitt et al., 2007); cystic fibrosis (Moss et al., 2007); rheumatoid arthritis (Boissier et al., 2007); FIX (Manno et al., 2003; Manno et al., 2006)
AAV	Mouse	Niemann-Pick type A (AAV2) (Dodge et al., 2005); phenylketonuria (AAV5) (Mochizuki et al., 2004), AAV8, liver-directed (Ding et al., 2006), AAV2, muscle-directed (Ding et al., 2008); α1-antitrypsin (AAV2/8) (Conlon et al., 2005); Limb-Girdle muscular dystrophy (AAV1) (Pacak et al., 2007); glycogen storage disease (AAV2/8) (Sun et al., 2005a), Factor VIII (AAV8) (Sarkar et al., 2004)
AAV	Dog	FIX (AAV8, AAV2/8) (Wang et al., 2005b); FVIII (AAV2) (Scallan et al., 2003)

1.2.2 Adeno-associated viral vectors

Adeno-associated viruses (AAVs) are non-enveloped Dependoviruses derived from the nonpathogenic Parvoviridae family first discovered as a contaminant in adenovirus preparations (Atchison et al., 1965). Wildtype virus can infect both non-dividing and dividing cells and can stably integrate into the human genome at a specific location on chromosome 19 (19q13.3-qter, AAVS1) without negative consequences which allows viral DNA to be perpetuated through host cell division (Samulski, 1993). All eleven naturally occurring serotypes currently known are replication-deficient requiring a helper virus, such as adenovirus or HSV, for viral replication (Atchison et al., 1965; Choi et al., 2005).

AAV viruses are one of the smallest viruses with only a ~26nm capsid and a linear single-stranded DNA genome of ~4.7kB (Lusby et al., 1980). The small genome contains two viral genes for *rep* (replication) and *cap* (capsid) proteins (Robbins and Ghivizzani, 1998), and two GC-rich 145-base pair inverted terminal repeats (ITR) that fold into a characteristic T-shaped hairpin structure at either end of the linear genome (Koczot et al., 1973). ITRs can act as a free 3'-OH for viral replication initiation and are required for DNA packaging; both plus and minus strands are packaged into capsids with equal efficiency. Through different *rep* promoters and alternative splicing (Mendelson et al., 1986), the *rep* gene encodes four proteins (*rep*78, *rep*68, *rep*52, and *rep*48), and similarly for the *cap* gene, capsid proteins VP1, VP2, and VP3 are encoded through alternative splicing and codon start sites (Trempe and Carter, 1988). The three capsid proteins are assembled in a 1:1:10 ratio, respectively (Linden and Berns, 2000).

To create a recombinant vector for gene therapy studies, *rep* and *cap trans*-acting genes are deleted leaving only the *cis*-acting ITRs required for capsid packaging. However, removal of all viral genes only allows for the packaging of up to 4.7kB of foreign DNA, one of the major

limiting factors of this viral vector (Dong et al., 1996). Also, since integration at the human AAVS1 locus requires interaction with *rep* proteins, removal of the *rep* gene results in a loss of the ability for AAV to integrate into the human host genome (Samulski, 1993), another disadvantage. However, it has been shown that free AAV DNA genomes can form stable episomal circular or linear concatemers (Nakai et al., 1996; Yang et al., 1999), and can result in long-term recombinant AAV (rAAV) transgene expression from a single dose (Koeberl et al., 1997; Mochizuki et al., 2004; Wang et al., 2005a), especially in nondividing cell types such as liver. Importantly, the helper-free triple transfection method, an efficient method for producing high titres of pure, uncontaminated rAAV now exists (Xiao et al., 1998b). Prior to its development, AAV stocks frequently became contaminated with helper virus; wildtype adenovirus was difficult to separate from AAV stock, and use of an E1-deleted replication-deficient adenovirus held the risk of recombination to recreate wildtype adenovirus (Goncalves, 2005; Xiao et al., 1998b). Any contaminating helper virus reduced rAAV stock safety due to an increased risk of eliciting an immune response in recipients.

One significant advantage associated with AAV vectors is their ability to infect both nondividing and dividing cells from a very wide variety of species and cell types due to attachment receptors and coreceptors. Heparin sodium proteoglyan and O- and N-linked sialic acids are among the few primary receptors that have been identified (Kaludov et al., 2001; Summerford and Samulski, 1998). Coreceptors are also required for efficient infection and can significantly influence a serotype's specificity, thus resulting in superior targeting of a certain cell type. For example, AAV8 selectively transduced hepatocytes 10-100 times more efficiently than all other serotypes (Sun et al., 2005b; Xiao et al., 1998a) and displayed high tropism for heart and muscle (Wang et al., 2005b), which may be related to currently unidentified

coreceptors on the AAV8 capsid surface (Nam et al., 2007). More recently, these differences in capsid receptors and cell specificity have been used to create new hybrid AAV serotypes in attempts to enhance cell targeting and improve gene therapy vectors (Choi et al., 2005). Methods for creating hybrid vectors that have been investigated are transcapsidation (packaging of one serotype's genome into another serotype's capsid), capsid adsorption modification (peptides are adsorbed onto the capsid surface), mosaic capsid (mixture of two different serotype's unmodified capsid proteins are assembled), and chimeric capsid (modified capsid proteins, in which peptide sequences are added to the VP sequence, are assembled) (Choi et al., 2005).

At the cellular level, there are several major steps involved in AAV infection (Choi et al., 2005; Ding et al., 2005): cell attachment by receptors and coreceptors, internalization by endocytosis, intracellular trafficking to the nucleus and endosomal escape, uncoating of virus [which may occur either within or prior to delivery to the nucleus, which is dependent upon the absence or presence of helper virus (Xiao et al., 2002)], and finally viral genome conversion from single to double-stranded DNA capable of acting as a template for transcription. Intracellular trafficking is a major rate limiting step in the absence of helper virus (Ding et al., 2005). In the presence of adenovirus, AAV and helper virus is quickly dynamin-dependently endocytosed through clathrin-coated pits (Bartlett et al., 2000), which translocates through the endosomal compartment to arrive inside the nucleus in less than an hour as intact viral particles (Xiao et al., 2002). Capsid was then shed within the nucleus and viral replication initiated. Conversely, translocation to the nucleus in the absence of helper virus required 12+ hours and initiation of replication, and thus expression of any transgene, required several days (Goncalves, 2005; Xiao et al., 2002). Capsid proteins were also shed before or during nuclear entry in the

absence of helper virus. Once endocytosed within a host cell the precise mechanism for AAV intracellular trafficking is unknown, though it was reported that AAV was unidirectionally transported to the nucleus by microtubule motors (Seisenberger et al., 2001). Several potential pathways of endosomal processing in the absence of helper virus have been suggested (Ding et al., 2005) stating that acidification of virus inside the late endosome or lysosome may be critical to ubiquitination of capsid and initiation of nuclear transport, and other viral processing inside the golgi or early endosome may also have significant roles.

Methods independent of coinfection with a helper to alter intracellular trafficking and speed up translocation to the nucleus have not been efficiently developed. Therefore attempts to speed other rate limiting steps were investigated, such as conversion from a single to double stranded viral genome. Once inside the nucleus, the single-stranded AAV DNA genome must convert to a double-stranded DNA to act as a template for transcription by either *de novo* DNA synthesis of a complementary strand using host cell DNA polymerase, or by annealing sense and antisense strands together (Choi et al., 2005; Goncalves, 2005), which are both very slow processes. Although self-complementary AAV (scAAV) viruses were able to quickly and efficiently fold back on themselves to form a double stranded DNA template which led to increased transcription-competent double-stranded genomes and improved expression yields, vector design required that AAV vector packaging be essentially cut in half greatly reducing an already limited packaging capacity (McCarty et al., 2001). Therefore, scAAV vectors would only be useful when therapeutic genes of interest were ~2.3kB or smaller.

Despite the high incidence of human seropositive screens [~80%+ for AAV2; other serotypes have not yet been tested on a grand scale (Xie et al., 2002)], AAV has not been linked to any disease (Blacklow et al., 1968; Goncalves, 2005; Moskalenko et al., 2000). However, a

humoral immune response to AAV exists, which signals the production of neutralizing antibodies (Hernandez et al., 1999). Although infection of targeted cells may still be possible in the presence of very large doses, neutralizing antibodies have been shown to significantly reduce or ablate AAV-mediated gene expression due to antibody targeting and destruction of AAV (Moskalenko et al., 2000). This may severely limit AAV-mediated gene therapy since much of the normal population is seropositive for many AAV serotypes, and readministration of a vector may not be possible. However, this issue was overcome through the use of transient immunosuppression following re-administration of a serotype (Halbert et al., 1997; Hernandez et al., 1999; Manning et al., 1998). Reverse genetic approaches are also being investigated as a way to circumvent AAV destruction by neutralizing *in vivo* allowing for initial therapy in seropositive individuals or repeated administration (Moskalenko et al., 2000). Reverse genetics approaches would involve administration of peptides mapped to identified regions of the AAV capsid containing immunogenic epitopes which could be used to block neutralizing antibody binding and thereby allow AAV infection without detection.

In some cases in gene knockouts, a cellular response was observed against the unfamiliar viral transgene product (Fields et al., 2001; Manno et al., 2006). However, more recently cellular immune responses to the capsid proteins of AAV were acknowledged in clinical trials (Mingozzi and High, 2007), which did not occur in previous animal studies with the vector (Li et al., 2007). The Mingozzi and High (2007) publication suggests that humans carry a population of antigen-specific memory CD8(+) T cells most likely arising from wild-type AAV infections which then expand upon re-exposure to capsid proteins. Around the same time these papers were published, something occurred that shocked the AAV gene therapy circuit – a patient in a Phase 1 clinical trial to cure active inflammatory arthritis died on July 24, 2007 (Weiss, 2007).

This resulted in the temporary halt of all AAV clinical trials and a federal investigation into this death and all other trials involving AAV (Kaiser, 2007). A link was not found between the viral vector and the recent death, but current and future AAV studies and clinical trials will most likely experience a more thorough screening prior to approval. This event also caused a significant black mark in the minds of the general public towards gene therapy, one of several since the death of Jesse Gelsinger after receiving adenovirus gene therapy in 1999, and three others developed leukemia following retroviral gene therapy (Marshall, 2002; Teichler, 2000).

Despite various disadvantages, such as questions involving a recent clinical death, a small packaging capacity, slow infection rates, and others summarized in **Table 3**, AAV's advantages over other viral gene therapy vectors, most notably their inability to produce disease, makes AAV a superior vector for treatment of monogenetic disease. AAV has already been used in a multitude of clinical trials, some progressing to Phase III, and treatment of various inherited diseases in animal models, some of which are summarized in **Table 4**. Also, due to AAV8's reported ability to transduce hepatocytes with a greater efficiency than other AAV serotypes (Sun et al., 2005b; Xiao et al., 1998a), and AAV concatemer persistence particularly in quiescent cells such as liver, it was determined to be a superior vector for delivering a therapeutic gene to liver in a mouse model of iMSUD.

1.3 CELL-BASED THERAPIES

Gene therapy and cell-based therapies could be considered overlapping fields since they involve similar therapeutic objectives. Gene therapy involves administration of a specific defective gene to a patient's endogenous cells, which produce long-term expression and restore

"normal" cell function to correct disease. Cellular therapy involves administration of healthy cells that will operate independently from defective endogenous cells, and increase tissue function and restore "normal" processes to correct disease. Cellular therapy may be advantageous since transplanted cells are self-contained, and therefore any issues involving compatibility or interaction of a transgenic protein with endogenous cellular machinery would not be a concern as it might be with gene therapy.

There exists a great deal of rationale for the use of cellular transplantation (CTx) in place of whole organ transplantation. CTx is a nonsurgical procedure with less associated morbidity and a reduced recovery time (Strom et al., 2006a). In patients with whole organ failure, CTx could be used as a bridging therapy to extend life until a donor organ becomes available, but for those with a metabolic disorder CTx could be administered on an outpatient basis or during a brief hospitalization to monitor the patient (Strom et al., 2006a). As with whole organ transplant, CTx requires donor cell matching to the recipient unless a patient's own cells can be used. Therefore, locating a suitable match could still pose a problem, but unlike whole organ transplant, one donor organ could provide cells to transplant many recipients helping to alleviate the high demand for donor organs. Following cell CTx, required long-term immunosuppressive therapy would be expensive, but initial cost of CTx is estimated to be ~5-10% of whole organ transplantation (Strom et al., 2006a). Conversely, without transplantation, metabolic disorders managed by special dietary formulas, such as MSUD, are also very costly and often require frequent and expensive hospitialization in times of metabolic crisis despite attentive life-long care. Thus, even partial corrections of metabolic disease by CTx could be a cost effective therapy expected to significantly improve quality of life for the patient.

Occasionally a patient's own cells can be harvested, expanded in culture, and transplanted for therapeutic use to bypass any immunologic issues. This is possible in the treatement of certain kinds of cancers or HIV/AIDS (Avunduk and Tekelioglu, 2006; Woffendin et al., 2001) or repairing a damaged cornea when the injury is unilateral (Quintarelli et al., 2008) to name a few. In other cases, such as with bone marrow, bone marrow stem cells (BMSC), or peripheral blood stem cells, family members are likely matches eliminating the need for many to wait an indeterminate amount of time for a suitable donor.

Although there are many significant advantages for the use of CTx as discussed above, disadvantages and limitations exist, which are summarized in **Table 5** for some of the most commonly transplanted cell types. One of the major limitations for hematopoietic stem cell and bone marrow transplantation, the majority of cells transplanted clinically, is the phenomenon of graft-vs-host disesase (Chu and Gress, 2008) which occurs in 25-80% of cases with varying presentations and a significant incidence of associated morbidity and death (Baird and Pavletic, 2006). Graft-vs-host disease, though poorly understood, is a result of immune reactions between donor-derived immune cells and the host's endogenous cell populations, which can result in the presentation of autoimmune-like or immunodeficient-like diseases. Embryonic stem cells (ESC) can be differentiated into various cell types *in vitro* and have been proposed as an alternate source of bone marrow cells (Burt et al., 2004). Although no evidence of graft-vs-host disease was found in major histocompatibility complex-mismatched ESC-derived hematopoietic cells transplanted into donor mice (Burt et al., 2004), the presence of both a humoral and cellular immune responses to transplanted ESC cells in non-syngeneic mouse myocardium suggested a need for immunosuppressive therapy following future transplants involving ESCs (Kofidis et al., 2005). However, the recent creation of induced pluripotent stem (iPS) cells, which could be

Table 5. Commonly transplanted cells and their limitations.

Cell type	Notes and limitations
Bone marrow (BM)	Requires immunologic cell matching to recipients, though family members are likely donors; ~25% rejection rate; invasive procedure to collect cells; can cause graft-vs-host disease
Bone marrow stem cells (BMSC)	Invasive procedure to collect from marrow; must be closely matched to recipient; can be differentiated into a variety of cell types, including blood forming, cardiac, and hepatic (controversial) cells; differentiation often requires strong *in vitro* pressure; cells have been shown to fuse with endogenous cells once transplanted; difficult to maintain *in vitro*; can cause graft-vs-host disease
Peripheral blood stem cells (PBSC)	Less invasive to collect than BMSC, though difficult to obtain enough for transplant due to their numbers in blood; must be closely matched to recipient; can cause graft-vs-host disease
Cord blood stem cells	Much easier to collect than either BMSC or PBSC since umbilical cord blood is often saved after birth; can obtain a large number of cells from a small amount of blood; less likely to be rejected or attack the host (graft-vs-host disease); relatively new source for hematopoietic stem cells still being characterized
T-lymphocytes	Patient's own cells can be expanded and transplanted for the treatment of certain types of cancers or HIV/AIDS; can cause graft-vs-host disease if a donor is used
Mesenchymal stromal stem cells	Must be closely matched to recipients; easily isolated from adult bone marrow, though requires an invasive procedure; mix of mature and immature cell types and though able to differentiate, requires strong *in vitro* pressure; can cause graft-vs-host disease
Hepatocytes	Requires cell matching by blood type to recipients; difficult to maintain in vitro; efforts to freeze cells for later use are not reliable; requires immunosuppression in most cases; already some success clinically
Embryonic stem cells (ESC)	Can be differentiated into theoretically any cell type, though differentiation protocols and user success varies greatly from report to report; limited useable human lines, limited private funding/opportunities to derive new lines; easy to maintain *in vitro*; transplantation of undifferentiated cells can develop into tumors

made from a patient's own cells, differentiated *in vitro*, and transplanted to cure disease, may solve immunogenic issues associated with traditional ESCs (Takahashi et al., 2007).

Cellular transplantation as a cure for liver disease, with a focus on metabolic liver disorders, is a thrilling concept. Initially, the ability for BMSCs to efficiently and effectively differentiate into functional hepatocyte-like cells caused a great deal of excitement in the field. BMSC-derived hepatocyte-like cells were reportedly able to correct a mouse model of tyrosinemia type 1 (Lagasse et al., 2000), and transplantation of highly purified human BMSCs could induce albumin-producing colonies of hepatocyte-like cells in mouse liver (Wang et al., 2003a). However, liver repopulation was hypothesized to be a slow, rare event due to few BMSC-derived cells in liver 7 weeks post-transplant (Wang et al., 2002). Later Wang et al. (2003b) determined transplanted cells were fusing with endogenous hepatocytes, not differenentiating from BMSC. Soon after, other groups independently reported no evidence of liver regeneration or the existence of hepatocyte-like cells following BMSC transplantation (Cantz et al., 2004; Kanazawa and Verma, 2003). Currently, two cell types hold the greatest promise for treatment of liver disease, differentiated ESC and isolated hepatocytes. Both are discussed in greater detail below.

1.3.1 Hepatocyte transplant

The need for donor organs greatly outweighs their availability. On average, greater than 20,000 patients are awaiting donor livers for transplantation in the United States alone, and within one year only roughly one third will receive them (Strom et al., 2006a). Many will die before a liver becomes available. Clearly there is a need for a therapeutic alternative to OLT to alleviate the overwhelming demand for donor organs.

The most likely source of hepatocytes for cell transplant are livers that have been rejected for use in OLT (Strom et al., 2006a; Strom et al., 2006b). Therefore, high transplant-quality cells must be harvested from organs deemed unsuitable for whole organ transplant due to >50% steatosis (i.e., fatty deposits) or other adverse factors. Other sources of hepatocytes, such as immortalized human liver lines (Kobayashi et al., 2000; Wege et al., 2003), xenotransplants (Nagata et al., 2003), hepatocytes from aborted fetuses (Habibullah et al., 1994; Malhi et al., 2002), and ESCs differentiated into hepatocyte-like cells discussed later (Fair et al., 2005; Hu et al., 2006; Ishii et al., 2007; Sharma et al., 2008; Soto-Gutiérrez et al., 2006; Tabei et al., 2005) have been suggested with exciting results in animal models. However, each alternate source carries their own disadvantages and concerns, most of which revolve around safety and ethics.

1.3.1.1 HTx in Use: proof of concept

Hepatocyte transplantation (HTx) has previously been investigated and implemented as a novel short-term "bridging" therapy to support liver function until a donor liver becomes available in times of liver injury or acute failure (Bilir et al., 2000; Fisher et al., 2000; Strom et al., 1999; Strom et al., 1997). In some cases of acute liver failure where regeneration of a few surviving hepatocytes is not enough to overtake the rate of massive cell loss, hepatocyte transplantation may provide enough relief for the patient's surviving liver cells, by working with transplanted cells, to regenerate the liver fully (Strom et al., 1999). This seemed to be the case in two patients with fulminant hepatic failure, who no longer required OLT after receiving HTx (Ott et al., 2004; Soriano, 2002).

HTx has had initial laboratory and clinical successes in the treatment of inherited and metabolic liver disorders such as Crigler-Najjar syndrome (Fox et al., 1998; Groth et al., 1977; Matas et al., 1976; Vroemen, 1986), glycogen storage disease, type 1 (Muraca et al., 2002a),

Wilson's disease (Irani et al., 2001), phenylketonuria (Hamman et al., 2005), Factor VII (Dahwan et al., 2004), an inborn error of fatty acid metabolism (Sokal et al., 2003), and a urea cycle disorder (Horslen et al., 2003). HTx has also been successful at improving metabolic defects, such as albumin secretion (Moscioni et al., 1989; Oren et al., 1999) and tyrosinemia (Overturf et al., 1996). Numerous studies in animal models have also indicated that hepatocytes transplanted into the spleen or the portal vein displayed normal hepatic function and survived for the life-time of the recipient (Gupta et al., 1991; Holzman et al., 1993; Mito et al., 1979; Ponder et al., 1991). Additionally, it has been demonstrated that within two minutes of injection, ~55% of cells migrated via the splenic and portal vein into the liver (Ponder et al., 1991). However, since hepatocytes are large enough to plug the liver microvasculature which may lead to greater repopulation of the liver over time (Strom et al., 2006b), it could also lead to micro emboli in the peripheral veins when large numbers of hepatocytes were infused (Horslen et al., 2003; Strom et al., 1997). Therefore, one group developed an efficient method to implant large numbers of hepatocytes into the subcutaneous cavity by first establishing a neovascularized network through gradual release of basic FGF, a potent agent of angiogenesis (Ohashi et al., 2005; Yokoyama et al., 2006). Prepared hepatocytes were later implanted inside; the establishment of a neovascularized space overcame previous hepatocyte survival issues following transplantation into the unprepared subcutaneous space (Ohashi et al., 2005; Yokoyama et al., 2006).

In addition to the above mentioned metabolic liver diseases that have been treated clinically by HTx with good success, there are several points that provide rationale for the use of HTx specifically for the treatment of MSUD. Human MSUD carriers expressing 50% of whole body BCKDH activity rarely display symptoms (Chuang and Shih, 2001), which was also true for iMSUD mice (Homanics et al., 2006). Furthermore, OLT was also recently determined to be

an adequate treatment for MSUD resulting in full or partial metabolic correction of disease phenotype (Strauss et al., 2006; Wendel et al., 1999), which was dependent upon the severity of initial disease presentation (Wendel et al., 1999). Taken together, these points confirm that 100% of whole body BCKDH activity would not be needed to correct a MSUD disease phenotype. In regards to this dissertation, I hypothesized that infusion of a small percentage of liver mass may be sufficient to correct metabolic function in a case of a mild MSUD variant, or at least partially correct severe cases to a milder and more manageable disease variant.

1.3.1.2 Small vs. large hepatocytes

When comparing various sources of hepatocytes for transplant, it has been suggested that small hepatocytes display a few advantages for their use in transplantation over larger mature cells. "Small hepatocytes", first described in 1992 in rat hepatocyte cultures (Mitaka et al., 1992), were later categorized somewhere between mature hepatocytes and "oval cells", small oval-shaped non-parenchymal cells found to have stem-cell like properties (Farber, 1956). Small hepatocytes, found in greater numbers in isolated human fetal hepatocyte populations, were favorable for transplantation and engraftment due to their smaller, more uniform size compared to mature hepatocytes (Shibata et al., 2006; Strom et al., 2006b). Small hepatocytes appeared to have a higher growth potential with the ability to proliferate better following transplantation (Michalopoulos et al., 1999; Shibata et al., 2006). Since cell isolations of small or fetal hepatocytes consist of a mixed population, this suggested that the full complement of all cell types in the liver were needed for efficient and long-term proliferation of hepatocytes; *in vitro* non-parenchymal cells eventually repopulated beneath hepatocyte colonies to maintain their growth and maturation (Michalopoulos et al., 1999). Additionally, fetal hepatocytes were shown to repopulate approximately 15-25% of the liver without selective regeneration pressure

(Lilja et al., 1997; Nierhoff et al., 2005) and upwards of 80% repopulation with selective regeneration pressure (Cantz et al., 2003; Nierhoff et al., 2005). This is impressive considering one major limitation of HTx for many metabolic liver diseases remains the low level of transplanted cell expansion most likely due to the low level of hepatocyte turnover present in the normal adult liver. The most exciting features of small hepatocytes, however, was their reported success in cryopreservation (Ikeda et al., 2002) and their ability to repopulate the liver using 10-fold fewer cells with the same efficiency as fresh unfractionated cells due to their higher growth potential (Shibata et al., 2006), both of which would be incredibily beneficial clinically.

1.3.1.3 Selective regeneration pressure of transplanted cells

Selective regeneration pressure would give transplanted hepatocytes, regardless of cell size or source, a selective growth advantage maximizing liver repopulation (Gupta et al., 1999; Laconi et al., 1998). It is known that in the presence of liver injury inducing massive cell loss, such as through chemical injury, accumulated toxicity, or surgical resection, there is a huge push towards liver regeneration (Gerber et al., 1983; Wolf and Michalopoulos, 1992); hepatocytes that are normally quiescent begin to divide very rapidly to repopulate the liver. Hepatocyte growth factors significantly increase both in the liver and circulation following partial hepatectomy in humans (Nishizaki et al., 1995) and have been used as a way to jumpstart liver regeneration and therefore stimulate a high proliferation rate in transplanted hepatocytes in animal models (Guha et al., 2001).

Chemical injury, as mentioned above, is another way to give transplanted cells a selective growth advantage. Retrorsine, a pyrrolizidine alkaloid that inhibits the cell cycle, and carbon tetrachloride (CCl_4) are two chemicals frequently used to induce liver injury in animals models (Guo et al., 2002). Retrorsine, given prior to cell transplantation, blocked proliferation of

endogenous liver cells while CCl_4, known to induce liver cell death near the central vein, was given after cell transplantation. Hepatocytes transplanted by intrasplenic injection engraft near the portal veins and thus were not affected by CCl_4 treatment (Guo et al., 2002). There was a high level of hepatic repopulation in normal mice with no method other than retrorsine/CCl_4 treatment to give transplanted hepatocytes any proliferative advantage (Guo et al., 2002).

1.3.1.4 Gene therapy of hepatocytes prior to transplantation

If unaltered hepatocytes can effectively engraft in the liver, it is logical to assume hepatocytes first treated *ex vivo* with a viral vector to express a specific gene to correct a metabolic disorder (e.g., fumarylacetoacetate hydrolase to correct tyrosinemia type I) or a hepatic growth factor could increase their selective advantage over endogenous liver cells. This method could also be used to eliminate the need for immunosuppressive therapy since a patient's own hepatocytes could be harvested and later retransplanted once the cells have been corrected through introduction of a therapeutic gene (Patel et al., 1989; Raper and Wilson, 1993). The first experiments testing this procedure was in a rabbit model to cure an low density lipoprotein (LDL)-receptor deficiency through retroviral gene transfer of explanted hepatocytes (Chowdhury et al., 1991). This resulted in long-term reduction of LDL serum levels This animal study prompted a clinical trial for humans with familial hypercholesterolemia (Grossman et al., 1995; Grossman et al., 1994), and although the treatment was determined to not be sufficient for significant therapeutic advantage, serum LDL levels showed a moderate decrease in some patients. More recently, Adachi et al. (2006) determined that there was no difference in gene transfer and expression of an adenoviral vector in rats by either traditional administration through the tail vein or by *ex vivo* graft perfusion. Other groups have also shown significant

liver repopulation following *ex vivo* infection of hepatocytes (Nguyen and Ferry, 2004; Parouchev et al., 2006) reaffirming the clinical applications *ex vivo* gene therapy may hold.

1.3.2 Embryonic stem cells

1.3.2.1 Establishment and culture

ESCs were first established in the early 1980's from the inner cell mass of preimplantation blastocyst-stage murine embryos (Evans and Kaufman, 1981; Martin, 1981). ESCs are pluripotent, meaning they can differentiate into all derivatives of the three primary germ layers: ectoderm (e.g., CNS, skin), mesoderm (e.g., blood, heart, skeletal muscle), and endoderm (e.g., liver, pancreas, lung) (Keller, 2005), and injection of human ESCs into immune deficient mice resulted in the formation of teratomas (Thomson et al., 1998). Some unique and important characteristics of pluripotent ESCs are their ability to be maintained and expanded in culture as pure populations of undifferentiated cells for repeated, possibly unlimited, passages and their ability to retain normal karyotypes throughout those passages unlike tumor cell lines (Keller, 2005).

To maintain stem cell pluripotentcy and prevent spontaneous differentiation, ESCs are typically cultured on feeder layers, such as mouse embryonic fibroblasts treated with mitomycin C which eliminates their ability to replicate, and in the presence of the pleitrophic cytokine leukemia inhibitory factor (LIF) (Hogan et al., 1994). It has been shown that feeder layers alone cannot maintain pluripotentcy of ESCs (Stewart et al., 1992), and ESCs cultured without feeder cells in the presence of LIF prevents differentiation (Suemori and Nakatsuji, 1987). ESCs can also be sustained, though less effectively, on plated gelatin or extracellular matrix proteins, such as collagen, while in the presence of LIF (Wiles, 1993). LIF, as well as related cytokines in the

interleukin 6 family, complex with glycoprotein 130 to induce signal transduction (Yoshida et al., 1994); cytokines related to LIF also maintain ESCs undifferentiated state (Conover et al., 1993; Nicholas et al., 1994; Rose et al., 1994). Interestingly, while establishment of pluripotent ESCs have been attempted in other animals (Hwang et al., 2005), no cells satisfying all the criteria to be considered true pluripotent ESCs were isolated from any species but mouse (Robertson et al., 1986), and germline chimeras were only successfully produced in mouse (Lee and Piedrahita, 2003), which makes possible the creation of gene targeted mouse models.

1.3.2.2 Human ESCs and U.S. federal policy

In 1997, human ESCs were first established from *in vitro* fertilized blastocysts (Thomson et al., 1998) and were initially cultured on mouse feeder cells in the presence of an animal-derived serum. However, it was later discovered that culturing by this method produced an immune response once the cells were transplanted into a human host (Martin et al., 2005). Therefore, feeder-free/serum-free culture methods devoid of all animal-derived products are being investigated, such as Matrigel and extracellular matrix proteins, as a culture medium for cells meant for transplantation into human patients. However, many of these feeder-free systems are still derived from animals (e.g., collagen from rat tails) (Hwang et al., 2005).

A major problem associated with human ESCs is availability of lines for research; though virtually hundreds of lines exist worldwide, only 22 human ESC lines are available to researchers, as listed by the National Institutes of Health (NIH, 2007), and are limited by the lack of proven data on distinct culture protocols and unique differentiation traits for each line. Derivation of new human ESC lines have been restricted through the implementation of a U.S. federal policy initiated at 9:00PM August 9, 2001, which stated that federal funding for human ESC research would be awarded only if derivation of the cell line was begun prior to the initiated

policy date, the cells were derived from a discarded embryo created for reproductive purposes, and that informed consent without monetary incentives was given prior to the donation of the embryo (NIH, 2006). Since implementation of the above federal policy, at least 14 laboratories across the world have derived new human stem cell lines using U.S. federal funding, though most lines are not currently available publicly (NIH, 2006; NIH, 2007). Derivation of new lines not in accordance with the federal policy is possible if using privately acquired funding rather than federal grants.

1.3.2.3 New methods to derive ESCs

In 1998, the possibility of deriving new human ESCs was made possible through somatic cell nuclear transfer (SCNT), an exciting development in stem cell research (Wakayama et al.). SCNT, or therapeutic cloning (as opposed to reproductive cloning), involved the injection of a somatic cell nucleus into the cytoplasm of an unfertilized egg whose own nucleus was removed, was first tested and proven in 1914 using newt eggs (Spemann). Once the cell begins to divide, pluripotent stem cells can be established from SCNT blastocysts for research purposes (Munsie et al., 2000). ESCs derived from therapeutic cloning have been reported to have the same unique characteristics as traditionally-derived ESCs, and have been used as a model to further prove the ability of ESCs to correct genetic disease (Markoulaki et al., 2008). The most exciting prospect of SCNT is the ability to produce "tailor-made" ESCs for transplantation from the patient's own adult cells and an unfertilized human egg thereby avoiding immune problems and the need for immunosuppression drugs. Importantly, SCNT is in accordance with applicable federal policies restricting research involving human ESCs, but because SCNT utilizes human egg cells, it does raise ethical concerns. Since the implementation of the President's policy in 2001, there have been several bills introduced to Congress whose content could either protect or ban therapeutic

cloning, though none yet have been passed (AAMC, 2007). It is important to recognize the difference between reproductive and therapeutic cloning and the importance of the continuation of research involving pluripotent ESCs.

In late 2007, a novel method to derive human ESCs, termed induced pluripotent stem (iPS) cells, has rocked the stem cell field (Takahashi et al., 2007). iPS cell production involved reprogramming adult fibroblast cells to a pluripotent ESC-like state through retroviral introduction of four defined transcription factors under ESC culture conditions. The method was first proven in mouse cells less than a year earlier (Takahashi and Yamanaka, 2006; Wernig et al., 2007). This solved the ethical objections to a previous nuclear reprogramming method, which involved fusion of somatic cells with a human ESC (Cowan et al., 2005; Tada et al., 2001). The four transcriptions factors were: (1) Oct3/4, a specific marker of undifferentiated embryonic stem cells, from the octamer-binding transcription factor family (Ryan and Rosenfeld, 1997), (2) Sox2, a SRY-related High Mobility Group-box transcription factor characterized by the presence of a HMG (Schepers et al., 2002), (3) Klf4 from the Kruppel-like factor zinc-finger family of proteins related to the Kruppel regulator in *Drosophila* (Dang et al., 2000), and (4) c-myc, a proto-oncogene, from the Myc family of transcription factors (Adhikary and Eilers, 2005). Resulting iPS cells, whose formation was a fairly rare event (<0.1%), displayed ESC morphology and growth patterns, and expressed ESC markers, although Nanog was absent from cells in this first report (Takahashi and Yamanaka, 2006). It was also later determined that induction of some, though not all, family members of the four transcription factors substituted for those listed above could also produce iPS cells (Nakagawa et al., 2008). Nanog-negative iPS cells resulted in teratoma formation characterized by differentiated tissue from all three germ layers after mouse transplantation, and could contribute to mouse embryonic development when

injected into a blastocyst, but chimeric embryos did not survive to term, and the stability of pluripotentcy was questionable (Takahashi and Yamanaka, 2006). However, when iPS cells selected for Nanog expression were used, DNA methylation, gene expression, and chromatin state were found to be more similar to ESCs than Nanog-negative cells, and pluripotency was more consistent (Wernig et al., 2007). Nanog, a homeoprotein found in ESCs and developing germ cells (Mitsui et al., 2003), was found to be the critical element in maintaining pluripotentcy and mediating germline transmission (Chambers et al., 2007), and accordingly Nanog-positive iPS cells were able to produce viable mouse chimeras and contribute to germ line (Okita et al., 2007; Wernig et al., 2007). Regrettably, ~20% of offspring generated from iPS germline mouse chimeras resulted in tumor formation from the reactivation of the c-myc transgene, thus providing rationale to avoid retroviral c-myc induction for production of iPS cells for clinical applications (Okita et al., 2007). Soon after, two groups were able to produce iPS cells from both mouse and human fibroblasts without c-myc, though generation times were much slower than with c-myc (Nakagawa et al., 2008; Yu et al., 2007). Extremely important for future clinical applications, iPS could be generated from a patient's own skin cells (Park et al., 2008), which could then be transplanted for correction of genetic disease or tissue repair avoiding immunoincompatibility concerns, much like SCNT. Recently iPS cells have been differentiated into hematopoietic and cardiovascular lineages (Schenke-Layland et al., 2008). Thus far an anemia mouse model has been corrected after transplantation of hematopoietic cells differentiated from fibroblast-derived iPS cells (Hanna et al., 2007) providing a convincing initial proof-of-principle study for the use of iPS cells to treat disease. However, there are issues and ethical concerns associated with iPS cells (Cyranoski, 2008), which are summarized in

Table 6, and since the science is still so very new, there are many unanswered questions regarding the true clinical potential of iPS cells.

Table 6. iPS cells: too good to be true?
Modified from (Cyranoski, 2008)

Claimed Benefits	Caveats
(1) Easy to produce	Each retrovirally introduced transcription factor has a ~15% transfection efficiency, and ~5% of those that receive all factors become fully reprogrammed; identified iPS cells require very specific culture conditions
(2) Custom-tailored cells for everyone	Alternate system of introducing factors are needed (currently retroviral); production of iPS cells are time consuming (see #1) followed by extensive testing to rule out possiblility of tumor formation (~2 year procedure); predicted to be extremely expensive
(3) Cures are coming soon	Disease models and proof-of-principle studies will be first; future of iPS cell therapies will likely depend on how well thay can be shown to differentiate into specific cell types and safe methods to administer them; clinical trials using embryonic stem cells will also decide the fate of iPS cell treatments
(4) iPS cells are identical to ESCs	There are no major differences yet; there are conflicting reports between iPS properties and cell marker expression; there will likely be differences between lines and therefore between iPS-derived cell types meaning each line will require rigorous testing
(5) iPS cells have no ethical issues	Production of gametes is possible from pluripotent iPS cells; iPS derived gametes could potentially meet demands for infertility procedures or clone a human, either of which may result in strange, unwanted outcomes.

1.3.2.4 Methods for initiating cell differentiation

ESCs are pluripotent, meaning they have the ability to form cells from any lineage, though *in vitro* this may require precise culture conditions, removal of LIF and/or feeder layers, and the addition of specific growth factors (Hwang et al., 2005; Keller, 2005; Palacios et al., 1995). Three general approaches for the initiation of ESC differentiation were developed. The first involved culture at high cell density on a non-adhesive surface resulting in the aggregation of ESCs and the formation of three-dimentional "embryoid bodies" (EB) characterized by an endoderm-like outer layer and inner cavity, which displayed many parallels to embryo

development *in vivo* (Doetschman et al., 1985). Once the EBs were allowed to reattach, they formed outgrowths which differentiated into various tissues. The second method involved ESC culture directly on stromal cells; specific growth factor secretion and other cellular signals from stromal cells were able to manipulate the ESCs to differentiate into lymphohematopoietic cells in a highly reproducible manner (Nakano et al., 1994). The third method involved differentiation in

Table 7. Initiation of ESC differentiation: advantages and disadvantages.

Differentiation Method	Advantages	Disadvantages
(1) EB formation	3-D structure much like *in vivo* development enhances cell-cell interactions, which may be important to differentiation of specific cell types	Complexity of the EB structure; generation of cytokines and other factors can confuse experimental interpretation
(2) Culture on stromal cells	Specific cell type/line used provides beneficial growth factors	Undefined or uncontrolled growth factors may differentiate cells to an undesired type; difficult to separate stromal cells from desired cells
(3) Culture on intracellular matrix proteins	Very simple protocol, eliminated influence and interactions encountered when using cell monolayers	Proteins used can greatly influence the generation and survival of differentiating cell types

culture of ESCs on a monolayer of extracellular matrix proteins (Nishikawa et al., 1998), which demonstrated differentiation into hematopoietic cells simply through direct interaction with collagen without the addition of any supplementary growth factors. All three differentiation methods have been used to produce a multitude of cell types from ESCs, however each method carries with it specific advantages and disadvantages, which are compiled in **Table 7,** as summarized by Keller, et al. (2005).

1.3.2.5 Differentiation into specific cell types

ESC differentiation into hematopoietic cells have been investigated since 1985 when the presence of hemoglobinized cells were identified in cultures containing embryoid bodies (Doetschman et al., 1985). From that time, many protocols to derive a hematopoietic lineage from ESCs have been reported (Bhatia, 2003; Lengerke and Daley, 2005; Nishikawa et al., 1998; Orlovskaya et al., 2008; Wang et al., 2006). Unfortunately, over the past 20+ years there has been very little progress, with most studies unable to identify differentiated cells from ESC culture (Müller and Dzierzak, 1993). However, in 2008 two reports stated the derivation of functional red blood cells and platelets through differentiation of ESCs *in vitro* (Hiroyama et al., 2008; Takayama et al., 2008). Therefore, there may be hope on the horizon for ESC-derived hematopoietic cell therapies. There are also many protocols for derivation of ESC to a cardiomyogenic lineage (Heng et al., 2004b), a neuronal lineage (Lee et al., 2000; Yan et al., 2005), a keratinocyte lineage (Heng et al., 2005), an osteogenic lineage (Heng et al., 2004a), a chondrogenic lineage (Bosnakovski et al., 2006; Heng et al., 2004b), a pancreatic ductal-cell lineage (Naujok et al., 2008; Soto-Gutiérrez et al., 2008), and a hepatic lineage (Chinzei et al., 2002; Hamazaki et al., 2001) to list a few.

Differentiation of ESC into hepatocyte-like cells has been thoroughly investigated as evident from the multitude of reviews comparing numerous differentiation protocols, culture methods, and growth mediums (Gouon-Evans et al., 2006; Ishii et al., 2008; Keller, 2005; Lavon and Benvenisty, 2005; Shirahashi et al., 2004; Soto-Gutiérrez et al., 2006); some of these protocols are summarized in **Table 8**. Basic differentiation protocols were based upon what was known of liver generation *in vivo*. During early mouse development (E8.25, 7-8 somites), liver formation initiates in the ventral foregut endoderm (Lemaigre and Zaret, 2004) through

fibroblast growth factor (FGF) expression from the nearby cardiac mesoderm (Jung et al., 1999) and bone morphogenic proteins 2 and 4 signals from the septum transversum mesenchyme cells (Ang et al., 1993; Rossi et al., 2001). Interaction of the budding cells [i.e., hepatoblasts – which yields both hepatocytes and cholangiocytes (ductal cells)] with mesoderm-derived endothelial cells was important to this stage in development (Matsumoto et al., 2001). Fetal liver is a major site of hematopoiesis, and it was discovered circulating hematopoietic cells that migrate to the liver express oncostatin M, an interleukin-6 family cytokine, which contributed to early liver development (Kamiya et al., 1999). Further growth and maturation of the liver required many more signal transduction and transcription factors (Duncan, 2003; Zaret, 2002). In 2003, Fair, et al. determined murine ESCs stimulated *in vitro* with chick cardiac mesoderm differentiated into cells with a putative endodermal phenotype. Fair et al. (2005) later determined murine ESCs stimulated *in vitro* with acidic FGF alone could cause differentiation into putative endodermal precursor cells (PEPs), which engrafted in the liver and functioned normally *in vivo*. Bone morphogenic protein 4 was later reported to be required for hepatic differentiation from ESCs (Gouon-Evans et al., 2006). However, since such a large array of transcription factors, growth factors, and specific interactions required at specific points during development for proper liver formation *in vivo* exists which have not yet been clearly defined, effective differentiation has proved difficult typically resulting in a low percentage of cell differentiation and cellular heterogeneity in culture (Wang et al., 2008). In order to improve upon current methods, one group developed a multistep protocol that involved adding specific growth factors during specific stages of cell differentiation in an attempt to mimic hepatocyte development *in vivo* (Hamazaki et al., 2001). Mouse ESCs differentiated by Hamazaki et al.'s protocol expressed albumin and produced urea, which are both hepatocyte-specific, and cells were able to maintain

Table 8. Small selection of ESC differentiation methods to produce hepatic cells.

[1]Protocol	[2]RNA Markers	[3]Protein Markers	Functional Assay	[4]In Vivo Assay	Reference
Spontaneous - EB	AFP, ALB, TTR, TCF1, TCF2, HNF4				(Abe et al., 1996)
Spontaneous - EB	AFP, ALB, TAT	ALB	Urea synthesis	IHC: ALB	(Chinzei et al., 2002)
Spontaneous - EB	ALB, AAT	ALB	Urea synthesis		(Shirahashi et al., 2004)
aFGF, TGF, AFP, HGF, OSM, dextrose	AFP, ALB, TTR, TAT, G6P, FOXA2	ALB, AFP, CK8, CK18			(Hu et al., 2004)
Insulin, dextrose, collagen type 1	AFP, ALB, CK19, G6P, GGT	ALB	Urea synthesis		(Shirahashi et al., 2004)
aFGF, HGF, OSM, dextrose, ITS, 3D scaffold	AFP, ALB, TAT, TTR, G6P	ALB		IHC: ALB, CK18	(Imamura et al., 2004)
Limited serum, dextrose, Act.A	AFP, ALB, TTR, AAT, TAT, CPS1			IHC: FOXA2, teratoma	(Kubo et al., 2004)
FOXA1, FOXA2	FOXA1, FOXA2, ALB, TTR, TCF2				(Levinson-Dushnik and Benvenisty, 1997)
FOXA2, bFGF, dextrose, asc., nicotinamide	ALB, C3, CYP1A1, PEPCK, PXMP1-L	ALB, CK18	Urea and lipid synthesis		(Ishizaka et al., 2002)

[1] EB – embryoid body, aFGF – acidic fibroblast growth factor, TGF – transforming growth factor, AFP – α-fetoprotein, OSM – oncostatin M, HGF – human growth factor, ITS – insulin transferring & selenius acid, Act.A – activin A, FOXA1/2 – forkhead box A1/2, asc. – L-ascorbis-2-phosphate

[2] TPP – transthyretin, TCF1/2 – transcription factor 1/2, HNF4 – hepatocyte nuclear factor 4, TAT – tyrosine aminotransferase, AAT – α1-antitrypsin, G6P – glucose-6-phosphatase, CPS1 – carbamyl phosphate synthetase 1, C3 – complement C3, CYP1A1 – cytochrome P450 1A1, PEPCK – phosphoenolpyruvate carboxykinase, PXMP1-L – peroxisomal membrane protein 1-like protein

[3] CK8/18 – cytokeratin 8/18

[4] IHC - immunohistochemistry

differentiation when transplanted *in vivo* (Chinzei et al., 2002). Other groups have also demonstrated the ability for hepatocyte-like cells to maintain differentiation *in vivo* (Imamura et al., 2004; Yamada et al., 2002), and still others have used these cells to treat disease, such as in correction of a FIX mouse model (Fair et al., 2005), induced hepatic failure (Hu et al., 2006; Ishii et al., 2007; Soto-Gutiérrez et al., 2006; Tabei et al., 2005), and a fumarylacetoacetate hydrolase deficiency model of tyrosinemia type I (Sharma et al., 2008). Although currently few reports exist which apply ESC-derived hepatocytes towards the treatment and correction of liver diseases, the results nonetheless serve as exciting early proof-of-principle studies for possible future clinical applications.

1.3.2.6 Common issues associated with ESC therapy

Although several problems are associated with the use of ESCs for cellular therapies, the most serious being lack of transplant function, homograft rejection due to host immune response, and teratoma formation, PEPs derived by Fair et al. (2005) produced significant liver engraftment *in vivo* with no evidence of rejection or antibody response although transplanted cells were not syngeneic. Conversely, another study found significant humoral and cellular responses following non-syngenic transplantation of ESCs to the myocardium in mice (Kofidis et al., 2005). However, if iPS cells can be used in place of ESCs, which could be made patient-specific, it would eliminate immunogenic concerns (Park et al., 2008). Teratoma rate in Fair et al.'s study, while not insignificant, was very low, ~6.2% (Fair et al., 2005); however, 129 mice, the source of ESCs used by this group, have been previously reported to be notorious for a high spontaneous teratoma rate (Stevens, 1973). Other groups have also reported teratoma formation after the transplantation of hepatocyte-derived ESC (Chinzei et al., 2002; Choi et al., 2002; Teramoto et al., 2005), though longer culture times prior to transplantation appeared to reduce

the incidence of teratoma formation presumably due to a greater percentage of cells in culture becoming differentiated (Chinzei et al., 2002). Specifically for hepatocyte-like cells derived from ESC, major issues focus on difficulties in differentiation due to a poor understanding of *in vivo* liver development (Wang et al., 2008), as mentioned above.

Prior to the use of ESCs for transplantation to correct liver disease in humans, these issues must be resolved. Steps must be taken to develop better screening methods for differentiated cells to avoid teratoma formation, develop more efficient/effective differentiation protocols to produce higher quality hepatocyte-like cells, which may also help increase transplant engraftment and function *in vivo*, and reduce the risk of host immune response to transplanted cells. Once efficacy and safety concerns have been quieted, ESC therapy may prove to be equal in its ability to correct metabolic liver disease as transplanted hepatocytes.

2.0 FURTHER CHARACTERIZATION OF iMSUD MICE

Hypothesis: I hypothesize that the iMSUD mouse model will closely mimic the human disease phenotype based upon previously published data, and thus provide an excellent model to test both gene therapy and cell-based therapies to cure MSUD.

2.1 BACKGROUND AND SIGNIFICANCE

Maple Syrup Urine Disease (MSUD) is a serious liver-based metabolic disease for which no universally satisfactory treatments exist. MSUD is caused by a deficiency in the BCKDH complex resulting in toxic accumulation of BCAA (leucine, valine, isoleucine) and their associated metabolites in the body (Chuang and Shih, 2001; Harris et al., 2004; Yeaman, 1989). This toxic accretion mainly affects the brain resulting in serious clinical manifestations, which in most cases results in permanent neurological dysfunction or death without treatment and life-long attentive care (Chuang and Shih, 2001; Cox and Chuang, 1993).

In order to test potential therapies, two novel mouse models were created using standard genetic engineering methods (Homanics et al., 2006), as described in greater detail in the Introduction section. The model for cMSUD was an E2 subunit gene knockout line. Although knockout pups appeared normal at birth (i.e., indistinguishable from wildtype and heterozygous littermates), they had no detectable BCKDH activity, extremely elevated BCAA levels, failed to

thrive, and ultimately died within a few days. This severely limited possible treatment options due to the extremely limited time the pups were alive. The model for iMSUD was created by transgenic expression of human E2 on the mouse E2 knockout background. The iMSUD mice had elevated levels of BCAA intermediate between wildtype and knockout mice, and many survived to adulthood without any special treatment. The phenotypes of these mouse models closely mimicked the pathophysiology of human MSUD. However, a more thorough understanding of how the iMSUD mouse model is affected by the disease will provide rationale towards determining the best and most effective therapy to correct it. Therefore, a more complete characterization of the iMSUD model was completed and is presented below.

2.2 METHODS

All studies involving animals were reviewed and approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

2.2.1 Mouse colony breeding

Production of iMSUD mice relied on the expression of two transgenes (LAP-tTA and TRE-E2; **Figure 4**) to produce human E2 on a mouse E2 knockout (KO; -/-) background (**Figure 3**). Two lines of iMSUD mice were used, A and 525A. The only difference between the two iMSUD mouse lines was the genomic location of the LAP-tTA transgene, which was introduced by breeding and not targeted insertion (Homanics et al., 2006). In order to facilitate the production of iMSUD mice, the transgenes were first bred to homozygosity. Mating pairs

consisted of mice homozygous for both transgenes and heterozygous for mouse E2 KO resulting in a 1:2:1 ratio of +/+ : +/- : -/- mouse E2. All offspring from these matings expressed human E2.

2.2.2 PCR genotyping

Some animals were PCR genotyped at 1 week of age to distinguish iMSUD animals from wildtype and heterozygous littermates. This method gave accurate genotyping data in 1-2 days. Pups were first identified using a toe microtattooer specific for mice (Ketchum, Brockville, ON), and genomic DNA was isolated from a small amount of blood using Instagene (BioRad, Hercules, CA). Each DNA sample was analyzed with two sets of primers specific either to mouse exon 5 of the E2 gene (present only in wildtype and heterozygous animals) or the PGKneo cassette (present only in heterozygous and homozygous iMSUD animals). Neo specific primers consisted of 5'GCATTCTGCACGCTTCAAAAG 3' and 5'GCAGCCGATTGTCTGTTGTG 3', which amplified an 186bp fragment. Mouse E2 specific primers were both contained within exon 5 and consisted of 5'CAGAGGAAGATGTTGTTGAACCC 3' and 5'TTGTTTTCCATTGCCAGCCGACGC 3', which amplified an 119bp fragment. This method of PCR genotyping was proven to be highly accurate and reproducible. Results were confirmed using Southern blot analysis.

2.2.3 Southern blot genotyping

Genomic DNA was isolated from 1cm mouse tail clips taken at 3 weeks of age. DNA was suspended in TE buffer, pH8 and each sample analyzed twice by digesting with two different enzymes: EcoRI and BglI. Digested samples were run through a 1% agarose/4x

Hellings gel for 950 minutes at ~20V, transferred to Hybond-N (Amersham, Piscataway, NJ) via capillary transfer, and DNA was fixed to the membrane by UV crosslinking. Prior to hybridization with a radiolabeled probe made via the Random Primed DNA Labeling Kit (Roche, Indianapolis, IN), membranes were incubated with prehybridization solution containing salmon sperm DNA for several hours at 42^{o}C. Southern blots with EcoRI digested samples were hybridized overnight with ^{32}P-labeled DNA probes specific to the LAP-tTA and TRE-E2 transgenes. BglI digested samples were hybridized overnight with a ^{32}P-labeled DNA probe specific to mouse E2 exon 6. Membranes were exposed to X-ray film for detection under nonsaturating conditions. E2 wildtype mice displayed a band at ~16kB, E2 knockout mice displayed a band at ~11kB, and heterozygous mice displayed bands of both sizes (**Figure 3**). Regarding the transgene-specific probes, transgene positive mice displayed a band (LAP-tTA = ~2kB, TRE-E2 =~15kB) and transgene negative mice did not.

2.2.4 BCKDH complex enzyme activity assay

Livers were removed, flash frozen in liquid N_2 and stored at -80^{o}C. On the day of analysis, livers were homogenized (1:9 w/w) in 0.25 sucrose, 10mM Tris-HCl and centrifuged 600xg for 10 minutes and the supernatant utilized for the enzyme assay. BCKDH activity was determined by measuring the release of $^{14}CO_2$ from α-keto [1-^{14}C] isocaproate (Amersham Biosciences, Piscataway, NJ) (Paul and Adibi, 1992). Assays were done in duplicate and carried out for 15 minutes at 37^{o}C; $^{14}CO_2$ was trapped in hydroxide of Hyamine (Sigma-Aldrich, St. Louis, MO) and radioactivity was determined by liquid scintillation spectrometry. Liver protein concentration was assessed by Bicinchoninic acid assay. Calculation: x μmol KIC oxidized = DPM $^{14}CO_2$/DPM ^{14}C-KIC initially added/15 minutes/x mg liver protein. Data was presented as

the mean +/- the standard error of the mean. Differences between genotypes were compared by the nonparametric Kruskal-Wallis test and a Mann-Whitney analysis (Kruskal and Wallis, 1952; Mann and Whitney, 1947).

2.2.5 BCAA levels

Blood or brain samples were collected at weaning. Blood samples were spotted on a Guthrie card and mailed to Pediatrix Screening Inc. (Bridgeville, PA) for processing by tandem mass spectrophotometry (MS-MS). Brain samples were immediately flash frozen in liquid N_2 and shipped to Dr. William Zinnanti (Pennsylvania State University of Medicine at Hershey, PA) for processing by HPLC analysis. Total BCAA levels were expressed as a ratio to alanine, a sensitive indicator of MSUD phenotype (Morton et al., 2002). Data was analyzed by Repeated Measures ANOVA and post-hoc Fisher's test.

2.2.6 Real Time qPCR

RNA was isolated from iMSUD and WT primary mouse hepatocytes and primary human hepatocytes using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA was converted to cDNA using the High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA). Real Time qPCR was performed using the TaqMan Assays-on-Demand Gene Expression Products for DBT (BCKDH E2) exon 2-3 and β-actin (ID# Mm00501651_m1 and Mm02619580_g1, respectively; Applied Biosystems, Foster City, CA) following the manufacturer's protocol. In short, 100ng of cDNA template, 12.5µl of 2x Taqman Universal Master Mix, and 1.25µl of 20x Target Assay Mix were combined into 25µl reactions.

Each sample was run in triplicate on an ABI 7000 Real-time PCR System (Applied Biosystems). Relative quantification of Real Time RT-PCR data was performed using the Comparative C_T (ΔC_T) Method. In brief, C_T values were first converted to an arbitrary number using a standard calculation [arbitrary number = $1000000000^{(-0.69077552 * CT)}$]. The three arbitrary numbers (AN) for each sample were then averaged. Target gene expression levels were normalized to the corresponding actin endogenous control by calculating the ΔC_T [ΔC_T = $_{ave}$AN $_{target\ gene}$ / $_{ave}$AN $_{actin}$]. Data was presented as the mean +/- the SEM and analyzed by ANOVA and post-hoc Fisher's test.

2.2.7 Northern blot analysis

RNA was isolated from liquid N_2 flash frozen liver tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA (20μg/sample) was size fractionated by electrophoresis for 4 hours at 100V through a 1% agarose/formaldehyde gel in MOPS buffer and transferred to Hybond-N via capillary transfer. RNA was fixed to the membrane by UV crosslinking, and briefly stained with 0.03% (w/v) methylene blue in 0.3M sodium acetate (pH 5.2) to verify a successful transfer. Prior to hybridization with a radiolabeled probe, membranes were incubated with prehybridization solution containing salmon sperm DNA for several hours at 42^{o}C. Northern blots were hybridized overnight with several ^{32}P-radiolabeled probes made via the Random Primed DNA Labeling Kit (Roche, Indianapolis, IN). These probes were specific to human β-actin cDNA (#9800-1, Clontech, Mountatinview, CA), and human E2 cDNA. Several human E2 probes were created through restriction enzyme digest

of the pLi-hE2 plasmid (human E2 cDNA in the pLitmus29 plasmid, **Figure 6**). Membranes were exposed to X-ray film for detection under nonsaturating conditions.

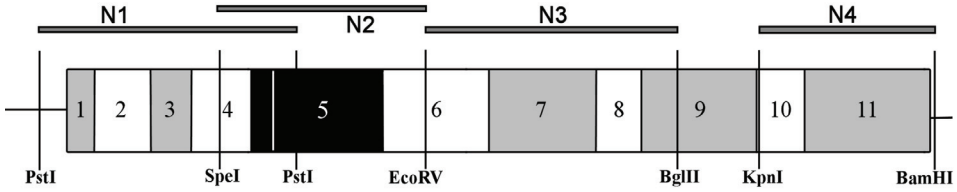

Figure 6. Human E2 cDNA probes for Northern blot.
pLi-hE2 was digested with various restriction enzymes to produce cDNA probes to screen mouse iMSUD RNA. The human probes cross-hybridized with mouse RNA. Probes were labeled N1-N4; the numbers 1-11 correspond to the 11 E2 exons. The portion depicted in black, exon 5 and part of 4, corresponds to the sequence deletion used to create an E2 gene knockout.

2.2.8 Western blot analysis

Protein extracts were isolated from liquid N_2 flash frozen homogenized liver. Protein (25μg per sample) was analyzed by electrophoresis for 2.5 hours at 76V through a 10% SDS-PAGE Ready Gel (Bio-Rad) and transferred to PVDF membrane (Bio-Rad) via electroblotting for 50 minutes at 400mA. Membranes were probed for E2 protein using polyclonal rabbit antisera (1:5000) which detects both mouse (~47kD) and human (~54kD) E2 subunits [(Hutson et al., 1998), a generous gift from Dr. S. Hutson]. A goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP; #NB730H, Novus Biologicals, Littleton, CO) was used for detection using the Western Lightning chemiluminescence reagent (Perkin Elmer) and exposed to X-ray film. Membranes were also reprobed with an antibody to c-myc (#ab9106-100; AbCam, Cambridge, MA) to detect transgene-derived E2. An antibody to β-actin (#ab8227-50; AbCam) and GAPDH (#NB300-327; Novus Biologicals) was also used to allow for loading control comparisons. E2 antibody was hybridized to the membrane for 1.5 hours and

washed for 4 x 15 minutes with phosphate buffered saline/0.1% Tween 20 (PBS-T). GAPDH and β-actin antibodies were hybridized to the membrane for 1 hour and washed for 3 x 10 minutes with PBS-T. Results were quantified using Kodak 1D software. With regard to the two different iMSUD mouse lines analyzed, A and 525A: The only difference between the two iMSUD mouse lines was the genomic location of the LAP-tTA transgene, which was introduced by breeding and not targeted insertion (Homanics et al., 2006). Data were presented as the mean +/- the standard error of the mean. Differences between genotypes were compared by ANOVA and post-hoc Fisher's test.

2.2.9 Immunofluorescence

Livers from iMSUD and WT mice were immediately flash frozen in liquid N_2 and stored at -80^{o}C until tissue sectioning. Frozen tissue was cryopreserved in Optimum Cutting Temperature Compound (OCT; Tissue-Tek), sectioned at 10μ using a Leica CM1850 cryostat, and mounted on slides. Mounted tissue was stored at -20^{o}C until analysis. Mounted sections were briefly fixed for 10 minutes in -20^{o}C methanol, washed 3 x 15 minutes in 0.1M PBS + 0.1% TritonX-100 for permeablization, and blocked in 10% normal goat serum (NGS) for 1 hour. Primary antibody hybridization occurred simultaneously in a dark humidity chamber at room temperature for 1 hour (or at 4^{o} overnight): 1 μg/mL rabbit polyclonal E2 antisera [(Hutson et al., 1998), a generous gift from Dr. S. Hutson] and 1μg/mL mouse monoclonal COX IV antibody (#ab14744; AbCam) in NGS. Liver sections were washed for 3 x 15 minutes in 0.1M PBS + 0.5% BSA + 0.15% glycine (Buffer A) and reblocked in NGS for 30 minutes. Secondary antibody hybridization occurred in tandem. Goat anti-rabbit-Cy3 (#ab6939; AbCam)

was hybridized at a concentration of 1μg/mL in NGS for 45 minutes at room temperature, followed by washes in Buffer A as described above. Tissue was reblocked in normal donkey serum (NDS) for 30 minutes, and donkey anti-mouse-Alexa Fluro 448 (generous gift from Dr. P. Monaghan, University of Pittsburgh) was hybridized at a concentration of 1μg/mL in NDS for 45 minutes at room temperature. Tissue sections were also tested for healthy liver morphology with a rhodamine-phalloidin stain, which hybridizes to cell cytoskeleton (i.e., actin), and a Hoechst stain for nuclear identification. Both the rhodamine-falloidin and Hoechst stains were generous gifts from Dr. Donna Stolz at the University of Pittsburgh Center for Biologic Imaging. After staining, sections were washed briefly in PBS and coverslipped for visualization with an aqueous-based mounting media. Livers were analyzed for E2 (E2, Cy3, red), mitochondria (CoxIV, Alexa Fluor 448, green), actin (rhodamine-phalloidin, red), and nuclei (Hoetchs, UV) with a Nikon Y-FL inverted microscope.

2.2.10 Histochemistry

Livers from iMSUD and WT mice were fixed in 4% paraformaldehyde for no more than 24 hours. Tissues were then incubated in 30% sucrose for at least 24 hours followed by cryopreservation and frozen sectioning by cryostat (30 or 6 microns). To analyze liver cell morphology, a standard hematoxylin and eosin stain was performed and sections were subjected to a dehydrating ethanol/xylene series wash and subsequently coverslipped with a xylene based mounting media. For analysis of fat deposits, a Red Oil O stain was performed. Liver sections were counterstained with hematoxylin for easier visualization of individual cells and coverslipped with an aqueous mounting media. Tissue was visualized with an Olympus Ix71 inverted microscope.

2.2.11 Body weight

Body weight was obtained at weaning (~21 days of age) of disease models and littermate controls for comparison. Data were analyzed by Student T-test.

2.3 RESULTS AND DISCUSSION

2.3.1 iMSUD mice had significantly stunted growth at weaning

iMSUD mice, though indistinguishable from WT littermates at birth, were previously observed to be substantially smaller than littermates at weaning (unpublished observations, Homanics). To determine whether the observed growth delay was significantly different from healthy WT littermates, mice were weighed at weaning (~21 days of age). It was determined that iMSUD mice suffered significant growth retardation compared to littermate controls ($p<0.0001$; **Figure 7**). Growth and developmental delay is a typical phenotype associated with human MSUD; BCAAs are essential amino acids, and therefore any decrease in the required amount causes problems with growth and development (Chuang and Shih, 2001). Growth restriction may also be partly due to the reduced production of β-hydroxy-β-methylbutyrate (HMB), a metabolite of leucine and KIC which is known to enhance muscle protein synthesis and thus maintain proper body composition (Nissen and Abumrad, 1997; Wilson et al., 2008). The majority of HMB is produced from KIC in the cytosol [BCKDH independent (Van Kovering and Nissen, 1992)], which occurs only in liver. However, a small amount of HMB is also produced from β-methyl-crotonyl-CoA in the mitochondria of all tissues [BCKDH dependent

(Nissen and Abumrad, 1997)]. Thus, mitochondrial HMB production would be inhibited in MSUD. At this time, it is unknown whether HMB contributed to decreased body weight at weaning of iMSUD mice. HMB levels in tissues of iMSUD mice have not been assessed, and therefore this requires further investigation.

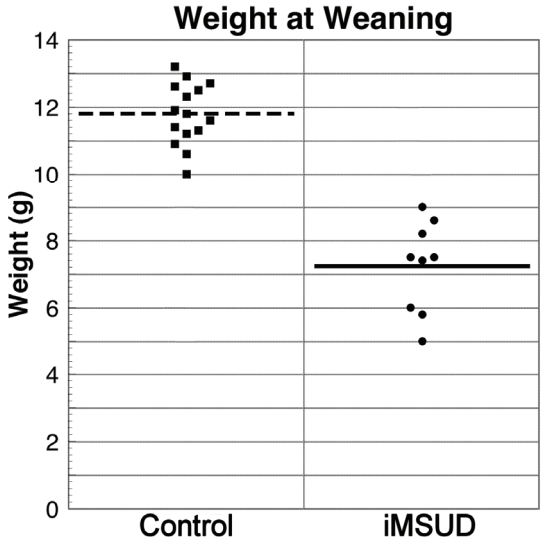

Figure 7. Weight at weaning of iMSUD mice and littermate controls.
Body weight of iMSUD and controls (WT) were collected at weaning (21 days of age). Weight at weaning of iMSUD animals were significantly different compared to Controls ($p<0.0001$). Each data point represents a single mouse in each group. The horizontal line indicates the mean value.

iMSUD mice also displayed other phenotypic differences at weaning that identified them as disease models. One subtle difference could be seen in their eyes. All iMSUD animals had squinted eyes, which were distinctly almond shape due to mice incompletely opening their eyes and not from incomplete eye/eyelid development; healthy littermates all had very round-shaped eyes. Interestingly, parents of human MSUD patients can tell when their child had high BCAA blood levels by their eyes, which looked squinted or "smaller" (personal communications, MSUD Symposium attendees, 2006 & P. Verma, 2008). iMSUD mice often displayed other phenotypic manifestations such as seizures, hypoactivity, uncoordinated gait, and hind limb

dystonia (Zinnanti et al., 2008). In severe cases, paralysis and muscle wasting was observed, which corresponded with severely high BCAA/ala levels (Zinnanti et al., 2008); animals found in that condition were considered moribund and were humanely sacrificed.

2.3.2 E2 protein in liver and other tissues

Transgenic human E2 was directed to the liver in iMSUD animals through use of the LAP promoter (Homanics et al., 2006). It was previously determined that iMSUD liver BCKDH enzyme activity was approximately 5-6% of control enzyme activity. Therefore, it was necessary to determine the amount of transgene derived human E2 protein in the liver as well as the specificity of the LAP-tTA liver specific transgene (Kistner et al., 1996). E2 protein in the liver, muscle, kidneys, and brain was analyzed by Western blot [**Figure 8** (Homanics et al., 2006)]. Surprisingly, E2 protein in liver of iMSUD mice, though variable between individuals, was expressed at levels similar to Control mice in some animals. C-myc expression was also determined as a positive control since a c-myc tag was incorporated on the C-terminal end of the transgenic human E2 protein. C-myc and human E2 expression in the liver of iMSUD mice were identical, as expected. E2 expression was found to be at undetectable or very negligible levels in other tissues suggesting that liver was the only tissue contributing to BCAA metabolism (Homanics et al., 2006). E2 expression in other tissues besides liver would have suggested those tissues were contributing to BCKDH activity and thus the enhanced survival seen in the iMSUD model. Therefore, if that had been the case, assessing enzyme activity in the liver alone would have been misleading.

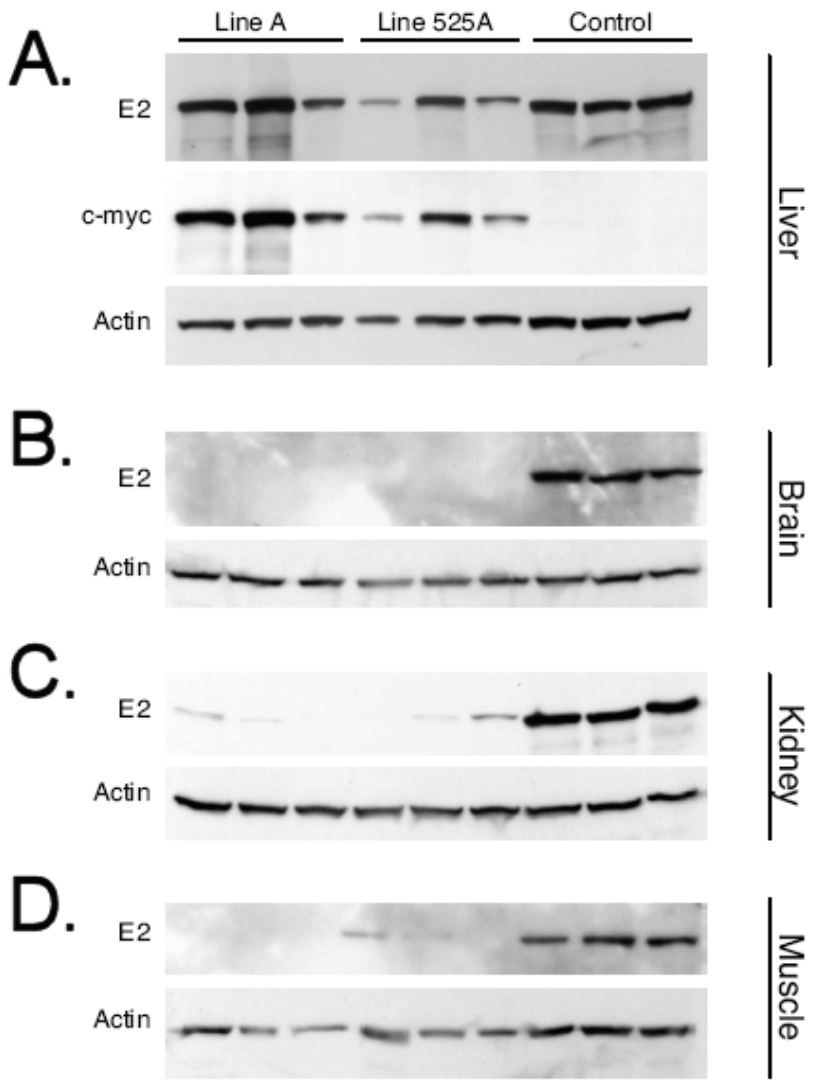

Figure 8. Transgenic E2 protein expression in iMSUD mice.
E2 transgenic protein expression was characterized in iMSUD A. liver, B. brain, C. kidney, and D. muscle by Western blot analysis. c-myc expression, incorporated on the C-terminus of transgenic E2, was identical to E2 expression in liver, as expected. Actin was used as a loading control. Line A and Line 525A are two iMSUD mouse lines which differ only in the genomic location of the LAP-tTA transgene. Reproduced with permission from (Homanics et al., 2006)

2.3.2.1 c-myc may hinder proper BCKDH enzyme assembly

The observation that near normal E2 protein levels in liver produced only 5-6% of BCKDH enzyme activity suggested that the BCKDH complex was operating at a suboptimal level. The reason for this may be the c-myc tag incorporated onto the carboxy-terminus of the human E2 cDNA in Transgene B (**Figure 3**). The presence of c-myc tagged protein was also assessed by Western blot, which was determined to be equal to E2 protein levels and was absent from control samples (**Figure 8A**) (Homanics et al., 2006). It was proven previously that the E2 C-terminal end was important for subunit assembly of two enzyme complexes in the same family as the BCKDH complex, the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes (Knapp et al., 2000; Mattevi et al., 1992). In Mattevi et al. and Knapp et al.'s studies, a 6xHis tag incorporated onto the C-terminal end of the E2 subunit reduced the normally occurring 24-mer to a trimer with significantly decreased activity. Therefore, since the BCKDH complex has the same protein structure as the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes, it is logical to deduce that a c-myc tag incorporated at the C-terminal end of the BCKDH E2 subunit interfered with proper enzyme assembly, thus reducing activity.

2.3.2.2 Human E2 may interfere with mouse BCKDH components

Another explanation for near normal E2 protein in the liver, yet only 5-6% BCKDH enzyme activity could be that human E2 is unable to assemble properly and thus is not fully functional when complexed with endogenous mouse E1 and E3 subunits. Although the BCKDH E2 subunit is very well conserved across all species, mouse and human E2 are 88% homologous when the leader sequence is included, and 91% homologous when omitted at the protein level [NCBI accession # NM 010022 (mouse E2), # NM 001918 (human)]. 91% is a more accurate

calculation of homology since leader sequences are cleaved off and degraded once the protein has reached its intended place of function, which in this case would be mitochondria.

2.3.3 Colocalization of E2 protein and mitochondria

BCKDH is a mitochondrial enzyme, yet each subunit is encoded at different locations in the nuclear genome (Chuang and Shih, 2001). Therefore, BCKDH proteins are nuclearly transcribed, translated in the cytoplasm, and then escorted to the mitochondria with the help of protein chaperones. Therefore, another explanation for near wildtype levels of E2 protein in liver but low enzyme activity could be a result of improper trafficking of transgene-derived E2 protein to the mitochondria. To assess the ability of E2 protein to successfully localize in the mitochondria of iMSUD mice, E2 colocalization with COX IV, an inner mitochondrial membrane protein, was attempted. Unfortunately, no punctuate (i.e., specific) labeling of E2 or mitochondria (**Figure 9A**, red and green, respectively) could be established in either iMSUD or Control tissue. Despite trying several antibody dilutions, tissue fixatives, IHC protocols, and enlisting the expertise of Dr. Donna Stolz (Center for Biologic Imaging, University of Pittsburgh), colocalization could not be established.

Hoechst dye, which specifically identifies genetic material (i.e., nuclei; **Figure 9A**, blue), suggested that tissue was inferior for use in IHC; nuclei did not appear intact, particularly in iMSUD liver sections. Tissue edges were also very ragged and the tissue in general appeared wispy. Therefore, a rhodamine-phalloidin stain, which specifically identifies cell cytoskeleton (i.e., actin) was used to better visualize tissue cell morphology and thus determine tissue quality.

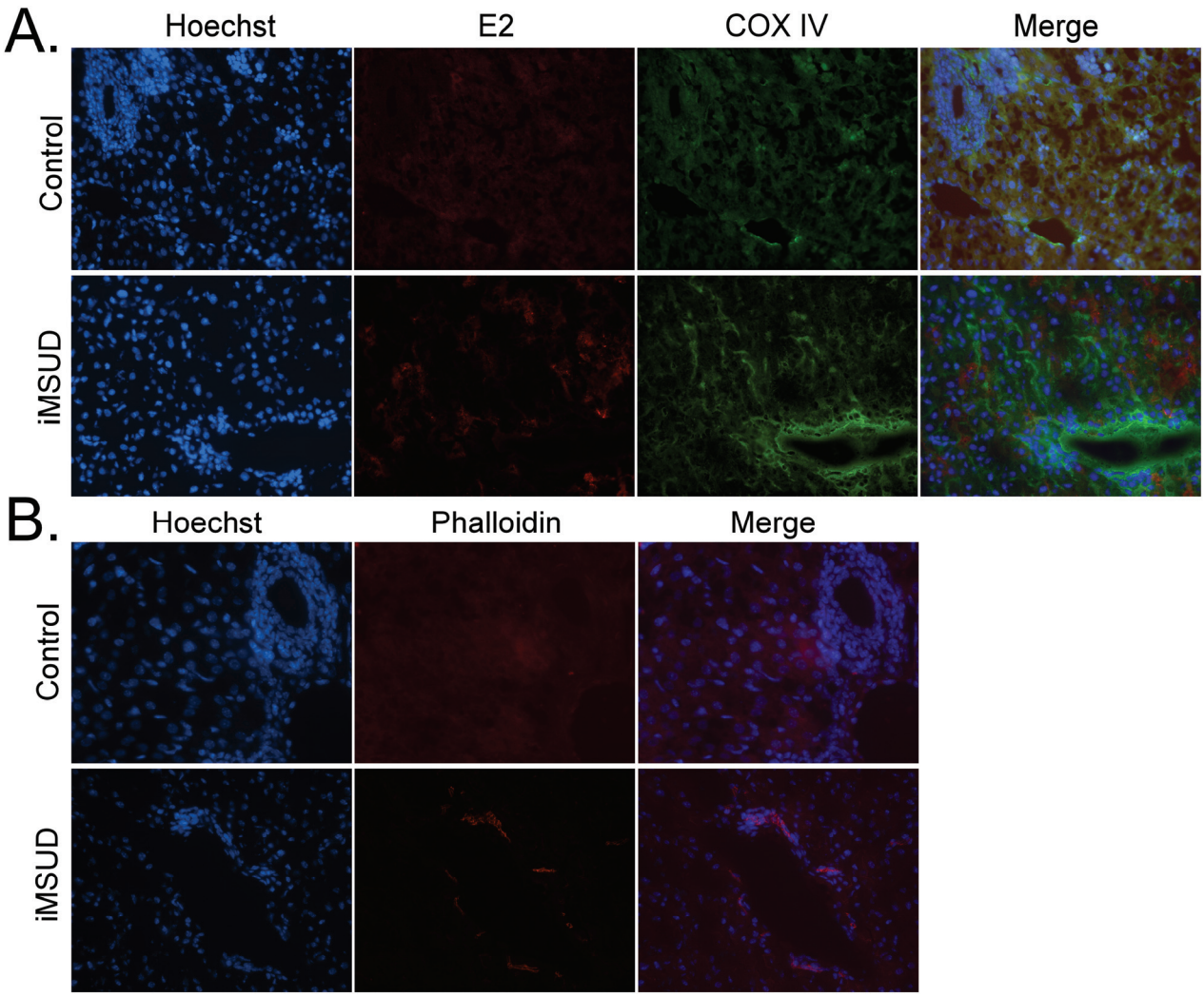

Figure 9. Colocalization of E2 protein and mitochondria.

A. Six micron liver sections from a Control and iMSUD mouse. Livers were treated with a Hoechst dye (nuclei, blue), and antibodies specific for E2-Cy3 (E2, red) and COX IV-Alexa Fluor 448 (COX IV, green). The three images were merged to identify E2/COX IV colocalization. E2 and COX IV staining was largely nonspecific or absent. Mitochondrial stain should appear as small punctuate dots within cells. Nuclei did not appear intact. **B.** Six micron liver sections from a Control and iMSUD mouse were treated with Hoechst dye (nuclei, blue) and rhodamine-phalloidin (actin cytoskeleton, red) to assess cell morphology and thus tissue quality. The two images were merged for orientation. Control and iMSUD tissues suffered a significant loss of cytoskeletal material. A very small amount of remaining actin was localized to the connective tissue capsule around a blood vessel, identified by tightly packed nuclei. Tissue most likely deteriorated significantly while in frozen storage and is not acceptable for use in IHC.

Phalloidin staining of both iMSUD and control liver tissue confirmed a loss of cytoskeletal material (**Figure 9B**, red). Phalloidin should identify the outer cell walls of all hepatocytes. It is also present in a greater concentration around biliary ducts and blood vessles, which are identified by tightly packed nuclei usually in a circular or oval shape (**Figure 9B**; Hoechst stain, top right of Control and center of iMSUD). Instead actin in Control tissue was absent, and a very small amount was identified in iMSUD tissue surrounding a blood vessel. The liver tissues analyzed were unfixed and cryogenically stored at -80^{o}C for 6+ months. Therefore, cell morphology likely deteriorated significantly while in frozen storage.

At this time (July 2008), the iMSUD colony has been significantly reduced and all breeding ceased. However, breeding pairs were transferred to both Dr. Strom and Dr. Gibson (University of Pittsburgh). Once new iMSUD colonies are established, colocalization of E2 and mitochondria will be completed to determine whether mitochondrial trafficking was altered.

2.3.4 Residual mouse E2 mRNA in iMSUD mice

BCKDH enzyme activity and mouse E2 protein was previously determined to be absent in the cMSUD mouse model (Homanics et al., 2006), which verified that removal of E2 exons 5 and part of 4 prevented the translation of the mouse E2 subunit creating an E2 KO. However, residual E2 mRNA transcription was never assessed. Therefore, to more completely characterize the iMSUD mouse, a Taqman ready-made probe and primer set for mouse E2 was used for Real Time quantitative PCR (qPCR). The Taqman assay was verified to be mouse specific as human hepatocyte control samples were not detectable (**Figure 10**), but surprisingly a signal for mouse E2 was detected in iMSUD mouse livers. A significant difference between groups was determined by ANOVA [$F(2,11) = 15$, $p<0.001$]. It was theorized that since exons 5 and part of

exon 4 were deleted to make the MSUD mouse models (**Figure 3**), it was possible mouse E2 mRNA was still being synthesized, though it was determined previously that protein was not translated (Homanics et al., 2006). Therefore, the Taqman assay, specific to E2 exons 2-3, was detecting the incomplete E2 mRNA message.

Efforts to corroborate the real time data by Northern blot analysis thus far have not been successful. iMSUD, human, and WT control RNA was successfully size fractionated and transferred to a nylon membrane. However, due to ongoing and unresolved issues involving the production of radiolabeled probes and subsequent membrane hybridization, all signals were extremely weak and therefore ambiguous for interpretation. Though initial hybridization produced a very weak signal, the human β-actin probe was still weakly detectable by X-ray film after membranes were stripped more than four times. RNA was not lost post-transfer, as verified by 0.03% methylene blue staining.

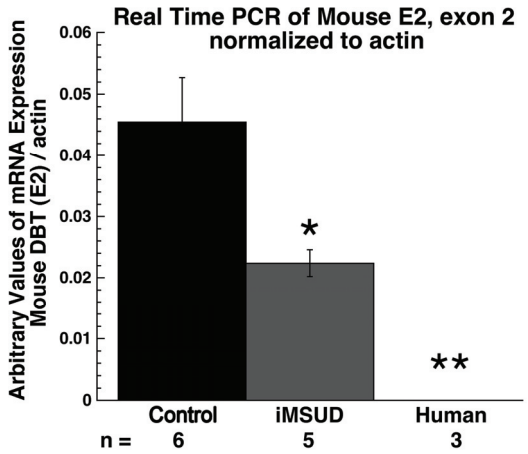

Figure 10. qRT-PCR of iMSUD mice and controls.
Livers from WT (Control) and iMSUD mice, as well as primary human hepatocytes (Human), were analyzed with a Taqman probe specific to E2 exon 2-3, which indicated mouse E2 mRNA was transcribed. E2 exons 5 and part of 4 were deleted from mouse genomic DNA to create the E2 KO mouse models. [*, ($p<0.01$) compared to human and Control; **, ($p<0.05$) compared to iMSUD and ($p<0.0005$) Control]. Mouse E2 RNA was not detectable in human samples verifying Taqman specificity.

2.3.5 Liver morphology

Liver morphology was also assessed to determine whether iMSUD livers, the site for directed novel therapies discussed in this dissertation, were overtly normal. Hematoxylin and Eosin (H&E) staining of liver sections revealed no difference in morphology between iMSUD and wildtype; iMSUD livers appeared normal and healthy (**Figure 11, left**). However, when a Red Oil O stain was applied, a difference in the liver fat content was revealed (**Figure 11, right**). iMSUD livers were noticeably less fatty compared to wildtype controls. This may be explained by iMSUD mice eating less than wildtype littermates, which would also explain their observed growth retardation since BCAAs are essential amino acids required for growth and overall good body health (**Figure 7**). When protein is ingested in an individual with MSUD, BCAA/BCKA's can quickly elevate to dangerous levels causing unpleasant symptoms (Chuang and Shih, 2001). Therefore, it was possible iMSUD mice were able to correlate ingestion of mouse chow with

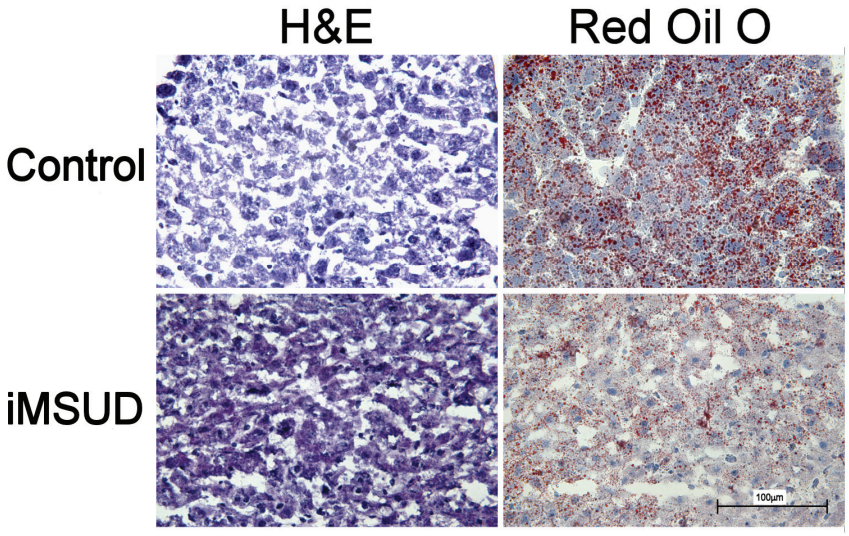

Figure 11. H&E and Red Oil O staining of iMSUD and Control livers.
H&E staining of iMSUD mouse liver illustrated no symptom of disease compared to Control (WT) mouse liver (left panels). Red Oil O treatment (right panels) revealed healthy Control livers to be fattier than iMSUD. Fat droplets were stained red. Magnification used was 40x.

feeling sick (BCAA crisis). To support this hypothesis, it was previously reported that mice displaying a MSUD-like phenotype caused by disruption of BCATm (She et al., 2007) and iMSUD mice (Zinnanti et al., 2008) were able to distinguish between a high and low BCAA diet, which resulted in their improved health and survival. Furthermore, mice with other diseases (e.g., renal failure, asthma) were consciously able to recognize and subsequently avoid stimuli which exacerbated their condition (Costa-Pinto et al., 2005; Gooderham et al., 2004).

2.3.6 Brain BCAA levels and neuropathology

Blood chemistry previously revealed an increase in BCAA levels and a decrease in alanine, glutamate, and glutamine in iMSUD mice compared to controls (Homanics et al., 2006). Brain analysis by HPLC (Dr. W. Zinnanti, Pennsylvania State College of Medicine, Hershey, PA) revealed that glutamate and glutamine were both reduced by ~50% and GABA was reduced by ~22% compared to Controls (Zinnanti et al., 2008). In addition to these changes, when compared to controls, dopamine was reduced by more than 60%, aspartate was reduced by more than 40%, and the free amino acids threonine, tryptophan and tyrosine were all significantly reduced while alanine and lysine were increased (Zinnanti et al., 2008). Amino acid changes were consistent with previous studies in rats (Araujo et al., 2001) and with human MSUD cerebrospinal fluid samples (Wajner et al., 2000). Glutamine is a substrate for the production of both glutamate and GABA, excitatory and inhibitory neurotransmitters in the brain, respectively (Waagepetersen et al., 2003). Depletion of neurotransmitters in MSUD was previously suggested based upon clinical observations of human patients and was consistent with previous studies with newborn MSUD calves (Dodd et al., 1992) and cultured human astrocytes (McKenna et al., 1998). GABA, glutamate, and glutamine rely on each other as well as BCAAs

to maintain a proper balance within the body (**Figure 12**). In the GABA-glutamate/glutamine cycle, glutamine is synthesized and released from astrocytes and taken up by neurons. GABA and glutamate is then synthesized from glutamine and released as neurotransmitters from GABAergic/glutamatergic neurons. In MSUD, BCAAs are increased which causes negative feedback and a paradoxical decrease of GABA, glutamate, and glutamine (Yudkoff, 1997).

Human MSUD is characterized by severe neurological manifestations such as encephalopathy, vacuolization and edema of the basal ganglia, brain stem, and cerebrum, lack of dendritic development in cortex, and dysmyelination (Chuang and Shih, 2001; Crome et al., 1961; Kamei et al., 1992; Silberman et al., 1961). Therefore, additional neuropathology of iMSUD mice was assessed through histology and immunohistochemistry (IHC) and MRI (Zinnanti et al., 2008). T_2 maps generated by MRI of iMSUD mice and controls revealed moderate (4 week old iMSUD mice) and severe (6 week old iMSUD mice) neuropathological deviations in the striatum, corpus callosum, and thalamus denoted by increased T_2 signals. Evidence of fluid build up was also noted. Hematoxylin and eosin (H&E) stained sections of forebrain (**Figure 13A-B**) and brain stem (**Figure 13C-D**) from control and iMSUD mice revealed areas of edema in iMSUD brain (white arrows, **Figure 13B&D**) (Zinnanti et al., 2008). Confocal images from similar sections as in **Figure 13A-D** were labeled with N52 for neurofilaments (green), GFAP for astrocytes (red) and DAPI for nuclei (blue) (**Figure 13E-H**) revealed moderate vacuolation and astrocytosis in the striatum of iMSUD sections (**Figure 13F&H**) (Zinnanti et al., 2008). Brain edema, along with vacuolation and astrocytosis of the striatum, are consistent with human cases of MSUD (Chuang and Shih, 2001; Crome et al., 1961; Silberman et al., 1961).

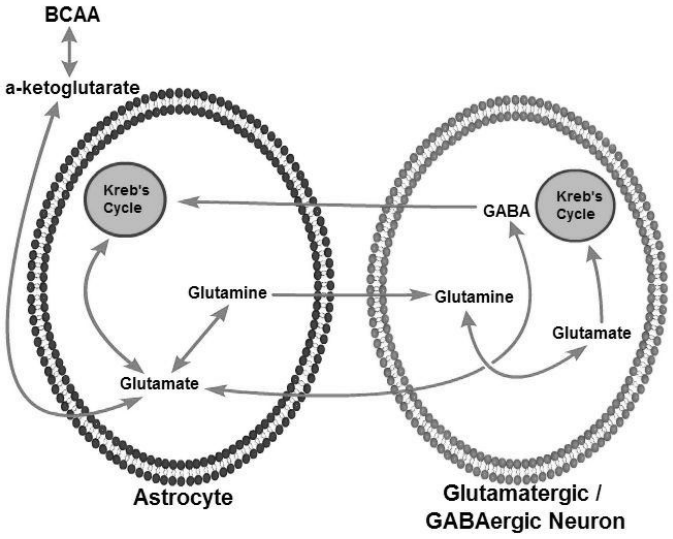

Figure 12. GABA-glutamate/glutamine cycle.
BCAA, GABA, glutamate, and glutamine all rely on each other to maintain proper balance in vivo. Glutamine is synthesized and released from astrocytes and taken up by neurons. GABA and glutamate is then synthesized from glutamine and released as neurotransmitters from GABAergic/glutamatergic neurons. In MSUD, BCAAs are increased, which promotes reverse transamination of glutamate back to α-ketoglutarate. Therefore, increased BCAAs causes a paradoxical decrease of glutamate, glutamine, and GABA. These interactions have heavy influence on the Krebs cycle and energy cycling. Figure made using Pathway Builder 2.0.

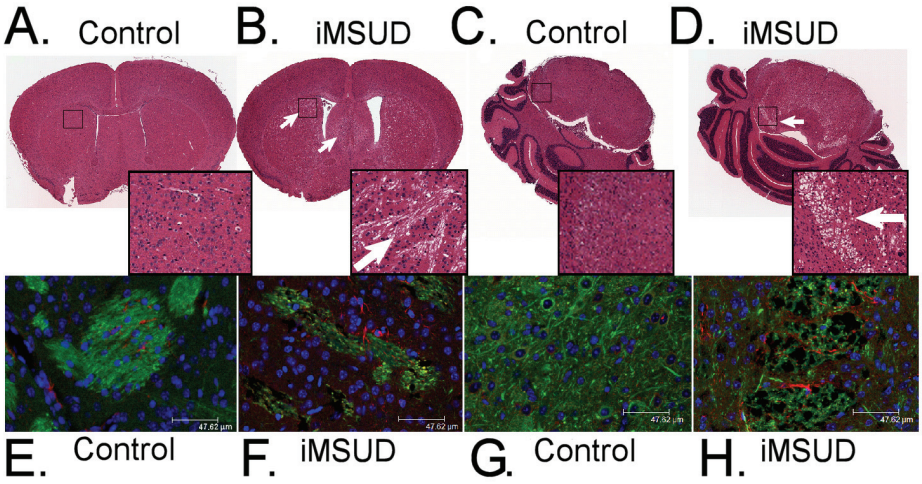

Figure 13. Neuropathology in the iMSUD mouse model.
White arrows indicate edema in the striatum of iMSUD animals in forebrain (**A-B**) and brain stem (**C-D**). The black boxes indicate the area of inset image (20x). **E-H**. IHC of similar brain sections labeled with N52 (neurofilaments, green), GFAP (astrocytes, red) and DAPI (nuclei, blue). All brain analyses were completed by Dr. W. Zinnanti (Penn State, Hershey, PA). Figure reproduced with permission from Dr. W. Zinnanti.

2.4 CONCLUSIONS

Previous characterization of the iMSUD mouse model revealed that it was remarkably similar to the human disease (Homanics et al., 2006). However, further characterization presented here increased our understanding of the mouse model and revealed additional similarities beweeen it and the human disease (summarized in **Table 9**). iMSUD mice suffered from reduced body weight at weaning, seizures, hypoactivity, and in severe cases, muscle wasting and paralysis. Brain BCAA levels agreed with previously assessed blood BCAA levels, and there was also a marked decrease in neurotransmitters and neurotransmitter precursors such as glutamate, glutamine, dopamine, aspartate, and GABA. iMSUD brains displayed abnormalities, such as evidence of edema, astrocytosis, and vacuolization in the striatum and brain stem, while iMSUD liver morphology was overtly normal, which was consistent with human cases of disease (Chuang and Shih, 2001; Crome et al., 1961; Silberman et al., 1961). However, iMSUD livers were less fatty than Controls, something not typically seen in the human disease. MSUD children and young adults are in many cases overweight since foods that are low or devoid of protein are high in fat and sugars (Breakout Session, MSUD Family Symposium, 2006). BCKDH enzyme activity was between 5-6% of normal, which was within the range of human iMSUD (3-30%) (Chuang and Shih, 2001). Therefore, I conclude that the novel iMSUD mouse model, which mimics an intermediate human MSUD phenotype, is suitable in which to test liver-directed treatment approaches, such as AAV and cellular therapies, to correct the disease.

Despite many similarities to the human disease (**Table 9**), there was one unexplained observation. In some animals, E2 protein levels in the liver were similar to wildtype controls despite low BCKDH enzyme activity. There were several hypotheses that might explain these

results. One possible explanation involved improper trafficking of human E2 protein to the mitochondria, could not be ruled out as a possibility due to inferior liver tissue quality for IHC analysis. Colocalization experiments will be completed once iMSUD animals have been generated either in Dr. K.M. Gibson's or Dr. S. Strom's laboratory (University of Pittsburgh). However, the most likely explanation for these data was the incorporation of a c-myc tag on the C-terminus of transgenic human E2. This hypothesis was based upon previous publications citing similar issues with BCKDH complex family members (Knapp et al., 2000; Mattevi et al., 1992). Enzyme activity may also be hindered by the transgenic expression of human E2 in a mouse system. In order to elucidate what may be occurring *in vivo*, a lipid-directed transfection assay using cultured E2 KO mouse embryonic fibroblast (MEF) cells could be explored. E2 KO fibroblasts could be transfected via lipid-DNA complexes packaged with one of three different mammalian expression vector constructs: (1) human E2 cDNA, (2) human E2 cDNA with incorporated C-terminal c-myc, and (3) mouse E2 cDNA. After allowing construct DNA to be delivered to KO MEF cells, BCKDH enzyme activity would be assessed (as described in the Methods section) to determine the ability of each to restore enzyme activity and then compared to enzyme activity in untransfected KO MEF cells and WT control MEF cells. Based upon my hypothesis, I would expect mouse E2 cDNA to restore activity to WT levels while a human E2 cDNA with incorporated C-terminal c-myc construct would significantly reduce enzyme activity to the levels seen in the iMSUD mouse. Due to similarities in human and mouse E2, both at the genomic and protein level, I would not expect the human E2 cDNA construct to significantly decrease enzyme activity, though a small decrease would not be surprising. A difference of 9% exists at the protein level, therefore depending on where the changes were and whether protein folding or function was slightly altered, it would be possible for activity to also change slightly.

Table 9. iMSUD disease phenotype vs. the human disease.

Disease Phenotype	iMSUD Mouse Model	iMSUD Human Patients[5]
Maple syrup smell in urine	Yes	Yes
"Squinty" eyes	Yes	Yes, in times when BCAAs are elevated
Able to produce normal offspring	Yes	Yes
Mental retardation	Not determined	Yes, after severe prolonged BCAA acidosis
BCAA/BCKA acidosis	Yes	Yes
Additional AA imbalances	Yes	Yes
Decreased BCKDH activity	Yes, 5-6% of normal	Yes, 3-30% of normal for iMSUD
Developmental/growth delay	Yes	Yes
Seizures	Yes	Yes
Hypoactivity	Yes	Yes
Paralysis, muscle wasting	Yes, in severe prolonged BCAA acidosis	Yes, in severe prolonged BCAA acidosis
Encephalopathy	Yes	Yes
Normal liver morphology	Yes, though less fatty	Yes
Hyperammonemia	Not determined	Yes
Neurotransmitter imbalances	Yes	Yes
Neurological damage (edema, vacuolization, dysmyelinization, decreased dendritic development, astrocytosis)	Yes	Yes, in severe prolonged or repeated BCAA acidosis
Coma	Not observed	Yes
Premature death	Yes	Yes

[5] The list in Table 9 is a compilation of possible clinical manifestations reported for human MSUD. With regards to human patients, many of the severe phenotypes listed can be effectively prevented through attentive management and care, though individuals can still go into crisis accompanied by permanent neurological trauma and death.

3.0 AAV GENE THERAPY IN iMSUD MICE

Hypothesis: I hypothesize AAV-directed gene therapy can correct E2 deficiency, restore liver BCKDH activity, and ultimately correct the disease phenotype of the iMSUD mouse model.

3.1 BACKGROUND AND SIGNIFICANCE

AAVs are derived from a nonpathogenic parvovirus, which are devoid of viral coding sequences making AAV replication-deficient; due to their small size, inability to replicate, and simple three-protein capsid structure, AAV has very low risk of immune response (Goncalves, 2005; Yang et al., 1995; Yang et al., 1994). The AAV vectors have also been shown to allow long-term expression of transgenes in transduced cells and their progeny from a single dose (Koeberl et al., 1997; Mochizuki et al., 2004; Wang et al., 2005a). To date, eleven AAV serotypes have been isolated, one of which shows the greatest promise for our application; AAV8 has been shown to selectively transduce hepatocytes 10-100 times more efficiently than all other serotypes currently analyzed (Sun et al., 2005b; Xiao et al., 1998a).

AAV was previously shown to be highly efficient at liver infection and subsequent trangene delivery (Jiang et al., 2001; Koeberl et al., 1999; Mount et al., 2002; Su et al., 1996; Xiao et al., 1998a). An albumin promoter can effectively direct expression in liver cells (Jiang et al., 2001; Koeberl et al., 1999; Su et al., 1996; Xiao et al., 1998a). In addition, AAV8 can be

used to achieve high levels of expression in many tissues, most notably hepatocytes and muscle, when injected in adult animals (Wang et al., 2005b; Zhu et al., 2005). AAV has also been approved for several clinical trials, such as Parkinson's disease, muscular dystrophy, α1AT deficiency, and cystic fibrosis (Goncalves, 2005; Kaplitt et al., 2007; Pacak et al., 2007), which have made some encouraging progress. Therefore, it was determined that AAV was a logical and appropriate gene delivery system for use with the novel iMSUD model due to low immune response and superior liver targeting.

3.2 METHODS

All studies involving animals were reviewed and approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

3.2.1 Mouse colony breeding

Production of iMSUD and control mice was carried out as previously described in Methods Section 2.2.

3.2.2 PCR and southern blot genotyping

PCR and Southern blot analysis for genotyping of iMSUD animals was completed as previously described in Methods Section 2.2.

3.2.3 AAV vector construction, packaging, and purification

The mouse albumin promoter (2.3kb; NCBI accession # J04738) was first subcloned into the KpnI and NotI sites of AAV vector plasmid pXX-UF1 to create pAAV-Alb. Human E2 cDNA (1.4kb) was then isolated from the retroviral vector MFG-E2 and inserted into the NotI and SalI sites of pAAV-Alb to generate the final vector construct, pAAV-Alb-hE2. The AAV vector construct also contained the 145bp inverted terminal repeat *cis*-acting element at either end of the viral genome, which was required for capsid packaging. AAV-Alb-hE2 viral vectors were packaged into AAV2 and AAV8 capsid proteins by the high-titer, helper-virus-free Triple Transfection Method, which was capable of producing high purity rAAV viral stocks of up to 10^{10} to 10^{11} transducing units (t.u.)/mL (up to 10^{13} viral particles (v.p.)/mL) and approaches wild type AAV yields (Xiao et al., 1998b). Resultant viral particles containing AAV-Alb-hE2 (**Figure 14**) were further purified by double CsCl ultracentrifugation and dialyzed against DMEM cell culture media. Viral titers were determined by dot-blot analysis. Briefly, vector DNA was extracted from 2μL of each fraction of the CsCl density gradient and dotted onto a nylon membrane along with a known amount of plasmid pAAV-Alb-hE2 DNA as a copy number standard. Hybridization of an E2 cDNA probe and visualization of resulting signals on X-ray film determined AAV-Alb-hE2 viral particle titers to be, in reference to the copy number, in the range of $5x10^{12}$ v.p./mL. Viral stocks were diluted to a working concentration of $1x10^{7}$ v.p/mL with filter sterilized PBS. All described work was done solely by Dr. Xiao Xiao and his staff (Dept. of Molecular Genetics and Biochemistry) while at the University of Pittsburgh.

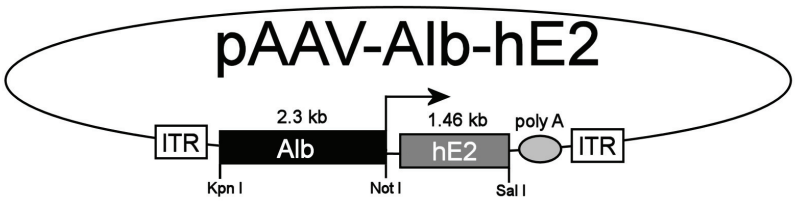

Figure 14. Recombinant AAV-Alb-hE2 gene therapy vector.
Viral DNA was packaged into AAV2 and AAV8 via the Triple Transfection Method. Human E2 (hE2, 1.46kB) transgene expression was driven by the mouse albumin (Alb) promoter (2.3kB). 145bp ITR's were included for correct packaging into AAV capsids as well as a poly A tail to protect against mRNA degradation.

3.2.4 AAV gene therapy

iMSUD mice were injected intraperitoneally (IP) with viral particals containing AAV8-Alb-hE2 or AAV2-Alb-hE2 (**Figure 14**) at a dose of $1x10^{11}$v.g/kg body weight per mouse or PBS at three weeks of age. Some mice were given a second dose at 7 weeks of age. Mice were sacrificed at 42 days post injection or earlier if animals were moribund, and the tissues analyzed.

3.2.5 Southern blot to detect AAV DNA

Genomic DNA was isolated from various tissues (liver, muscle, kidney, heart) and cultured mouse embryonic fibroblasts (MEFs). Each sample (25μg) was analyzed by digestion with the restriction enzyme EcoRI. Digested samples were run through a 1% agarose/4x Hellings gel for 950 minutes at ~20V, transferred to Hybond-N (Amersham, Piscataway, NJ) via capillary transfer, and DNA was fixed to the blot by UV crosslinking. Prior to hybridization with a radiolabeled probe made via the Random Primed DNA Labeling Kit (Roche, Indianapolis, IN), membranes were incubated with prehybridization solution containing salmon sperm DNA for several hours at 42^{o}C. Southern blots were probed with a ^{32}P-labeled probe specific to mouse E2 exon 6, which detects only mouse E2, and a probe specific to human E2 cDNA, which detects

only human E2 (transgene derived E2 and AAV derived E2), and a probe specific to the TRE element of the TRE-E2 transgene. Membranes were exposed to X-ray film for detection under nonsaturating conditions.

3.2.6 BCKDH complex enzyme activity assay

iMSUD-PBS, iMSUD-AAV8, and iMSUD-AAV2 treated mouse livers were removed, flash frozen in liquid N_2 and stored at -80^{o}C. BCKDH activity was determined as previously described in Methods Section 2.2.

3.2.7 Blood BCAA levels

Blood samples were collected from the tail at weaning, immediately prior to therapeutic injection, and then ~weekly for 12 weeks post-treatment. Blood samples were spotted on a Guthrie card and mailed to Pediatrix Screening Inc. (Bridgeville, PA) for processing by tandem mass spectrophotometry (MS-MS). Total BCAA levels were expressed as a ratio to alanine, a sensitive indicator of MSUD phenotype (Morton et al., 2002). Data were analyzed by Repeated Measures ANOVA or post-hoc Fisher's test.

3.2.8 Western blot analysis

Protein extracts were isolated from liquid N_2 flash frozen homogenized liver or cultured mouse hepatocytes. Protein (25μg per sample) was analyzed as previously described in Methods Section 2.2.

3.2.9 Survival

Date of birth, date of AAV or PBS treatment, and date of death was recorded for iMSUD mice. Data were analyzed using the Kaplan-Meier method and the Log Rank test (Kaplan and Meier, 1958; Mantel, 1966).

3.2.10 Adult hepatocyte isolation

Adult mouse hepatocytes (~8-10 weeks of age) were isolated by collagenase perfusion (Strom et al., 1996). In brief, wildtype donor mice were sedated and a midline laparotomy performed. The inferior vena cava was then perfused and the portal vein sectioned within 5-10 seconds of the start of perfusion to allow the solution to flow through the liver. The liver was sequentially perfused at a rate of 2.5 mL/min with EGTA solution (HBSS without calcium or magnesium, EGTA 0.5 mM, HEPES 10 mM, pH 7.5 supplemented with penicillin/streptomycin 1%) for 5 minutes followed by collagenase solution for 10 min (EMEM with calcium/magnesium plus collagenase 0.1 mg/ml, HEPES 10 mM, pH 7.5 and penicillin/streptomycin 1%). Hepatocytes were removed by mechanical disassociation and filtered through sterile 150 mM nylon mesh. Cells were washed 3 times in EMEM media by centrifugation at 50 x g for 5 min, finally resuspended at a concentration of 10^7 cells/ml in HMM media (Cambrex) and plated on collagen-coated plates for culture.

3.2.11 In vitro AAV infection

Adult mouse hepatocytes isolated from wildtype mice were seeded in 12-well plates (BD Falcon, Franklin Lakes, NJ) coated with 0.05mg/mL rat tail collagen (~$5x10^5$ cells per well). These cells were either mock-infected with PBS or incubated with AAV2-Alb-hE2 or AAV8-alb-hE2 vectors at 10^3 genomic particles/cell in a minimal volume in the presence of serum. One hour after the addition of serum, cells were washed and media was replaced with serum-free media. Cells were washed and the media replaced daily. Mock infected cells were harvested at days 1, 3, 5, and 7 and AAV infected cells were harvested at days 3, 5, and 7 post-infection. AAV infected cells were not collected at day 1 post-infection since AAV without helper virus requires several days to infect, translocate to the nucleus, and begin therapeutic gene delivery (Xiao et al., 2002). The presence of human E2 protein was assessed by Western blot analysis.

3.3 RESULTS AND DISCUSSION

3.3.1 BCKDH enzyme activity and E2 protein in the liver

Following IP AAV gene therapy injection ($1x10^{11}$v.g/kg body weight) into iMSUD mice, hepatic E2 protein and BCKDH enzyme activity were analyzed. BCKDH enzyme activity, expressed as a percentage of total WT activity, initially appeared to be elevated in AAV8 treated animals compared to PBS control and AAV2 treated animals (**Figure 15A**). Since AAV8 has been shown to selectively transduce hepatocytes 10-100 times more efficiently than all other serotypes, these data were not unexpected (Sun et al., 2005b; Xiao et al., 1998a). However, due

to a great deal of variability between samples, which was also present in PBS control treated animals and untreated iMSUD animals analyzed previously, this difference was not statistically significant as determined by ANOVA [$F(2,9)=2$, $p>0.3$]. Baseline variability of BCKDH enzyme activity between individuals is characteristic of MSUD due to its associated clinical heterogeneity (Chuang and Shih, 2001) which therefore makes small changes in enzyme activity more difficult to detect.

E2 protein in liver was analyzed by Western blot (**Figure 15B**) and quantified. No significant difference in E2 protein levels were detected between PBS, AAV2, or AAV8 treated animals [$F(2,7)=2$, $p>0.2$]. There were several factors complicating the detection of any change in E2 protein levels. The first was the same issue encountered for BCKDH enzyme activity – base protein levels varied greatly between individuals which made detection of a small increase in E2 protein levels from gene therapy difficult. The second was that both transgene- and AAV-derived E2 protein were human and therefore indistinguishable by Western blot. Therefore, a substantial increase in E2 protein would be needed in AAV-treated animals over PBS-treated controls for the quantification software to distinguish a statistical change in band density, especially since there were large error values due to sample variability.

3.3.2 BCAA blood levels and survival

Logically, a small change in BCKDH enzyme activity in AAV treated animals, even if such a change was statistically insignificant, may be sufficient to decrease blood BCAA levels and therefore improve survival. However, no difference in survival between groups was observed (**Figure 15C**). For all treatment groups, approximately 50% of animals died by 7 days post-injection while ~22% (n=2/9) of AAV2-treated animals and ~7.6% (n=1/13) of AAV8-

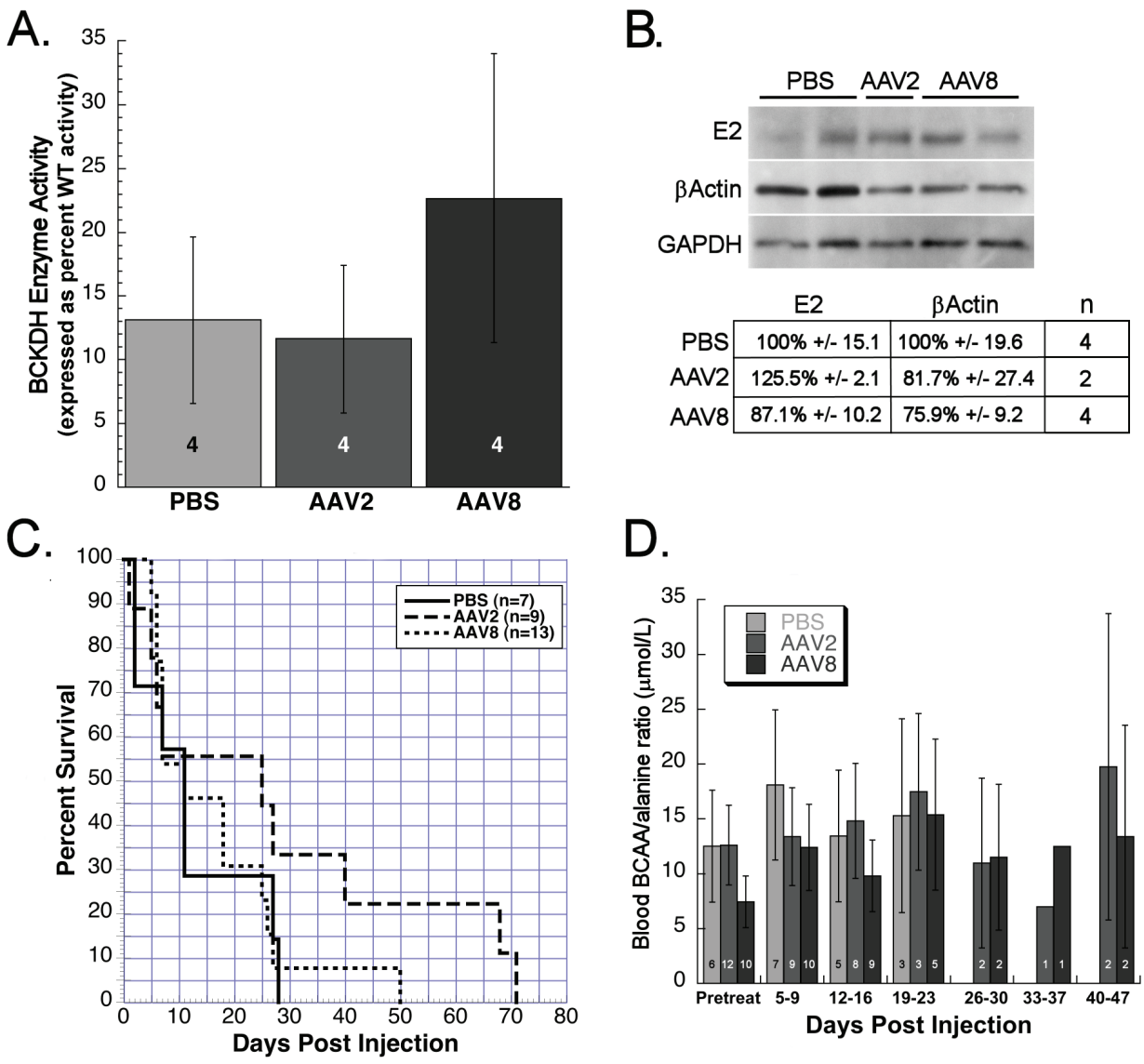

Figure 15. AAV gene therapy results in iMSUD mice.
iMSUD-PBS, iMSUD-AAV2, and iMSUD-AAV8 animals were analyzed for differences in **A.** BCKDH activity (expressed as a percentage of WT control activity), **B.** E2 protein by Western blot, **C.** Survival, and **D.** BCAA/alanine levels in bood. Normal BCAA/alanine levels are ~1μmol/L. High variability between samples was observed in **A.** & **D.** There was no significant difference between treatment groups in panels **A-D.** The numbers on the bars of **A.** & **D.** indicate n values. All data in **A.**, **B.**, & **D.** were expressed as the mean +/- SEM.

treated animals survived longer than 27 days post-treatment, the maximum survival of PBS treated animals.

BCAA levels in serum, analyzed by MS-MS, also did not yield a significant difference between treatments likely due to substantial variability between samples (**Figure 15D**). The high variability between individuals agreed with iMSUD blood analysis completed previously (Homanics et al., 2006). High variability between individuals is not an uncommon attribute in patients with a metabolic disease displaying clinical heterogeneity such as MSUD (Chuang and Shih, 2001), but this phenomenon could be exacerbated by when the mouse last ate. Mice are nocturnal. Therefore, all blood samples were taken at ~1pm, which should correspond to low activity levels, to minimize fluctuations from ingested protein. However, only removing food several hours before blood collection would reliably eliminate that factor.

3.3.3 AAV DNA detection by Southern blot

Since no effect of AAV gene therapy was apparent in iMSUD treated mice, various tissues were analyzed by EcoRI restriction enzyme digest and subsequent Southern blot analysis for the presence of AAV genomic DNA. Three DNA probes were used: one specific for endogenous mouse E2, one specific for human E2 cDNA, and one specific for the TRE element of the TRE-E2 transgene. iMSUD mouse lines A and 525A differ only in the genomic location of the TRE-E2 transgene, which results in a difference in the DNA fragment size corresponding to TRE-E2 by Southern blot analysis (Homanics et al., 2006).

The ability of AAV to transduce cells was first tested in MEF cells. MEFs were infected with AAV2-Alb-hE2 *in vitro*. Uninfected MEF cells possessed only endogenous mouse E2 DNA while AAV-infected MEFs possessed both mouse and human E2 DNA (**Figure 16**, small

blot on right), which suggested that AAV vectors were functional. In addition, species specificity of E2 probes to human or mouse was verified. Infected MEFs were washed thoroughly prior to cell collection for analysis; therefore detected human E2 DNA was from AAV genomic DNA transduced inside of MEFs rather than from AAV particles present in the media external to cultured cells.

Genomic DNA isolated from kidney, liver, heart and muscle from iMSUD animals were analyzed by Southern blot for the presence of AAV DNA. However, AAV genomic DNA was not detectable in mouse tissue samples. A strong single band was identified when analyzed with a mouse E2 probe (**Figure 16**, top large blot) which corresponded to endogenous mouse E2. The mouse E2 probe hybridized to E2 exon 6 present in all iMSUD mice, thus serving as a positive control to determine DNA quality. The human E2 probe, specific to human E2 cDNA present in the TRE-E2 transgene and AAV viral vectors, identified one strong band, which differed in size by mouse line, with multiple less intense bands (**Figure 16**, middle large blot). The faint multiple bands may be the result of incomplete digestion of the genomic tissue DNA. A single strong band accompanied by multiple less intense bands in the same patterns as presented here for both mouse lines were routinely observed in untreated genomic tail DNA after digestion with EcoRI for Southern blot genotyping. The pattern of multiple bands was more distinct in iMSUD line A mice, which also agreed with prior results observed in Southern blot genotyping. Furthermore, DNA isolated from AAV- and PBS-treated animals (525A line) detected identical bands. Taken together, these data suggested the DNA fragments identified by the human E2 probe corresponded to E2 DNA present in the TRE-E2 transgene and not AAVs. The TRE element probe detected identical bands as the human E2 specific probe (**Figure 16**, bottom large blot), which verified human E2 DNA was transgenic in origin and not viral.

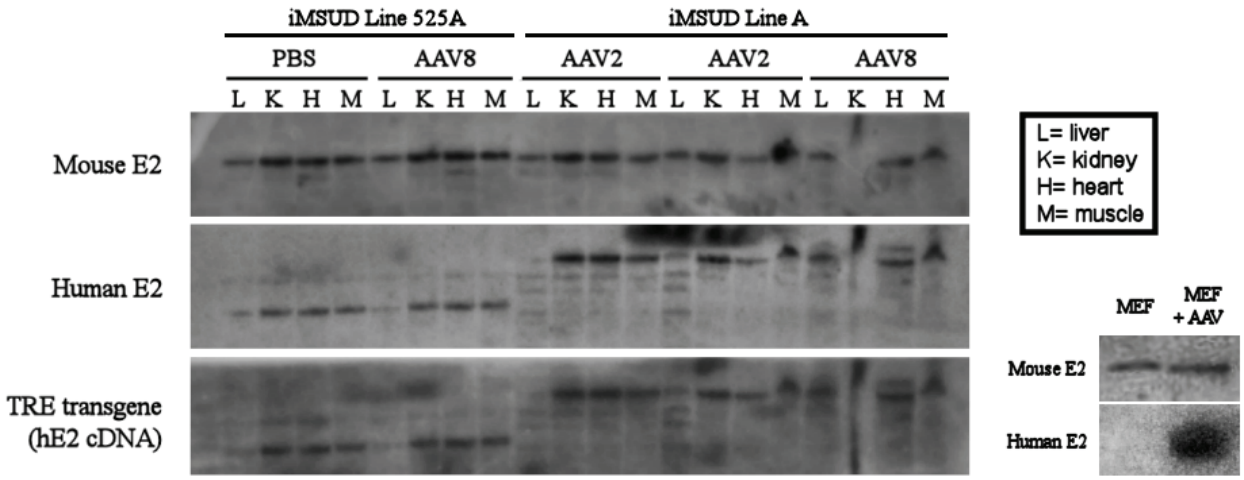

Figure 16. AAV DNA Southern blot.
Liver, kidney, heart, and muscle genomic DNA isolated from iMSUD animals treated with PBS, AAV2, or AAV8 were digested with EcoRI and analyzed for the presence of AAV DNA containing human E2. Probes for mouse E2 exon 6 (Left, top panel; positive control present in all mouse DNA), human E2 (Left, middle panel; specific for human E2 present in the TRE-E2 transgene and AAVs), and the transgenic TRE element (Left, bottom panel; specific for the TRE element present in the TRE-E2 transgene). iMSUD mouse lines A and 525A differ only in the genomic location of the TRE-E2 transgene. The ability for AAV to infect cells and the specificity of the human E2 probe was verified in cultured MEF cells (Right, bottom blot).

The inability to detect AAV DNA in tissue samples by Southern blot analysis could be explained in several ways. The AAV vectors were possibly not functional and were therefore unable to infect cells once injected *in vivo*. However, due to the presence of an intense human E2 band by Southern analysis following *in vitro* infection of MEF cells, this explanation is unlikely. AAV DNA was possibly lost over time after cell infection. However, hepatocytes are largely quiescent; therefore dilution of AAV DNA due to hepatocyte turnover is also unlikely. Finally, AAV DNA was possibly present at a concentration below the threshold of Southern blot analysis. This explanation is highly likely since it was later learned the dose administered to iMSUD animals was ~1 v.g./cell (Dr. H. Nakai, personal communication Sept 2007, University of Pittsburgh). This and other issues are discussed in greater detail in the Conclusions section of this chapter.

3.3.4 AAV hepatocyte infection

Freshly isolated adult mouse hepatocytes were infected *in vitro* to determine if AAV vectors were able to manufacture E2 protein in albumin expressing cells. Cell lysates were analyzed by Western blot and quantified. Unfortunately, no human E2 protein (54kD) was visible in cultured hepatocytes following infection with either AAV2 or AAV8 therapeutic vectors (**Figure 17**). Cultured hepatocytes normally expressed only endogenous mouse E2 (47kD). A heterozygous liver sample was included for direct comparison of AAV-infected hepatocytes since mouse and human E2 were present as a doublet. A statistical significance was observed for total E2 protein between controls [No AAV (n=6)] and AAV8 or AAV2 (n=9 for both) infected hepatocytes as determined by ANOVA [$F(2,19)=7$, $p<0.005$], though it was not in the expected direction. AAV-infected hepatocytes displayed a decrease in total E2 protein compared to No AAV controls; total E2 protein at 3 days post-infection was 52.7% +/- 4.7 SEM of control for AAV2 ($p<0.001$) and 64.4% +/- 4.4 SEM of control for AAV8 ($p<0.01$). E2 protein continued to decrease slightly at 5 and 7 days post-infection, though protein fluctuation between AAV-infected 3, 5, and 7 day cultures were not significant to each other. To further complicate interpretation of Western results, E2 protein also appeared to slightly decrease from day 0 to day 5 in No AAV controls, which suggested time in culture was negatively affecting endogenous E2. Significance could not be determined possibly due to small sample size (n=3 for each timepoint), however a decrease in E2 protein would not be surprising since culture media was not supplemented with BCAAs. In addition, visual comparison of the E2 band on day 5 in No AAV control and AAV-treated hepatocytes suggested AAV interaction was negatively affecting E2 expression, though this difference was not significant possibly due to small sample size (n=3 for No AAV control at day 5). Uninfected cell lysates were also collected from

cultured hepatoctyes at 3 days but were not analyzed due to space restrictions on the Western gel. Collection of pre-infection hepatocytes was attempted at 7 days as well, however due to difficulties surrounding hepatocyte survival *in vitro*, cell numbers and quality were unsatisfactory for analysis. Each sample was analyzed in triplicate.

In addition to concerns suggesting AAVs were negatively affecting endogenous E2 expression, there were several possible explanations for the apparent lack of human E2 in infected cells as assessed by Western blot analysis. The most likely explanation was that AAVs required more time to translocate to the nucleus and begin production of human E2. In nondividing cells such as hepatocytes, AAV infection in the absence of helper virus is very slow – approximately 7-10 days (Dr. H. Nakai, personal communication Sept. 2007, University of Pittsburgh), and hepatocytes are difficult to sustain in culture beyond 7 days. If an established human hepatocyte cell line such as HepG2 was infected instead (since these cells divide rapidly in culture), AAV vectors would be able to more quickly infect cells and express protein. However, AAV-derived human E2 expression would likely not be robust enough to distinguish itself from baseline human E2 present in HepG2 cells or other immortalized liver lines to be convincing. Unfortunately established mouse hepatocyte cell lines are not available. Furthermore, AAV8 is not very effective *in vitro* regardless of cell type (Dr. H. Nakai, personal communication Sept. 2007, University of Pittsburgh). Other possible explanations were that human E2 protein was below the threshold level of the assay or that AAVs were unable to infect hepatocytes in culture. The latter explanation is unlikely since these vectors were able to infect cultured MEFs (**Figure 16**).

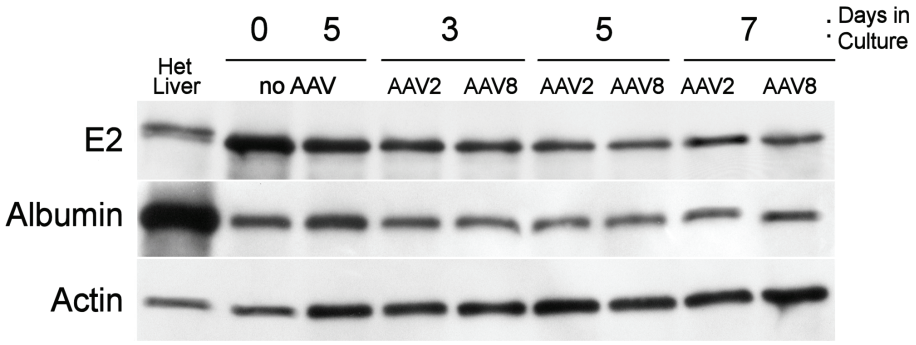

Figure 17. AAV infection of mouse hepatocytes *in vitro*.
WT adult mouse hepatocytes seeded onto cell culture plates at 5 x 10^5 cells/well were either mock infected (No AAV, n=6) or infected with 10^3 v.p/cell of AAV8- (n=9) or AAV2-Alb-hE2 (n=9). No AAV hepatocytes cultured for 0 and 5 and AAV-infected hepatocytes cultured for 3, 5, and 7 days were analyzed for E2 protein, albumin (functional hepatocyte control), and actin (loading control). Mouse liver heterozygous for the mouse E2 KO was also analyzed as a control: human (54kD) and mouse (47kD) E2 were visible as a doublet. No human E2 protein was visible in AAV infected cells. E2 was decreased in AAV-infected cells ($p<0.01$, AAV8; $p<0.001$, AAV2) when compared to No AAV controls suggesting AAV was negatively affecting endogenous mouse E2 expression.

3.4 CONCLUSIONS AND FUTURE DIRECTIONS

I hypothesized that AAV-directed gene therapy could restore liver BCKDH activity to ultimately correct the disease phenotype in the iMSUD mouse model. Due to earlier studies that involved AAV8 specifically (Sarkar et al., 2004; Sun et al., 2005b; Xiao et al., 1998a), it was determined to be the vector of choice for our study. However, no significant improvement was successfully determined in AAV-treated iMSUD mice for survival, blood BCAA/ala levels, liver BCKDH enzyme activity compared to PBS-treated mice (**Figure 15**). AAV DNA was not detected in analyzed tissues from AAV-treated animals by Southern blot analysis (**Figure 16**). A significant difference was determined between E2 protein in mock-infected and AAV-infected hepatocytes in culture, however AAV-infected cells displayed a decrease in protein, not an increase as expected. The most likely reasons this study was unsuccessful was due to multiple issues, which involved a low treatment dose, use of the weak albumin promoter, and possible

competition and interaction between AAV-derived and transgene-derived human E2. These issues and others are discussed in greater detail below.

AAV-derived human E2 was perhaps unable to improve the iMSUD phenotype due to a low administered treatment dose. AAV virus, injected IP, will infect a wide range of cell types; however, expression of E2 will only occur in hepatocytes due to transgene direction from the mouse albumin promoter. Therefore, a low dose would not be able to infect a significant number of hepatocytes and thus express a significant amount of E2 protein to correct the disease in iMSUD mice. The dose administered, $1x10^{11}$ v.g./kg body weight, correlated to approximately 1 v.g./cell (Dr. H. Nakai, personal communication Sept 2007, University of Pittsburgh). In addition, IP injection may be too general an injection site for sufficient viral load to infect the liver and improve iMSUD phenotype, although preliminary data revealed high albumin-driven GFP expression in the liver from an AAV vector following IP administration (Xiao Xiao, UNC School of Pharmacy, unpublished data).

It was previously shown that an albumin promoter in AAV can effectively direct expression in liver cells (Jiang et al., 2001; Koeberl et al., 1999; Su et al., 1996; Xiao et al., 1998a). However, the albumin promoter was suggested to be a weak liver promoter. One such publication compared fifteen promoters for driving expression of luciferase in the mouse liver; the mouse albumin promoter placed 15th out of 15 tested liver-specific promoters (Al-Dosari et al., 2006). Furthermore, the mouse albumin promoter had ~5% the activity of the ApoHCR-α1AT liver promoter commonly used in Dr. Nakai's laboratory (personal communication Sept. 2007, University of Pittsburgh).

The presence of c-myc tagged transgenic human E2 may have hindered AAV-derived human E2 function due to interaction or competition. It was reported that the carboxy terminal

end of E2 was important for subunit assembly of two enzyme complexes in the same family as the BCKDH complex, the pyruvate dehydrogenase and α-ketoglutarate complexes, which was discussed previously (Knapp et al., 2000; Mattevi et al., 1992). In both of these studies, a 6xHis tag incorporated onto the C-terminal end of the E2 subunit reduced the normally occuring 24-mer to a trimer with significantly decreased activity. Therefore, since the BCKDH complex has the same protein structure and function as its two family members, it was logical to deduce that a c-myc tag at the C-terminal end of the BCKDH E2 subunit may also interfere with proper enzyme assembly reducing activity. Since c-myc tagged transgenic E2 and AAV treatment-derived E2 would compete for inclusion into the enzyme complex in the cell, any incorporated c-myc tagged E2 may disturb proper BCKDH complex formation and function. In addition, this may mean any treatment that uses the endogenous BCKDH complex machinery would also not be successful in this particular iMSUD mouse model.

As discussed in a previous section, human E2 may also not be compatible for optimum activity with endogenous mouse E1 and E3 subunits. The BCKDH E2 subunit is very well conserved across species, mouse and human E2 are 88% homologous when the leader sequence is included and 91% homologous when omitted at the protein level. 91% is a more accurate calculation of homology since leader sequences are cleaved and degraded once a protein has reached its intended place of function. However, even 9% difference in protein sequence homology may be sufficient to disrupt optimal assembly and function.

It was also possible that the AAV vector expressed E2 protein at a very low level or not at all. One potential issue was the fact that the AAV-Alb-hE2 vectors lacked an intron. The benefits for the presence or absence of an intron in AAV vectors are somewhat controversial. It has been suggested that sustained expression of AAV *in vivo* was sometimes strengthened by the

presence of an intron (Ostedgaard et al., 2005). However, it has also been suggested that an intron was not required for expression and benefits are generally minimal (Wang et al., 2005b); many investigators omit it in order to conserve precious space.

Despite these negative data and many potential issues, it was likely the AAV-Alb-hE2 viral vectors were functional, albeit at a low level. Therefore, possible plans for the future will be to construct a new AAV viral vector using a stronger promoter to drive expression of E2, i.e., human alpha-1-antitrypsin (AAT). The human AAT promoter has been shown to be capable of directing high expression specifically in hepatocytes (Knapp and Liu, 2004; Nash et al., 2004). Incorporation of an intron to help strengthen sustained AAV expression *in vivo* is also planned. Hydrodynamic viral administration via the tail vein, which can have a 40% or greater hepatocyte transfection efficiency with a single injection (Liu et al., 1999; Zhu et al., 2006), should also help to transfect a greater number of hepatocytes in the iMSUD mouse model. In addition, a dose of 10^{12} v.g./animal would be an optimal starting dose (Dr. H. Nakai, personal communication Sept. 2007, University of Pittsburgh).

4.0 HEPATOCYTE TRANSPLANT (HTx) THERAPY IN iMSUD MICE

Hypothesis: I hypothesize that liver-directed hepatocyte transplant (HTx) therapy can correct E2 deficiency, restore liver BCKDH activity, and ultimately correct the disease phenotype of the iMSUD mouse model.

4.1 BACKGROUND AND SIGNIFICANCE

MSUD, caused by a deficiency in the BCKDH complex, is a serious liver-based metabolic disorder with limited options for reliable treatment. However, it is known that 100% of whole body BCKDH activity is not needed to correct the disease phenotype. Human MSUD carriers expressing 50% of whole body BCKDH activity rarely display symptoms (Chuang and Shih, 2001), which was also true for iMSUD mice (Homanics et al., 2006). In MSUD patients, >30-40% residual whole body BCKDH activity (variant dependent; **Table 1**) is also rarely associated with disease phenotype. Importantly, transplantation of liver alone in MSUD patients was an adequate treatment for MSUD resulting in full or partial metabolic correction of disease phenotype (Bodner-Leidecker et al., 2000; Netter et al., 1994; Strauss et al., 2006; Wendel et al., 1999). The MSUD phenotype following orthotopic liver transplantation (OLT) was dependent upon the severity of initial disease presentation (Wendel et al., 1999). Therefore, it is reasonable to hypothesize that infusion of a small percentage of liver mass (i.e., HTx) may be sufficient to

correct metabolic function in the case of a mild MSUD variant, or partially correct severe cases to a milder and more manageable variant.

HTx has had initial clinical and laboratory successes in the treatment of inherited and metabolic liver disorders such as Crigler-Najjar syndrome (Fox et al., 1998; Groth et al., 1977; Matas et al., 1976; Vroemen, 1986), glycogen storage disease, type 1 (Muraca et al., 2002a), Wilson's disease (Irani et al., 2001), Factor VII (Dahwan et al., 2004), and a urea cycle disorder (Horslen et al., 2003). HTx has been successful at improving metabolic defects, such as albumin secretion (Moscioni et al., 1989; Oren et al., 1999) and tyrosinemia (Overturf et al., 1996). It has also been investigated and implemented as an exceptional "bridging" therapy to support liver function in times of liver injury or acute failure as the result of a variety of liver diseases (Bilir et al., 2000; Fisher et al., 2000; Strom et al., 1999; Strom et al., 1997).

HTx therapy holds many benefits over whole organ transplantation, and may serve as a therapeutic alternative to OLT. HTx is a nonsurgical procedure with less associated morbidity, has fewer less serious complications, and a reduced recovery time (Strom et al., 2006a). HTx also has a much lower economic cost (~5-10% of OLT). In patients with whole organ failure, HTx has been used to extend life until a donor organ becomes available, but for those with a metabolic disorder such as MSUD, HTx could be administered on an outpatient basis (Strom et al., 2006a). Transplantation of up to 5% of patient liver mass has been accomplished thus far with no adverse effects (Dhawan et al., 2004) with multiple HTx treatments being possible (Strom et al., 2006a). Cryopreservation of donor hepatocytes could also be employed so that cells are readily available upon demand. However, one disadvantage of HTx is that the most likely source for hepatocytes are rejected livers meant for whole organ transplantation (Strom et al., 2006a; Strom et al., 2006b). Therefore, high transplant-quality cells must be harvested from

organs deemed unsuitable for OLT due to >50% steatosis or other adverse factors. However, one donor liver could be used to transplant multiple HTx recipients, which would greatly help to relieve overwhelming donor organ shortages.

In light of these points, HTx was determined to be logical and appropriate for use with the novel iMSUD mouse model to restore liver BCKDH activity and perhaps ultimately correct the liver metabolic disorder of MSUD.

4.2 METHODS

All studies involving animals were reviewed and approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

4.2.1 Mouse colony breeding

Production of iMSUD mice was carried out as previously described in Methods Section 2.2, however only iMSUD mouse line A was used for this section's described experiments.

ROSA26 [B6.129S7-*Gt(ROSA)26Sor*/J; Jackson Laboratory stock #2192 (Friedrich and Soriano, 1991)] colony breeding was carried out by mating a heterozygous (+/-) ROSA26 mouse with a WT C57BL/6 mouse. This breeding strategy resulted in approximately 50% of offspring being ROSA26 +/- (LacZ positive), which were used as donors for hepatocyte transplantation.

4.2.2 PCR genotyping and analysis

ROSA26 +/- mice were genotyped at birth by PCR of genomic DNA isolated from a small amount of blood using Instagene (BioRad, Hercules, CA). The PCR primers and cycling parameters were outlined by Jackson Laboratories on the #2192 data sheet (JAX, 2008); reactions were carried out using PCR Supermix HiFi (Invitrogen, Carlsbad, CA). In summary, each DNA sample was analyzed with two sets of primers specific either to WT mouse DNA (present only in wildtype and heterozygous animals) or the LacZ/ROSA locus (present only in heterozygous and homozygous ROSA26 animals). WT primers (5' GGCTTAAAGGCTAACCTGATGTG 3' forward and 5' GGAGCGGGAGAAATGGATATG 3' reverse) amplified a 374 bp fragment. ROSA26 primers (5' AATCCATCTTGTTCAATGGCCGATC 3' forward and 5' CCGGATTGATGGTAGTGGTC 3' reverse) amplified a ~500bp fragment. Results were confirmed using a β-galactosidase (β-gal) Staining Kit (Mirus, Madison, WI) of a small tail clip (~0.1-0.2mm) at birth.

DNA (20ng/sample) from flash frozen mouse liver samples were also isolated for PCR analysis via the DNeasy kit (Qiagen, Germantown, MD), or using a standard phenol:chloroform purification protocol following tissue lysis. Primers and PCR cycling parameters were identical to those used for genotyping.

4.2.3 Southern blot genotyping

Southern blot analysis for genotyping of iMSUD mice was completed as previously described in Methods Section 2.2.

4.2.4 β-galactosidase activity assay

β-gal activity was quantified from ROSA26 +/- (LacZ positive), iMSUD-HTx, and WT control (LacZ negative) liver tissue homogenates using the High Sensitivity β-galactosidase Assay Kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Data was normalized to newborn ROSA26 +/- activity and expressed as the mean percent +/- SEM. Differences between groups were compared by ANOVA and post-hoc Fisher's test.

4.2.5 ROSA26 +/- hepatocyte isolation

Hepatocytes from 1.5-2.5 week old ROSA26 +/- mice (Friedrich and Soriano, 1991) were isolated by collagenase perfusion (Strom et al., 1996). In brief, donor mice were sedated with 100mg/kg ketamine (Fort Dodge Animal Health, Ft. Dodge, IA) + 10mg/kg xylazene (Vedco, St. Joseph, MO) and a midline laparotomy performed. The inferior vena cava was then perfused and the portal vein sectioned within 5-10 seconds of the start of perfusion to allow the solution to flow through the liver. The liver was sequentially perfused at a rate of 2.5 mL/min with EGTA solution (HBSS without calcium or magnesium, EGTA 0.5 mM, HEPES 10 mM, pH 7.5 supplemented with 1% penicillin/streptomycin) for 5 minutes followed by collagenase solution for 10 min (EMEM with calcium/magnesium plus collagenase 0.1 mg/ml, HEPES 10 mM, pH 7.5 and 1% penicillin/streptomycin). Hepatocytes were removed by mechanical disassociation and filtered through sterile 150 mM nylon mesh. Cells were washed 3 times in EMEM media by centrifugation at 50 x g for 5 min and finally resuspended at a concentration of $2x10^6$ cells/mL in PBS. The viability of donor hepatocytes was estimated by Trypan blue exclusion; only

suspensions with viability >80% were used for transplants. One mature liver was sufficient for 30+ transplant recipients.

4.2.6 Hepatocyte transplantation

Isolated donor ROSA+/- hepatocytes with viability >80% were prepared at a concentration of $2x10^6$ cells/mL PBS as described above. A sterile 30 gauge needle was used to administer 50μL of PBS alone or PBS containing 100,000 hepatocytes (approximately 1% of liver mass) directly through the abdomen of neonatal mice into the liver pulp; one injection was administered between the ages of 1-7 days and a second injection was administered approximately 3-7 days later. At this early age, the liver was clearly visible through the skin.

4.2.7 Histochemistry

Livers from iMSUD or ROSA26 +/- mice were either immediately flash frozen in liquid N_2 or incubated in 30% sucrose for at least 24 hours. Tissue was cryopreserved in Optimum Cutting Temperature Compound (OCT; Tissue-Tek), sectioned at 30μ using a Leica CM1850 cryostat, and mounted on slides. To visualize LacZ positive cells, liver sections were stained using the β-galactosidase Staining Kit (Mirus, Madison, WI) following the manufacturer's instructions for tissue. Sections were fixed for 2 minutes at room temperature with Cell Fixative Reagent, stained with Cell Staining Solution at 37°C for 2 hours, and washed briefly in PBS. Sections were then subjected to an ethanol dehydration series, xylenes, and coverslipped with Permount Mounting Medium (Fisher Scientific, Pittsburgh, PA).

4.2.8 BCKDH complex enzyme activity assay

iMSUD-PBS, untreated iMSUD, iMSUD-HTx, ROSA26 +/-, and WT control mouse livers were removed, flash frozen and stored at -80^{o}C. BCKDH enzyme activity was completed as previously described in Methods Section 2.2.

4.2.9 Ion-exchange chromatography

Serum was isolated from whole blood collected by cardiac puncture at sacrifice through centrifugation for 10 minutes at 3000 x g. Whole brains were collected and immediately flash frozen in liquid N_2. Serum and brains was stored at -80^{o}C until analysis. Amino acids from mouse serum and brains were quantified using standard ion-exchange chromatography (IEC) with post-column ninhydrin derivatization (Slocum and Cummings, 1991). Data were analyzed by ANOVA and post-hoc Fischer's test. All analyses of whole brains and serum were completed by Dr. K.M. Gibson, University of Pittsburgh.

4.2.10 Blood BCAA levels

Blood samples were collected from the tail at weaning and approximately weekly until sacrifice. Blood samples were spotted on a Guthrie card and mailed to Pediatrix Screening Inc. (Bridgeville, PA) for processing as previously described (Methods Section 3.2) or given to Dr. Donald Chace (Pediatrix Screening Inc.) for analysis by MS-MS at the National Institute of Health.

4.2.11 HPLC detection of monoamines

Whole mouse brains were collected immediately after sacrifice and flash frozen in liquid N_2. Brain tissue was stored at -80^{o}C until time of analysis. Monoamine concentrations were determined by HPLC as described previously (Ogburn et al., 2006). Briefly, tissues were thawed and deproteinized with 5 volumes of perchloric acid (0.1M) containing DETAPAC (1mg/ml) and DTE (0.1mg/ml). After centrifugation 10µL of clear perchloric acid extract was injected directly into the HPLC system. Dopamine (DA), serotonin (5-HT) and their metabolites (DOPAC, HVA, 3-MT and 5-HIAA) were separated on an HPLC column (C18 250 x 3 mm, 5µ, Phenomenex, CA); at a flow rate of 0.5 ml/min with a mobile phase consisting of potassium phosphate (0.05 mM), octylsulfate (8.5 mg/50ml) and 14% methanol, adjusted to pH 2.65. Detection was performed using a coulometric electrochemical cell (model 5014B, ESA Inc, Chelmsford, MA) set to a potential of +400 mV. Data were acquired and processed using Coularray for Windows software (ESA Inc, Chelmsford, MA). All analyses of whole brains for monoamines were completed by Dr. T. Bottiglieri (Baylor College of Medicine, Houston, TX).

4.2.12 Western blot analysis

Protein was isolated from liquid N_2 flash frozen livers or cultured adult hepatocytes. Protein (25µg per sample) was analyzed as previously described in Methods Section 2.2.

4.2.13 Real Time qPCR

RNA was isolated from primary mouse hepatocytes (obtained from ROSA26 +/-, iMSUD, iMSUD-PBS, iMSUD-HTx, and WT controls) and primary human hepatocytes (obtained from liver disease patient resections) using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA was converted to cDNA using the High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA). Real Time qPCR was performed using the TaqMan Assays-on-Demand Gene Expression Products for β-actin (ID# Mm02619580_g1; Applied Biosystems, Foster City, CA) following the manufacturer's protocol. In short, 100ng of cDNA template, 12.5µl of 2x Taqman Universal Master Mix, and 1.25µl of 20x Target Assay Mix were combined into 25µl reactions. Real Time qPCR was also performed using the SYBR Green system. Mouse E2 primers for SYBR Green qRTPCR (5' GGAAACCACTGATCGACATTGAGA 3' Forward; 5' ATTGCCAGCCGACGCACT 3' Reverse) were custom designed using a BLAST alignment (**Figure 18**) of the cMSUD deletion site in mouse E2 mRNA (bp 444-617; NCBI accession # NM 010022) and the corresponding human E2 sequence (bp 415-588; NCBI accession # NM 001918). Primers were chosen based upon the number of mismatches to human to ensure specificity to mouse only, and verified using BiosearchTech's (Novato, CA) free online RealTimeDesign software for primers. In short, 100ng of cDNA template, 12.5µl of 2x SYBR green PCR Master Mix, and 0.075µl of each 0.1mM primer were combined into 25µl reactions. Each sample was run in triplicate on an ABI 7000 Real-time PCR System (Applied Biosystems). Relative quantification of Real Time RT-PCR data was performed using the Comparative C_T (ΔC_T) Method. In summary, C_T values were first converted to an arbitrary number using a standard calculation [arbitrary number = $1000000000^{(-0.69077552 *}$

$^{CT)}$]. The three arbitrary numbers (AN) for each sample were then averaged. Target gene expression levels were normalized to the corresponding actin endogenous control by calculating the ΔC_T [$\Delta C_T = {}_{ave}AN_{\text{target gene}} / {}_{ave}AN_{\text{actin}}$]. Data were presented as the mean +/- the SEM and analyzed by Student's T-test or ANOVA and post-hoc Fisher's test.

```
            Exon 4       Forward Primer →                        Exon 5
Mouse  444  ATCGCTTATGTGGGGAAACCACTGATCGACATTGAGACAGAAGCTTTAAAGGATTCAGAG  503
            || || |||||||||||| ||| |  | ||||| || || |||||||||||| |||||||||
Human  415  ATTGCCTATGTGGGGAAGCCATTAGTAGACATAGAAACGGAAGCTTTAAAAGATTCAGAA  474

Mouse  504  GAAGATGTTGTTGAAACCCCCGCTGTGTCCCATGATGAGCACACTCACCAAGAGATAAAA  563
            |||||||||||||||| || || ||||| |||||||| || || ||||||||||||||
Human  475  GAAGATGTTGTTGAAACTCCTGCAGTGTCTCATGATGAACATACACACCAAGAGATAAAG  534
                                      ← Reverse Primer
Mouse  564  GGCCAGAAAACACTAGCAACGCCTGCAGTGCGTCGGCTGGCAATGGAAAACAAT  617
            ||||  |||||||| ||||| |||||||| || || ||||||||||||||||||
Human  535  GGCCGAAAAACACTGGCAACTCCTGCAGTTCGCCGTCTGGCAATGGAAAACAAT  588
   84% identity
```

Figure 18. Mouse and human E2 mRNA alignment and SYBR Green qRTPCR primers.
The E2 mouse sequence (top, bp 444-617), which was deleted to create the cMSUD model, was aligned to the corresponding E2 human sequence (bottom, bp 415-588; 84% identity to mouse). Vertical lines connecting human and mouse base pairs indicate a match while the absence of a line indicates a mismatch. Primers for SYBR Green Real Time qPCR analysis are bolded and highlighted in grey. The bolded ATT codon designates the start of E2 Exon 5.

4.2.14 Weight and survival

Date of birth, date of treatment, weight at weaning (~21 days of age), and date of death were recorded for iMSUD-HTx, iMSUD-PBS, untreated iMSUD, and WT control mice. Data were analyzed via the Kaplan-Meier method and Log-Rank Test [survival (Kaplan and Meier, 1958; Mantel, 1966)], or ANOVA and post-hoc Fisher's test (weight).

4.3 RESULTS AND DISCUSSION

4.3.1 ROSA26+/- hepatocyte characterization

Prior to injecting HTx into iMSUD mice, it was necessary to first characterize ROSA26 donor hepatocytes. Information acquired from the Jackson Laboratory ROSA26 data sheet (#2192) stated that while all cells expressed LacZ in the developing embryo, only "most cells" expressed LacZ in the adult animal. It was necessary to verify whether adult liver cells expressed LacZ, as well as determine the intensity of LacZ expression from ROSA +/- cells.

Liver sections from newborn (1 day old) ROSA +/- mice revealed significant β-gal enzyme activity, evidenced by a dark blue color in most liver cells with an apparent lack of β-gal activity in the connective tissue capsule (cells around the liver's outer edge and surrounding blood vessels; **Figure 19A**). However, β-gal activity was greatly reduced in adult (8-12 week old) ROSA26 +/- livers compared to newborn. Adult liver sections revealed patches of weakly β-gal positive hepatocytes and a more significant blue stain in the connective tissue capsule than was seen in newborn livers (**Figure 19A**). Compared to newborn livers, a reduction in hepatocyte β-gal activity was also observed in young (4-6 week old) ROSA26 +/- mice, although loss was not as extensive as in adult livers (data not shown). To confirm these results, β-gal activity was quantified using a High Sensitivity β-gal assay kit. A significant difference existed between genotypes as determined by ANOVA [$F(2,9)=1247$, $p<0.0001$]. Adult liver β-gal activity was determined to be ~50% ($p<0.0001$) of newborn liver activity (**Figure 19B**). Background expression in LacZ negative Control mice was negligible (<0.002% of newborn

activity +/- 0.001 SEM). Taken together, these data confirm that as ROSA26 mice aged, detectable LacZ expression and thus β-gal activity was gradually lost in hepatocytes.

BCKDH enzyme activity and E2 protein expression of newborn and adult ROSA26 +/- livers were also analyzed. Liver BCKDH enzyme activities were not significantly different between the two age groups (**Figure 19C**). However, Western blot analysis revealed E2 protein expression in ROSA26 +/- newborn liver to be reduced ~48% ($p<0.01$) when compared to adult livers (**Figure 19D**). These data suggested the BCKDH complex functioned at an increased rate in newborn liver hepatocytes possibly due to a decrease in E2, and presumably other BCKDH subunits; E1α, E1β, and E3 protein levels were not assessed. Therefore, despite changes in protein availability at the analyzed timepoints, enzyme activity remained at a constant "normal" level. Another possible explanation for these data could be that newborn BCKDH activity was stimulated by a recent meal. BCKDH enzyme activity can reportedly increase after a high protein diet (Harris et al., 1985; Soemitro et al., 1989). This phenomenon was hypothesized to be linked to increased glucagon in the portal circulation given that glucagon action is mediated by cAMP, which was also shown to upregulate BCKDH liver mRNA and increase enzyme flux (Chicco et al., 1994; Eisenstein et al., 1979).

In summary, despite a ~48% decrease in E2 protein in newborn liver tissue compared to adult, no significant difference between BCKDH enzyme activities were determined. Thus, ROSA26 +/- hepatocytes from either age would be equally beneficial for transplant to correct iMSUD mice due to similar BCKDH activities. However, detectable LacZ expression in ROSA26 +/- livers decreased as mice aged with a significant decrease apparent as early as 4 weeks. Therefore, it was concluded that hepatocytes for HTx were to be isolated from young (newborn to <3 week old) ROSA26 +/- mouse livers. LacZ expression would be used to identify

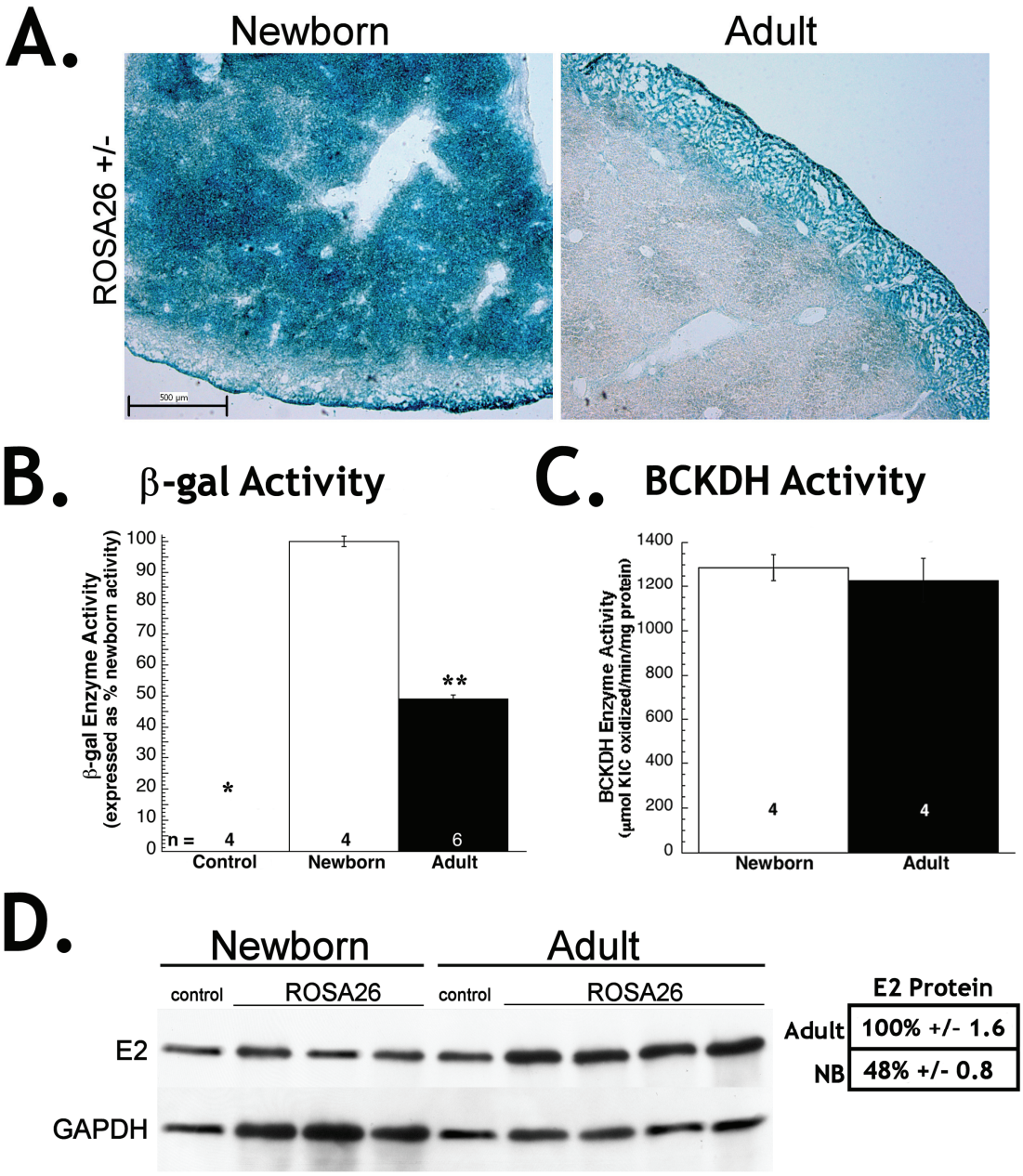

Figure 19. ROSA26 +/- hepatocyte characterization.
A. Newborn (1 day old) and adult (8-12 week old) mouse liver sections (30µ) were treated with a β-gal staining kit (10x magnification). Newborn LacZ expression was strong in most hepatocytes (dark blue staining) while expression in connective tissue capsule cells (outer liver edge and surrounding blood vessles) was reduced compared to hepatocytes. LacZ expression in adult hepatocytes was reduced compared to newborn with a high level of β-gal staining in the connective tissue capsule. **B.** Adult β-gal enzyme activity was reduced by ~50% compared to newborn livers (**, $p<0.0001$ compared to control and newborn). Control liver β-gal activity was negligible (*, $p<0.0001$ compared to newborn and adult). The numbers on the bars indicate n values. **C.** Newborn and adult liver BCKDH enzyme activities were similar. The numbers on the bars indicate n values. **D.** Western blot analysis of newborn (n=6) and adult (n=8) ROSA26 +/- liver determined newborn (NB) liver E2 protein to be reduced ~48% +/- SEM when compared to adult ($p < 0.01$). Control protein lanes were for reference only. GAPDH was used as a protein loading control. All data were expressed as the mean +/- SEM.

transplanted ROSA26 +/- donor cells in the host liver, which would allow for quantification of percent engraftment.

4.3.2 ROSA26 +/- HTx and long-term β-gal expression

Isolated ROSA26 +/- cells were verified to express LacZ prior to HTx injection, as indicated by a blue color when stained with a β-gal staining kit (**Figure 20**). Donor hepatocytes ($1x10^5$ cells in 50μL PBS) were injected directly into the livers of neonatal mouse pups 1-7 days of age and a second injection administered approximately 3-7 days later. HTx-treated mouse livers, all analyzed greater than 1 week post-transplantation, revealed no detectable β-gal staining and β-gal enzyme activity did not differ from untreated LacZ negative controls (data not shown).

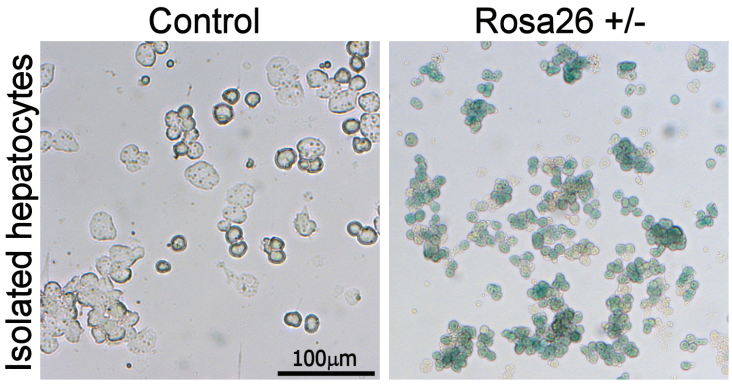

Figure 20. Isolated Control and ROSA26 +/- hepatocytes.
ROSA26 +/- hepatocytes for HTx and control hepatocytes were isolated by collagenase perfusion and stained with a β-gal staining kit. Greater than ~80% of ROSA26 +/- hepatocytes expressed LacZ (blue cells). A blue color could not be visualized in any Control (LacZ -) hepatocytes. The difference in cell size was due to a difference in age (Control: ~2.5 weeks old; ROSA: ~1.5 weeks old). 100x magnification was used.

The lack of β-gal staining and activity in HTx treated livers may have been due to loss of LacZ expression as the engrafted donor hepatocytes aged, similar to what was seen in ROSA +/- livers (**Figure 19A**). Small clusters or single β-gal positive hepatocytes may have also been

difficult to identify. It is also possible ROSA26 +/- hepatocytes died or simply donor hepatocytes leaked into the peritonium following direct injection into the liver parenchyma. In an effort to determine the most likely explanation, a mouse was sacrificed 24 hours post-HTx and the liver examined. β-gal positive (blue) cells in liver sections (**Figure 21**, some identified by black arrows) determined that a large quantity of injected donor hepatocytes remained in the liver following HTx and that small clusters of engrafted LacZ +/- cells could be identified by a β-gal staining kit. One caveat of this experiment was that only a single mouse was analyzed 24 hours post-HTx; what was observed in this one mouse may not be representative of what occurred in all HTx-treated mice. However, the identification of blue cells verified that ROSA26

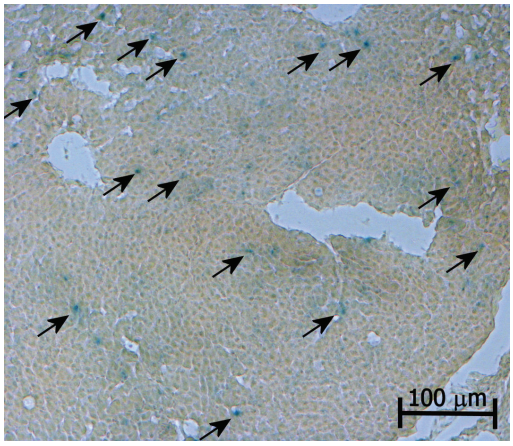

Figure 21. Mouse liver analyzed 24h post-HTx.
1.5 week old ROSA26 +/- hepatocytes (**Figure 20**) were isolated by collagenase perfusion and injected through the abdomen directly into the liver parenchyma of a neonatal mouse pup ($1x10^5$ cells in 50µL PBS). 24 hours post-HTx, the liver was sectioned (30µ) and analyzed for the presence of β-gal positive (blue) cells with a β-gal staining kit. Selected blue cells are indicated by black arrows. 100x magnification was used.

+/- hepatocytes were delivered to and stayed in the liver following a direct injection. Cells were not leaking into the peritonium to a significant degree. HTx-treated livers were analyzed when ROSA26 +/- donor cells were 5-6+ weeks old, an age that corresponded to significant loss of LacZ gene expression in ROSA26 +/- livers. Therefore, inability to detect β-gal positive cells in

recipient livers several weeks after HTx may be due to gradual inactivation of the LacZ gene or donor cell death. In summary, determination of β-gal activity was concluded to not be a reliable method for assessing percent engraftment in the liver of HTx mice.

4.3.3 HTx effects on blood amino acids of iMSUD mice around weaning

A highly significant difference in BCAA/ala ratios obtained from blood spots was determined between treatment groups by ANOVA analysis [$F(3,20)=242$, $p<0.0001$]. BCAA/ala serum levels were decreased by approximately 75% in iMSUD-HTx mice compared to untreated iMSUD mice (**Figure 22A**; $p<0.0001$). However, mean iMSUD-HTx blood BCAA/ala levels were not normalized to control levels; iMSUD-HTx BCAA/ala levels still suffered a 4-fold increase over Controls ($p<0.0001$). iMSUD-PBS values (15.9μmol/L blood +/- 6.4 SEM, n=8; data not shown) were not different from untreated iMSUD mice. The mean blood BCAA/ala ratio of Controls, either HTx-treated or untreated, was ~1μmol/L, which agreed with data reported previously (Homanics et al., 2006). These data suggested HTx had significantly improved BCAA turnover in iMSUD mice, but was unable to correct it to Control levels.

Blood was collected for analysis at weaning (~21 days of age), and some animals had blood taken at addition timepoints, ~1-2x/week, up to when mice were ~30 days of age. **Figure 22B** displays each analyzed blood spot as an individual data point for each iMSUD-HTx mouse (e.g., D7332, D7334, etc.). At a few analyzed timepoints iMSUD-HTx BCAA/ala blood ratios were at or very close to control levels (**Figure 22B**); however a great deal of variability existed between individual mice as well as between individual analyzed timepoints from the same mouse. Variability between data was not surprising since similar results were observed previously (Homanics et al., 2006). Blood BCAA variations were likely related to how much

time had passed between protein ingestion and blood collection; for example, iMSUD-HTx mouse #D7332 had BCAA/ala levels of 4.3μmol/L blood at weaning age (21 days), 5.7μmol/L blood at 26 days of age, and 1.1μmol/L blood at 30 days of age (**Figure 22B**). Control values did not vary significantly between mice or timepoint. Therefore, although HTx by this protocol greatly improved BCAA turnover in iMSUD mice, it was unable to compensate for fluctuating BCAA levels and thus was not completely effective resulting in BCAA/ala spikes in blood levels dependent upon collection timepoint. Additional HTx administrations may completely or more effectively correct disease phenotype.

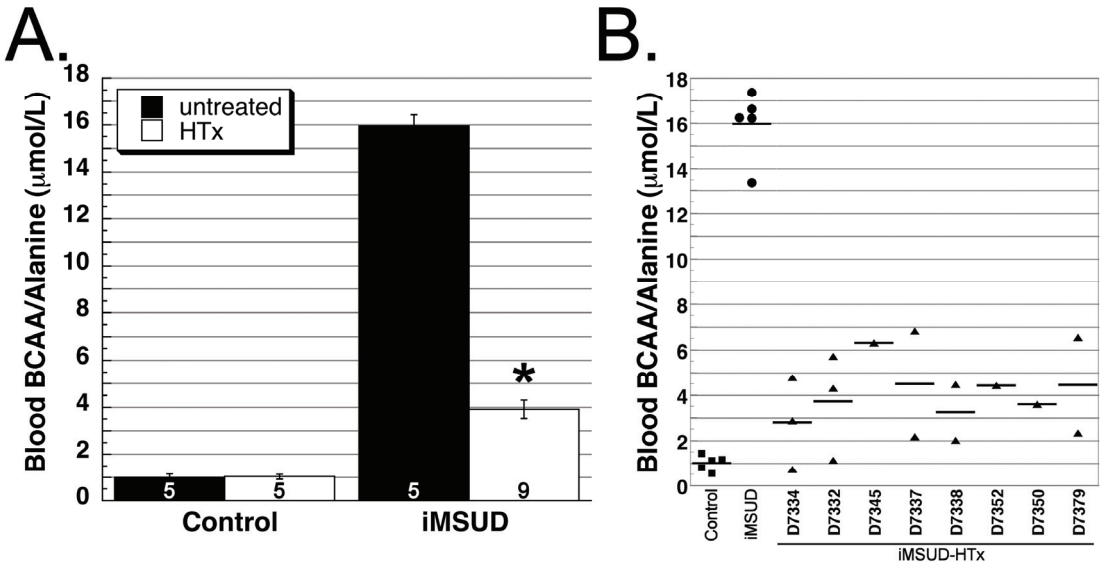

Figure 22. Blood BCAA/ala ratios from HTx mice collected around weaning.
A. Mean BCAA/Ala values +/- SEM of untreated and HTx-treated iMSUD and Control mice were determined by MS-MS. iMSUD-HTx BCAA/ala ratios were reduced 75% compared to untreated iMSUD, but were still increased 4-fold above control values (*, $p<0.0001$). Untreated iMSUD ratios were also significantly different from iMSUD-HTx and control values ($p<0.0001$). The number displayed on each bar indicates the n value. **B.** Some iMSUD-HTx mice had blood taken multiple times; once at weaning and then ~1-2x/week up to mice were 30 days of age. Each analyzed blood spot averaged in panel A was displayed as an individual data point for each iMSUD-HTx mouse (e.g., D7332). Individual iMSUD and Control data points represent a single mouse and correspond to the untreated (black) bars from panel A. The horizontal line indicates mean value. Blood was analyzed by Pediatrix Screening, Inc. (Bridgeville, PA).

4.3.4 HTx effects on blood amino acids of iMSUD mice at sacrifice

Preliminary data were obtained by ion exchange chromatography (IEC) from total serum collected at the time of sacrifice (33-35 days of age). These data suggested that blood amino acid levels of iMSUD-HTx mice (n=2, 33 days of age at sacrifice) were not normalized to control values (**Figure 23**). However, due to a low sample size for iMSUD-HTx statistical significance between groups could not be determined. These data were very different from results obtained by MS-MS analysis at weaning, which was discussed above. Although blood spots and total serum were analyzed by different methods as well as by different investigators, both investigative groups were highly skilled, and MS-MS and IEC are able to provide sensitive and reliable results for serum amino acids. Therefore, these issues were unlikely to have contributed to differences in calculated results.

The preliminary results from iMSUD-HTx serum may be dependent on the time of death at day 33. The two iMSUD-HTx mice analyzed by IEC were also analyzed at weaning by MS-MS (**Figure 22B**; D7550 & D7552); a mean BCAA/ala level was determined to be 4.0μmol/L blood +/- 0.4 SEM at 21 days of age. At 33 days of age, IEC determined mean serum BCAA/ala levels to be ~12.5μmol/L blood. Therefore, since immunosuppression therapy was not used, the most likely explanation for these data was that perhaps at the time of sacrifice rejection of donor cells had begun to occur, thus pushing iMSUD-HTx mice into BCAA crisis.

Muscle protein mobilization into the blood stream around the time of death may also have compounded serum amino acid load. There were similarities between amino acid changes that occur during fasting of MSUD patients (Haymond et al., 1978; Schwahn et al., 1998) and iMSUD-HTx IEC results. This suggested that perhaps mice were not eating normally (i.e.,

fasting) because they were feeling sick. iMSUD-HTx animals displayed an increase in leucine, isoleucine, and valine, and a reduction in serum alanine compared to controls (**Figure 23**). Mobilized protein from muscle would be readily metabolized into BCAA/BCKAs, and alanine was shown to be reduced in fasting human MSUD patients (Haymond et al., 1978; Schwahn et al., 1998). However, interpretation was confounded since elevation of BCAAs are hallmark indicators of BCAA crisis in human patients (Morton et al., 2002; Wajner et al., 2000) and reduced alanine is also an expected amino acid aberration in human MSUD patients with elevated BCAAs (Morton et al., 2002). Glutamine was also reportedly readily released from muscle into the blood during times of fasting (Felig et al., 1976), and although serum glutamine in iMSUD-HTx mice appeared to be increased compared to untreated iMSUD mice, it was reduced compared to controls. Since so few iMSUD-HTx samples were analyzed, direct comparisons cannot be reliably made between groups. However, the presence of high levels of alloisoleucine in iMSUD-HTx serum, a sensitive and specific diagnostic marker for all MSUD variants (Schadewaldt et al., 1999), suggested that HTx treated mice were suffering from symptoms of their disease.

Stress was also reported to increase BCAA/BCKA levels in the human disease (Chuang and Shih, 2001). Amino acid anomalies found in serum at sacrifice may be due to stress triggered by excess noise and vibration from being wheeled down the mouse facility hallway. This experience occasionally triggered seizures which often resulted in death in untreated iMSUD animals (unpublished observations). However mice were subjected to the same experience when blood for MS-MS analysis was collected at weaning. Therefore, if stress was causing a spike in BCAA levels, iMSUD-HTx BCAA/ala levels from MS-MS data becomes even more impressive (**Figure 22**).

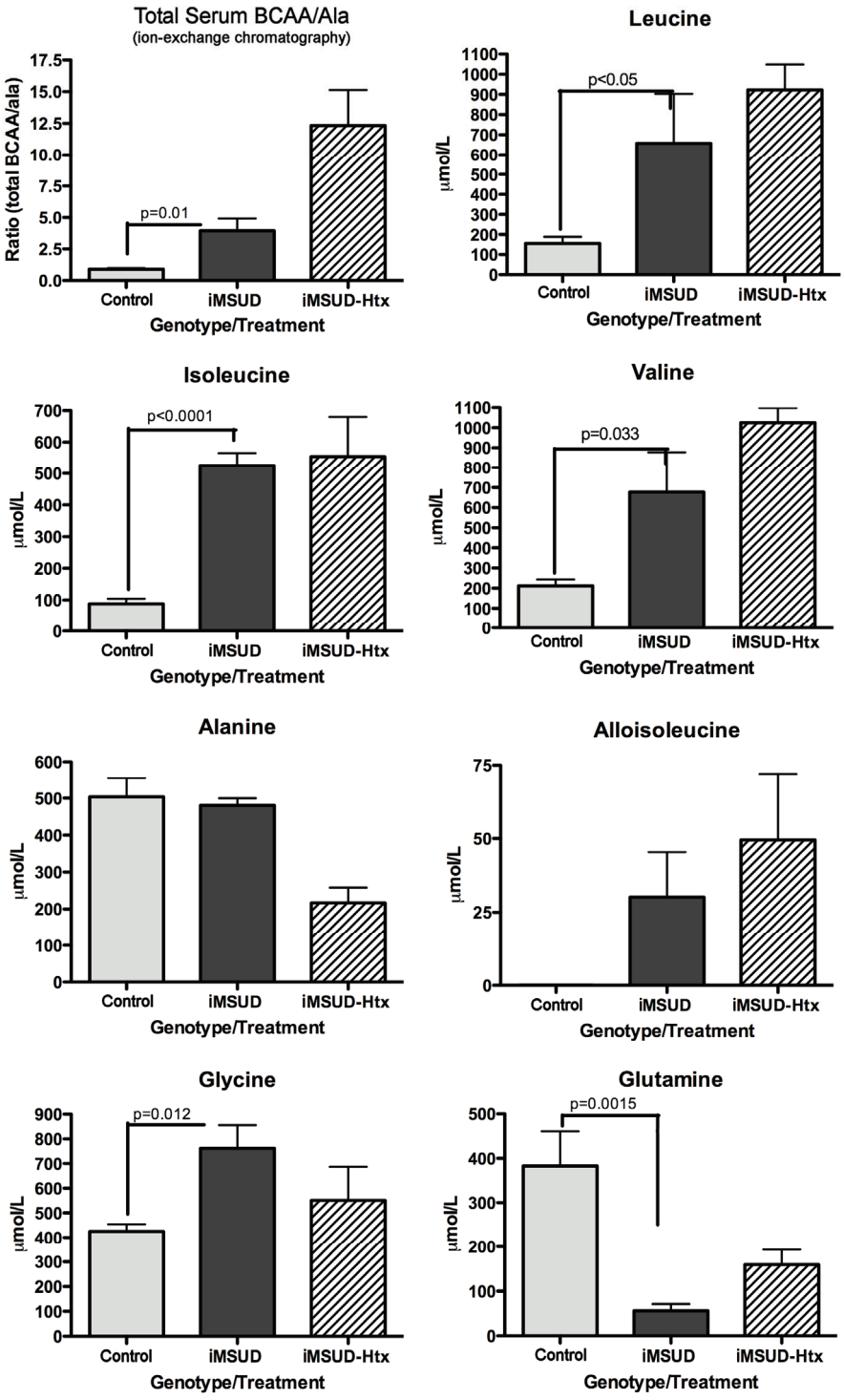

Figure 23. Serum results from total serum collected at the time of sacrifice.
Total serum was collected from Controls (WT; n=5), untreated iMSUD (n=5), and iMSUD-HTx (n=2) mice by cardio puncture and analyzed by IEC. Results are reported as the mean +/- SEM. Statistical analysis comparing iMSUD-HTx mice to other groups could not be completed due to a small sample size, though trends suggested iMSUD-HTx blood AAs were not improved by HTx. Analyses were completed by Dr. K.M. Gibson, University of Pittsburgh.

In summary, preliminary data from IEC analysis of serum at sacrifice suggested HTx was no longer able to reduce BCAA/ala levels (**Figure 23**) despite a significant improvement observed in blood amino acid levels at weaning (**Figure 22**). Several explanations could explain these data, such as donor cell rejection, mobilizing protein from muscle, and stress. Nevertheless, the low number of HTx serum samples analyzed by IEC requires that more iMSUD-HTx be examined to elucidate the appropriate conclusion.

4.3.5 HTx effects on liver BCKDH and E2 protein levels

MS-MS results suggested that ROSA26 +/- donor hepatocytes were able to functionally engraft in the liver and participate in amino acid turnover to significantly reduce toxic BCAAs in iMSUD-HTx mouse blood at the time of weaning. Therefore, donor cells must have contributed to BCKDH enzyme activity at a discernable level over untreated iMSUD mice. To verify this, a BCKDH enzyme assay was performed with fresh liver homogenate.

Mean liver BCKDH enzyme activity of HTx treated animals was significantly improved over iMSUD-PBS mice (**Figure 24A**). iMSUD-HTx activity was increased ~2-fold over iMSUD-PBS animals from ~6.2% of Control activitiy to ~14.4% ($p<0.005$). BCKDH enzyme activity of untreated iMSUD mice (6.8% of Control activity, data not shown) was not different from PBS-treated iMSUD mice. These data confirmed that HTx treated animals indeed had a distinguishable and significant increase in BCKDH activity compared to iMSUD-PBS and untreated iMSUD mice. Supplemental BCKDH was hypothesized to be derived from engrafted donor hepatocytes, which supports the dramatic improvement assessed in iMSUD-HTx amino acid profiles from bloodspots (**Figure 22**).

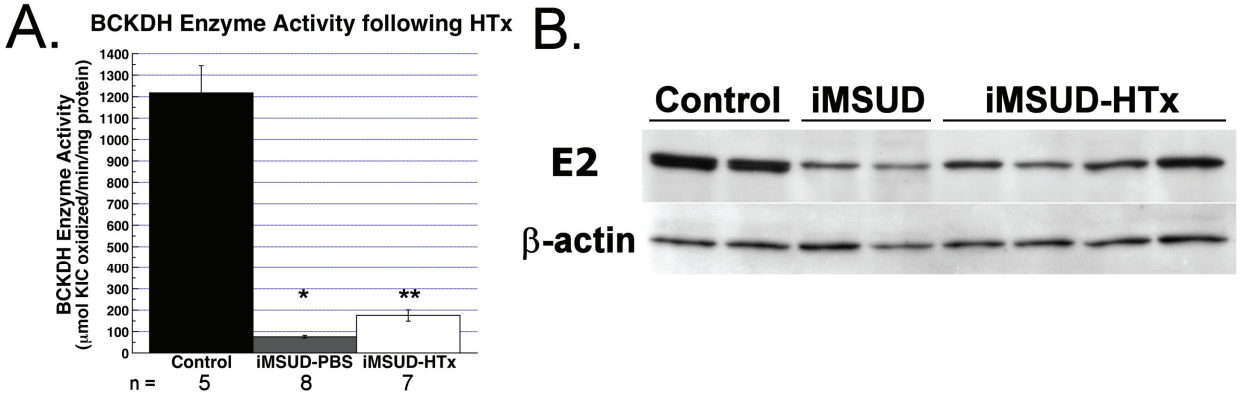

Figure 24. HTx effects on liver BCKDH activity and E2 protein levels.
A. BCKDH enzyme activity was assessed for Controls, iMSUD-PBS, and iMSUD-HTx mice. iMSUD-HTx enzyme activity was significantly different from Control and iMSUD-PBS activities (**, $p<0.005$). iMSUD-PBS enzyme activity was also significantly different from Control (*, $p<0.005$). **B.** Western blot analysis assessed E2 protein in livers of Control (n=2 pools of 2 mice), untreated iMSUD (n=2 pools of 2 mice) and iMSUD-HTx (n=4 pools of 2 mice). Each lane represents a pool of two mouse protein samples. Under optimal conditions, a doublet corresponding to mouse (47kD) and human (54kD) E2 proteins can be visualized.

It was therefore theorized that since BCKDH enzyme activity of iMSUD-HTx had increased ~8% over untreated iMSUD mice, E2 protein may have also increased to a level that would be detectable by Western blot analysis. However, E2 liver protein levels, expressed as a percent of Control (n=4 pools of 2 mice, each analyzed in duplicate), were not significantly different between untreated iMSUD animals [64.9% +/- 3.5% SEM (n=2 pools of 2 mice, each analyzed in quadruplicate)] and iMSUD-HTx mice [72.9% +/- 4.0% SEM (n=4 pools of 2 mice, each analyzed in quadruplicate)]. Control samples contained a mixture of both human (54kD) and mouse (47kD) E2. E2 protein in iMSUD-PBS livers [n=4 pools of 2 mice, each analyzed in quadruplicate (data not shown)] was not statistically different from untreated iMSUD. These data were expected since injection of PBS should have no effect on protein levels. Pooled samples were analyzed since previously executed Western blots of iMSUD liver showed a large degree of variability between individuals (**Figure 8**). Pooling samples theoretically average the variability present in individual samples and should create a more representative example from each group to analyze.

I expected to see the presence of a small amount of mouse E2 protein, derived from donor ROSA26 +/- hepatocytes, in iMSUD-HTx mouse livers. However, only a single band corresponding to transgenic human E2 was discernable by Western blot. This may be due to minimum threshold limits of the assay. For example, anything below a measured 5% change acquired with the Kodak 1D quantification software can be considered background error. Therefore, it was possible E2 protein was increased below this set minimum threshold. Efforts to reveal mouse E2 protein by Western, such as with longer exposure times to X-ray film or loading more protein per lane, resulted in the eclipse of any mouse E2 that may have been present by the high concentration of human E2 protein in iMSUD-HTx samples. Therefore, first subjecting total liver homogenate to an affinity column to remove transgenic c-myc tagged human E2 protein may allow mouse E2 to be visualized. Loading double or triple the collected protein elutant (50 - 75μg) followed by exposure to x-ray film for an extended time may also help to resolve a mouse E2 band in iMSUD-HTx samples.

4.3.6 Confirmation of engrafted donor hepatocytes following HTx

Mouse E2 protein was not detected by Western blot analysis. Therefore, Real Time qPCR was employed to analyze for the presence of mouse E2 RNA in iMSUD-HTx livers. A significant difference between groups was determined by ANOVA [$F(3,20)=9$, $p<0.0001$]. iMSUD-HTx liver samples contained a significantly different, though small, amount of mouse E2 RNA [**Figure 25**, ~2.8% of mouse Control levels, ($p<0.01$)] compared to untreated iMSUD samples. A mean 2.8% of mouse E2/β-actin RNA in iMSUD-HTx livers would suggest a ~2.8% repopulation by HTx. However an increase of ~8% in BCKDH enzyme activity was determined over iMSUD-PBS mice (**Figure 24A**). These data would suggest each engrafted ROSA26 +/-

mouse cell was contributing to more enzyme activity than it would under normal conditions, an occurrence also described in Muraca et al. (2002b), a case of human HTx to treat glycogen storage disease type 1a. Stimulation by various hormones, or the presence of high protein, has also been reported to increase BCKDH activity (Chicco et al., 1994; Eisenstein et al., 1979). Human liver RNA samples, as well as untreated mouse iMSUD (**Figure 25**) and iMSUD-PBS liver samples (data not shown) all had undetectable levels of mouse E2 confirming SYBR Green primer specificity to mouse RNA. In addition, a comparison of WT controls determined no significant difference between ROSA26 +/- and Control mouse E2/β-actin RNA levels (data not shown).

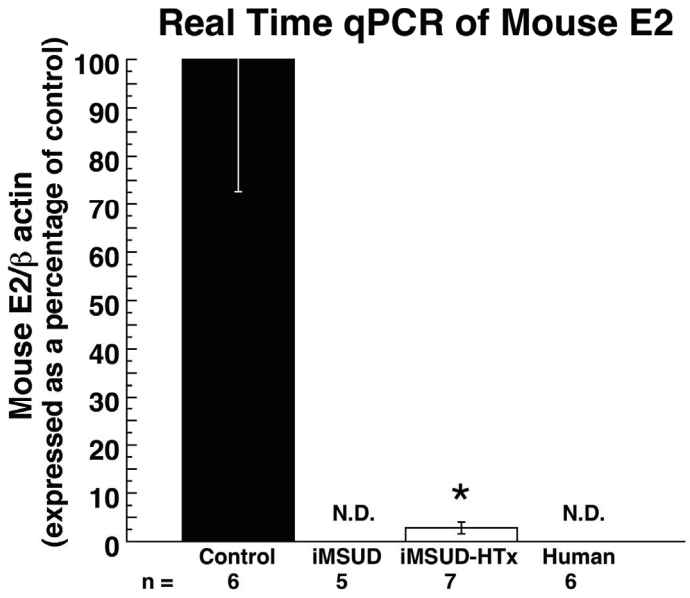

Figure 25. Real Time qPCR of iMSUD treatment groups and controls.
qRTPCR SYBR Green primers spanned mouse E2 exons 4/5, and were contained within the E2 deletion used to create cMSUD mice (Figure 18). Mouse E2 RNA levels were normalized to mouse β-actin. Quantified E2/β-actin RNA from Controls (WT), iMSUD-HTx, untreated iMSUD (contains only transgenic human E2), and human hepatocyte samples were normalized to Control. [*, significantly different from iMSUD and human ($p<0.01$) and Control ($p<0.0001$)]. N.D. = RNA levels were not detectable confirming primer specificity to mouse E2.

As a secondary method to verify ROSA26 +/- donor cell engraftment in iMSUD livers, a standard PCR using DNA isolated from liver was performed using Wildtype (WT, 374bp band) and LacZ (~500bp band) specific primers. WT primers served as a control to verify the quality of the DNA; all analyzed DNA samples displayed a band from WT primers. Control lanes produced the expected results: ROSA 26 +/- (R, **Figure 26)** exhibited a band at ~500bp indicating the presence of the LacZ gene, while LacZ negative liver DNA samples [Control (C), untreated iMSUD (U), and iMSUD-PBS (P); **Figure 26**] did not exhibit a LacZ specific band. All but one iMSUD-HTx DNA sample displayed a fainter but apparent LacZ specific band at ~500bp, which verified the presence of ROSA26 +/- cells in HTx livers in 6 of 7 DNA samples. The LacZ negative iMSUD-HTx DNA sample corresponded to the same sample determined to contain a markedly lower level of mouse E2/β-actin RNA by qRTPCR compared to other HTx samples. Therefore, it was likely ROSA26 +/- DNA present in that sample was below the level of detection for the assay.

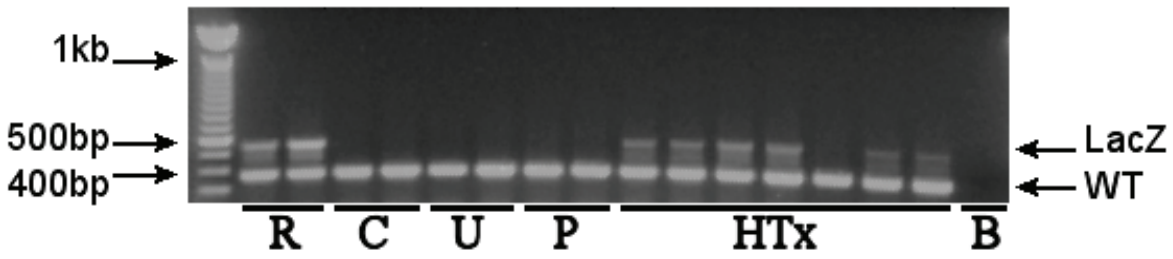

Figure 26. PCR of HTx and control DNA for ROSA26 LacZ gene.
Rosa26 +/- **(R)**, WT control **(C)**, untreated iMSUD **(U)**, iMSUD-PBS **(P)**, and iMSUD-HTx **(HTx)** DNA (20ng/reaction) from livers, and a Blank no DNA control **(B)**, were analyzed. PCR primers specific for WT (~374bp) and LacZ (~500bp) were used. WT primers amplified a common DNA segment and were used as a control for DNA quality. LacZ primers were able to amplify the LacZ gene in 6/7 iMSUD-HTx DNA samples verifying the presence of the gene. The 1 unable to produce a band corresponded to a sample determined to have much lower RNA by qRTPCR compared to other iMSUD-HTx samples. LacZ DNA from ROSA26 +/- was therefore most likely below threshold level of the assay. Band size was determined with the 100bp Ladder.

4.3.7 HTx effects on brain AA, neurotransmitters, and monoamines

To complement the blood spot and total serum amino acid levels, brain amino acids were also analyzed by IEC. Amino acid data obtained from brain suggested HTx was not having any effect at the time of sacrifice (~35 days of age), which supported total serum data obtained by the same methodology (**Figure 23**). Leucine, isoleucine, and valine were all significantly elevated in iMSUD-HTx brains while alanine, synthesized from either BCAA's or pyruvate and important to the Krebs cycle, was reduced compared to controls (**Figure 27A**). This produced a significant elevation in the brain BCAA/ala ratio in iMSUD-HTx mice, though BCAA/ala and individual leucine, isoleucine, and alanine values were not significantly different from untreated iMSUD. Of the dysregulated amino acids outlined in **Figure 27B**, aspartate (a metabolite of the Krebs Cycle which is able to interact with excitatory neurotransmitter receptors in the brain, and is a precursor of isoleucine), glutamine (a precursor of the inhibitory and excitatory neurotransmitters GABA and glutamate, respectively), and serine (a precursor of serotonin and glycine) concentrations in iMSUD-HTx brains were reduced compared to Controls. However, concentrations of these amino acids were unchanged relative to untreated iMSUD. Concentration of glycine (an inhibitory neurotransmitter in the CNS) and taurine (an amino acid able to interact with neuroinhibitory transmitter receptors) were both elevated in iMSUD-HTx brains compared to controls, however neither were significantly different relative to untreated iMSUD values. Brain phosphethanolamine, a precursor of the neurotransmitter acetylcholine, was also unchanged in iMSUD-HTx mice compared to untreated iMSUD animals. However, alloisoleucine (**Figure 27C**), a stereoisomer of isoleucine purported to be the most specific and sensitive diagnostic marker for all MSUD variants (Schadewaldt et al., 1999), and ornithine, a

metabolite important to the function of the urea cycle, were significantly reduced in HTx brains compared to untreated iMSUD.

Taken together, cumulative amino acid data shown in **Figure 27** suggested globally altered brain bioenergetics; disrupted energy metabolism was previously suggested to be secondary to BCAA and BCKA accumulation in the brain (Patel et al., 1973; Yudkoff et al., 2005). As BCAAs elevate, BCKAs, namely KIC, can inhibit pyruvate and α-ketoglutarate dehydrogenases (Patel, 1974; Patel et al., 1973). Despite persistent amino acid abnormalities in iMSUD-HTx brains (**Figure 27A-B**), the observation that ornithine and alloisoleucine were normalized to control levels following HTx (**Figure 27C**) was nonetheless encouraging for the clinical implications of HTx.

IEC analysis of brain homogenate allowed for the discovery of several neurotransmitter and neurotransmitter precursor imbalances (**Figure 27B**). Considered with collaborative data (Zinnanti et al., 2008) revealing additional changes in GABA, dopamine (DA), and tryptophan [a precursor of serotonin (5-HT, 5-hydroxytryptophan)], we wanted to determine if any of these changes were resolved after HTx treatment. For reference, a schematic depicting some of the neurotransmitters and their related precursors and metabolites discussed in this section was shown in **Figure 28A**. HPLC analysis revealed a statistically significant reduction in the neurotransmitters GABA, glutamate, 5-HT, and DA (**Figure 28B**) in untreated iMSUD animals compared to control. Compared to untreated iMSUD mice, a significant change was determined for 5-HT ($p<0.01$) and DA ($p<0.001$) in iMSUD-HTx mice, although 5-HT was not normalized to control levels. In addition, DOPAC (dihydroxyphenylacetic acid, a metabolite of DA) was normalized in iMSUD-HTx mice (**Figure 28C**). Interestingly, the monoamines determined to have been corrected through HTx reside primarily in the synaptic cleft. Tetrahydrobiopterin

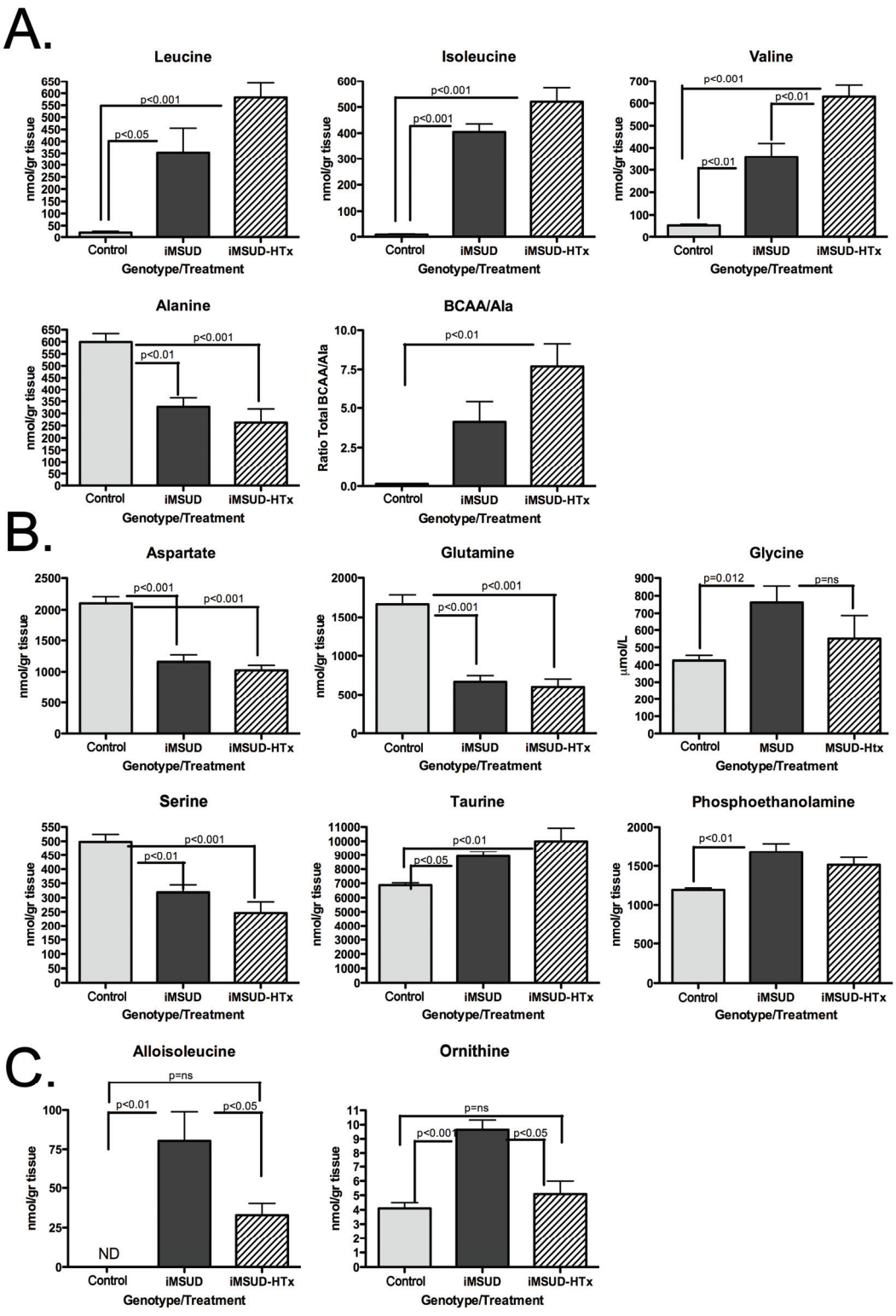

Figure 27. AAs, neurotransmitters, and relevant MSUD markers following HTx in brain.
IEC analysis of Control (WT; n=5, age 35d), iMSUD (n=6, age ~18d) and iMSUD-HTx (n=5, age ~35d) brains. **A.** Changes in BCAA (leu, ile, val) and alanine. All are hallmark aberrations of MSUD. A ratio of BCAA/ala is a sensitive indicator of disease. **B.** Changes in neurotransmitters and neurotransmitter precursors. **C.** Changes in diagnostic markers of MSUD. Analyses were completed by Dr. K.M. Gibson, University of Pittsburgh.

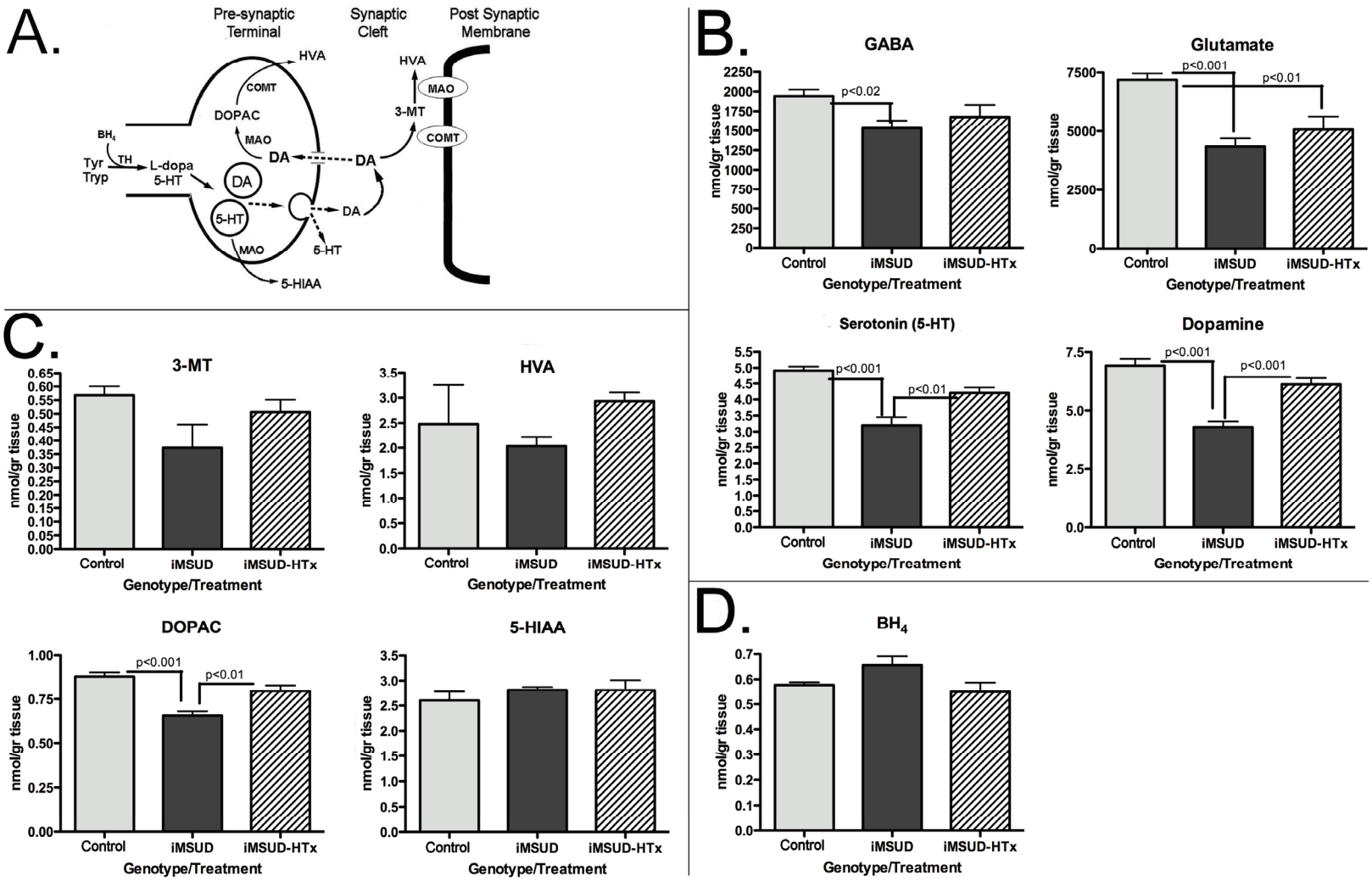

Figure 28. Neurotransmitters and related precursors/metabolites following HTx.
HPLC analysis of Control (WT; n=5, age 35d), iMSUD (n=6, age ~18d) and iMSUD-HTx (n=5, age ~35d) brains. **A.** Diagram displaying the metabolic pathway of the neurotransmitters and their related biochemicals outlined in **B-D**, with regard to the synaptic cleft. **B.** Major brain neurotransmitters differed between groups. **C.** Changes in metabolites of dopamine and serotonin. Only DOPAC levels were significantly different between groups. **D.** There was no difference in BH_4 levels, a cofactor required for the production of DA and 5-HT. Analyses were completed by Dr. T. Bottiglieri, Baylor College Medicine, Houston, TX.

(BH_4, a cofactor required for the production of both DA and 5-HT), 3-MT (3-methoxytyramine, a metabolite of DA), HVA (homovanillic acid, a metabolite of DA), and 5-HIAA (5-hydroxyindoleacetic acid, a metabolite of 5-HT) were all not significantly changed between treatment groups (**Figure 28C-D**).

4.3.8 HTx increases iMSUD weight at weaning and survival

Following HTx, animals were monitored closely for changes in health. A statistical difference between groups was determined by ANOVA [$F(2,28)=59$, $p<0.0001$]. Body weight of iMSUD-HTx animals were significantly increased compared to iMSUD-PBS ($p<0.0001$) but were significantly less than controls ($p<0.001$; **Figure 29A**). It should be noted that at the time of weaning (~21 days of age) PBS-treated iMSUD disease models were typically very thin and sickly-looking with unkempt fur; mice sat hunched and unmoving. All iMSUD-PBS animals became moribund at or shortly after weaning and were humanely sacrificed (**Figure 29B**). HTx-treated animals behaved similarly to healthy control animals actively exploring with bright eyes and a smooth coat.

Survival of HTx animals [70% survival at 37 days (n = 7/10)] was significantly improved over PBS treated animals [0% survival at 37 days (n = 0/9); $p<0.0001$] and untreated animals [0% survival at 37 days (n = 0/9); $p<0.0001$, **Figure 29B**]. All PBS and untreated animals had died or were found moribund and humanely sacrificed on or before day 24. There was no statistical difference in survival between untreated and PBS-treated iMSUD animals.

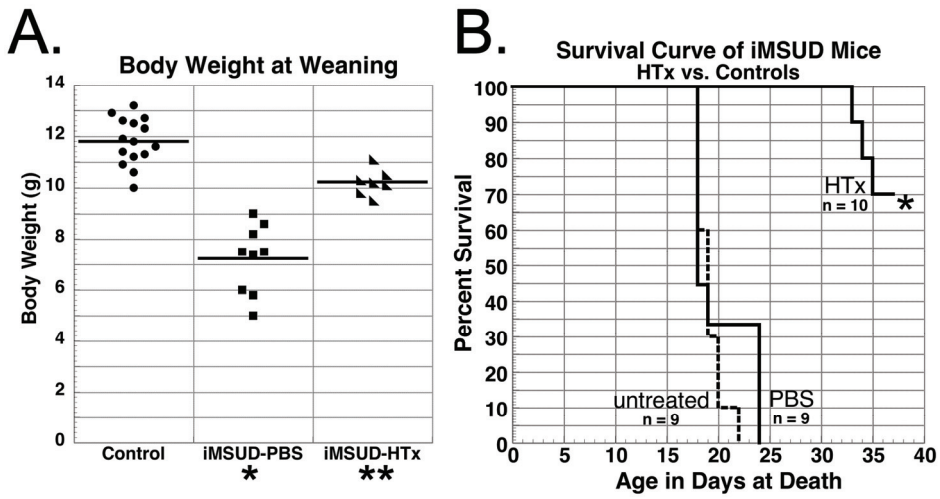

Figure 29. Effect of HTx on body weight and survival.
A. Body weight at weaning of Control (WT; n=15), iMSUD-PBS (n=9) and iMSUD-HTx (n=7) was assessed. [*, ($p<0.0001$) compared to iMSUD-HTx and Control; **, ($p<0.001$) compared to Control]. **B.** Survival was compared between iMSUD-PBS (PBS), iMSUD (untreated), and iMSUD-HTx (HTx) mice. PBS animals (n=9) all died by day 24. Untreated animals (n=9) all died by day 22. HTx animals (n=10) displayed 70% survival at 37 days, which was significantly different from untreated and PBS (*, $p<0.0001$). There was no difference in survival between untreated and PBS-treated animals.

4.4 CONCLUSIONS AND FUTURE DIRECTIONS

I hypothesized that liver-directed HTx therapy would correct the E2 deficiency, restore liver BCKDH activity, and correct the disease phenotype in the iMSUD mouse model. Excitingly, analysis at weaning (21 days, n = 9) revealed a dramatic 75% reduction in BCAA/ala levels compared to untreated and PBS-treated iMSUD controls. BCKDH enzyme activity in iMSUD-HTx animals was increased ~8% over untreated iMSUD and iMSUD-PBS controls, though an increase in mouse E2 protein could not be detected by Western blot analysis. Real Time qPCR determined mouse E2 RNA to be present at 2.8% of control levels in iMSUD-HTx liver samples suggesting a 2.8% liver engraftment. However, since enzyme activity increased ~8% over disease controls, this suggested each engrafted cell contributed more activity than it would under normal circumstances. This phenomenon was documented in a case of human HTx

in 2002 (Muraca et al., 2002b). PCR was performed as a secondary method to confirm ROSA26 +/- hepatocyte engraftment, and LacZ specific bands were detected in 6 of 7 samples. The negative sample corresponded to one with significantly lower mouse E2 RNA as determined by qRTPCR, and therefore the negative result by PCR was most likely due to limits of the assay. Brain analysis revealed an increase in BCAAs and a decrease in alanine compared to controls. The neurotransmitter alterations (Glu, Gly, 5-HT, GABA, DA) and neurotransmitter precursor alterations observed in brain mirrored imbalances in the human disorder (McKenna et al., 1998; Wajner et al., 2000). Following HTx, DA and 5-HT, along with a related metabolite (DOPAC), were corrected to control levels. Interestingly, all of these reside primarily in the synaptic cleft. Finally, and perhaps most importantly, body weight at weaning, a sensitive measure of health, and survival were significantly improved in iMSUD mice that received HTx. In conclusion, these findings of partial metabolic correction of iMSUD in a mouse model by HTx were very encouraging. HTx has already been used clinically to treat metabolic diseases as an alternate treatment to OLT, and multiple manipulations are possible to further enhance engraftment with HTx (Fisher and Strom, 2006; Strom et al., 2006a). Therefore, we suggest HTx represents a promising therapeutic intervention for human MSUD which should be investigated further.

HTx at birth was determined to be the most beneficial timepoint for treatment of iMSUD mice for several reasons. Although iMSUD mice were indistinguishable from healthy littermates at birth (Homanics et al., 2006), by weaning (~21 days of age) disease models have become sickly and fragile. Weanling iMSUD mice were significantly smaller than healthy littermates (**Figure 7**) and had high BCAA/ala serum and brain levels accompanied by neurological dysfunction (Homanics et al., 2006; Zinnanti et al., 2008). Seizures were also observed in disease models at this age, which were often triggered by loud noise or excess handling

presumably from an increase in animal stress, which occasionally resulted in death (unpublished observations). Stress is also a documented trigger for BCAA crisis in human MSUD patients (Chuang and Shih, 2001). If treatment was delayed until weaning, when mice would be large enough to undergo surgery to transplant hepatocytes into the spleen, the neurological damage HTx was meant to prevent may already be irreversible. It was hypothesized that the reduced metabolic function and excess BCAAs/BCKAs present in the iMSUD mouse model would provide a sufficient growth advantage for transplanted cells over endogenous hepatocytes. Therefore toxic chemical treatments, such as retrorsine and CCl_4, which would likely negatively affect survival after treatment, were not used for these first experiments. Once HTx significantly improved disease phenotype and survival beyond weaning age, additional splenic HTx injections would be possible.

Plans for the future include a more aggressive HTx treatment of iMSUD mice. HTx injections would begin at birth, as described previously in this section, but donor cells per injection would be increased. In this study approximately 1% of newborn liver mass ($1x10^5$) was directly injected into the liver parenchyma. However human clinical cases of HTx have determined that a much larger percentage of liver mass (up to 5%) can be tolerated without incident (Dhawan et al., 2004). HTx was able to significantly extend iMSUD lifespan and improve health past weaning; therefore additional HTx injections would be given around that time (~21 days of age) into the spleen to attempt to more completely correct disease phenotype and improve survival out several months. Numerous studies in animal models have indicated that hepatocytes transplanted into the spleen or the portal vein display normal hepatic function and can survive for the life-time of the recipient (Gupta et al., 1991; Holzman et al., 1993; Mito et al., 1979; Ponder et al., 1991). It has also been demonstrated that within two minutes of

injection, ~55% of cells migrate via the splenic and portal vein into the liver (Ponder et al., 1991), which is presumably much more accurate than a direct injection into liver through the abdomen.

Selective regeneration pressure may also give transplanted hepatocytes a greater growth advantage. For example, fetal hepatocytes have been shown to repopulate the liver ~15-25% without selective regeneration pressure (Lilja et al., 1997; Nierhoff et al., 2005) and upwards of 80% with selective regeneration pressure (Cantz et al., 2003; Nierhoff et al., 2005). Therefore, preconditioning with retrorsine and CCl_4 treatments are possible (Gupta et al., 1999; Laconi et al., 1998), as well as partial hepatectomy to stimulate liver repopulation. Finally, the presence of immune response markers following HTx would be investigated since some results discussed previously (IEC serum and brain amino acid data) suggested that cell rejection may be occurring. Therefore, immunosuppression would be administered if needed to prevent cell rejection.

5.0 EMBRYONIC STEM CELL (ESC) THERAPY

Hypothesis: I hypothesize liver-directed differentiated embryonic stem cell (ESC) therapy can correct E2 deficiency, restore liver BCKDH activity, and ultimately correct the disease phenotype of the iMSUD mouse model.

5.1 BACKGROUND AND SIGNIFICANCE

ESC therapies to treat disease are fairly new advances that have generated a great deal of attention in the scientific community. ESCs are derived from early embryos, can be maintained and expanded *in vitro* indefinitely, are pluripotent, and hold great therapeutic promise (Trounson, 2005). Recently, proof of principal for using ESCs to correct an inherited liver defect, Factor IX (FIX), was reported (Fair et al., 2005). To date, differentiated ESCs have also been transplanted into mice to correct induced hepatic failure (Hu et al., 2006; Ishii et al., 2007; Soto-Gutiérrez et al., 2006; Tabei et al., 2005) and a fumaryl acetoacetate deficiency (Sharma et al., 2008).

During development, it has been suggested that the liver develops from the ventral foregut endoderm through fibroblast growth factor (FGF) and bone morphogenic protein signals expressed from cardiac mesoderm (Ang et al., 1993). Fair, et al. (2003) determined that murine ESCs stimulated *in vitro* with chick cardiac mesoderm differentiated into cells with a putative endodermal phenotype. Fair et al. (2005) later determined that murine ESC stimulated *in vitro*

with FGF caused differentiation into putative endodermal precursor cells (PEPs), which engrafted in the liver following direct injection into liver parenchyma. These cells functioned normally *in vivo* and cured a FIX deficiency in a mouse model (Fair et al., 2005).

Although several problems are associated with the use of ESCs for cellular therapies, the most serious being lack of transplant function, homograft rejection, and teratoma formation, Fair et al. (2005) overcame two of these three problems: Following liver injection of non-syngenic PEPs, no evidence of rejection or antibody response was detected while transplant function was high enough to correct the disease phenotype. In addition, teratoma rate was very low, ~6.2% (Fair et al., 2005). However, strain 129 mice, the source of ESCs used by this group, are notorious for a high spontaneous teratoma rate relative to other laboratory mouse strains (Fair et al., 2005; Stevens, 1973). Therefore, C57BL/6J derived ESCs may reduce teratoma rate to negligible levels, which formed the rationale behind using ESCs derived from both 129/Sv and C57BL/6J mice for the studies presented in this section.

In summary, ESC therapy is logical and appropriate for use with the novel iMSUD model to increase liver BCKDH activity and effectively correct MSUD. Proof of principle experiments, described previously in Section 4.0, determined that two injections of $1x10^5$ healthy donor hepatocytes given at birth were able to increase enzyme activity ~8% over untreated iMSUD controls (**Figure 23A**) resulting in partial metabolic correction of the disease phenotype. Differentiation of ESCs into liver-like cells has the potential to work equally well. The use of differentiated ESCs for transplantation could potentially reduce, or perhaps eliminate, the need for precious donor livers for cellular transplantation. As a first step toward using differentiated ESCs to correct MSUD, PEPs were first characterized *in vitro*, and wildtype mice were used to determine the engraftment efficiency of transplanted cells *in vivo*.

5.2 METHODS

All studies involving animals were reviewed and approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

5.2.1 Mouse colony breeding

Production of iMSUD and control mice was carried out as previously described in Methods Section 2.2.

5.2.2 Southern blot genotyping

Genotyping by Southern blot analysis was completed as previously described in Methods Section 2.2.

5.2.3 Differentiation of ESC

R1 ESCs, which were derived from the 129/Sv mouse strain (Nagy et al., 1993), or C57BL/6 mouse strain-derived ESCs expressing green fluorescent protein [GFP; both generous gifts from Dr. Khillan, University of Pittsburgh, Pittsburgh, PA)] were cultured on p100 plates (BD Falcon, Franklin Lakes, NJ) with a mouse embryonic fibroblast (MEF) feeder layer. Cells were cultured in "R1 Media," which is DMEM media (Gibco, Grand Island, NY) supplemented with 0.3% leukemia inhibitory factor (LIF; Gibco), 15% fetal bovine serum (FBS; Gibco), 1% L-glutamine (Chemicon International, Temecula, CA), and 4μL β-mercaptoethanol (Sigma-

Aldrich, St. Louis, MO)/500mL media] until ESC colonies reached ~75% confluency. ESCs were then trypsinized, centrifuged briefly, and the media removed. Then ~5mL "MEF media" (R1 Media without the addition of LIF) was added, the cells vortexed briefly, and incubated at room temperature for 10 minutes to allow the MEF cells to settle to the bottom. The top 1-2mL of media containing ESCs were then cultured on a 0.05 mg/mL rat tail collagen-coated 6-well plate (BD Falcon) and differentiation was induced *in vitro* with 100μg/mL FGF (Sigma-Aldrich, St. Louis, MO) or 100μg/mL FGF + 100μg/mL Activin A (Sigma-Aldrich) in MEF Media for 0, 3, 6, 7, 9, or 12 days. PEPs were then either lysed with RadioImmuno Precipitation Assay Lysis and Extraction Buffer (Pierce, Rockford, IL) for Western blot analysis or harvested for differentiated ESC therapy.

5.2.4 Differentiated ESC therapy

Adult WT iMSUD mice (6-8 weeks of age) were anesthetized with 100mg/kg ketamine (Fort Dodge Animal Health, Ft. Dodge, IA) + 10mg/kg xylazene (Vedco, St. Joseph, MO) and the spleen or liver exposed by laparotomy. Mouse ESCs were stimulated *in vitro* with 100μg/mL FGF or 100μg/mL FGF + 100μg/mL Activin A as described above for 7 days to create PEPs. A single injection of PEPs was administered directly into the spleen or liver parenchyma (~$1x10^5$ cells/50μL PBS) and the incision was sutured and stapled. Mock-treated control animals were subjected to the same surgical procedure and injected with sterile PBS. ESC therapy was administered at 28 days of age. Mice were sacrificed at 3 hours or 1 month and the livers analyzed.

5.2.5 Western blot analysis

Protein isolated from PEP and mouse livers were analyzed as previously described in Methods Section 2.2. In addition to what was previously described, blots were individually probed with several additional primary antibodies: rabbit anti-α1-antitrypsin (#sc-30121; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-albumin (#ab19196; AbCam, Cambridge, MA), goat anti-α-fetoprotein (#sc-8108; Santa Cruz Biotechnology), and rabbit anti-OCT4 (#AB3209; Chemicon International, Temecula, CA). Corresponding commercially available secondary antibodies conjugated to HRP [goat anti-rabbit-HRP (#NB730H; Novus Biologicals, Littleton, CO) and donkey anti-goat-HRP (#NB7357, Novus Biologicals)] was used for detection via the Western Lightning chemiluminescence kit.

5.2.6 Immunohistochemistry

Following PEP transplant, livers from WT mice were fixed in 4% paraformaldehyde overnight for no more than 24 hours. Tissue was then incubated in 30% sucrose for at least 24 hours followed by cryopreservation in Optimum Cutting Temperature Compound (OCT; Tissue-Tek), and sectioned at 30μ using a Leica CM1850 cryostat. Tissue sections were mounted on slides, blocked in 10% normal goat serum (NGS) in PBS, and treated with an Avidin Biotin Blocking Kit (Vector Labs, Burlingame, CA). This step was necessary since anti-biotin was reported to be a highly specific detection method for mitochondria, which results in high background staining when using a 3,3-diamino-benzidine tetrahydrochloride (DAB) detection method (Hollinshead et al., 1997). Sections were then incubated with a polyclonal rabbit anti-GFP primary antibody (#NB600-303, Novus Biologicals, Littleton, CO) overnight at 4^{o}C in a

humidified chamber and later incubated in a goat-anti-rabbit biotin-congugated secondary antibody (#NB730B; Novus Biologicals). Sections were then treated with Avidin Biotin Complex (Vector Labs, Burlingame, CA) for 30 minutes followed by DAB (Sigma-Aldrich) for 10 minutes. Sections were then subjected to a series of dehydrating ethanol and xylene washes and coverslipped with Permount Mounting Medium (Fisher Scientific, Pittsburgh, PA). Tissue was visualized with an Olympus Ix71 inverted microscope.

5.2.7 BCKDH complex enzyme activity assay

In addition to determination of BCKDH activity from liver tissue as described previously in Methods Section 2.2, ESCs and PEPs were also analyzed. Following differentiation for 12 days with 100μg/mL of FGF and PEP formation, cells were collected from a p100 plate (BD Falcon), spun down and the media removed. Cells were then homogenized and further prepared for the BCKDH enzyme assay as previously described in Methods Section 2.2.

5.3 RESULTS AND DISCUSSION

5.3.1 Verification of GPF expression in ESCs

Prior to any cellular manipulations, R1-GPF and C57BL/6-GFP ESCs were first verified to be intense expressers of GFP by visualization by fluorescence microscopy. This step was important since GFP expression was used as a marker to identify cells once they were injected into mouse liver. GFP expression was determined microscopically to be present in 100% of

ESCs of both cell lines (**Figure 30**). Expression was not limited to any specific organelle, such as the nucleus, but instead present throughout the cell. This suggested that GFP was translocated to the cytoplasm upon translation.

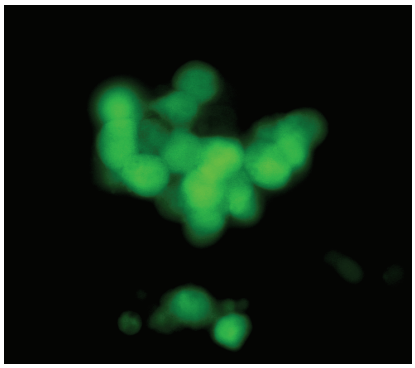

Figure 30. R1-GFP ESC prior to differentiation.
R1-GFP embryonic stem cells were visualized under a fluorescence microscope to verify GPF expression. Expression was present in 100% of R1-GPF cells. Intensity was strong and appeared to be present throughout the entire cell, most likely localized to the cytoplasm.

5.3.2 Differentiated ESCs expressed liver-specific markers

There was no determinable difference between the two tested differentiation protocols (FGF or FGF + activin A), or between mouse ESC lines (R1-GFP or C57BL/6); therefore, R1-GFP ESCs differentiated with FGF will be focused upon in this section (**Figure 31**). Albumin, a hepatocyte specific marker, was abundantly expressed in liver tissue controls as expected, and an apparent band was detected in ESCs following the first timepoint (3 days) of FGF differentiation. Albumin levels appeared to remain constant with longer differentiation timepoints. α-fetoprotein (AFP), a marker of immature hepatocytes, was robustly expressed in day 1 mouse liver tissue and was undetectable in 20 and 56 day old livers. AFP was also detectable at a high level in R1-GFP ESCs after only 3 days in differentiation media and appeared to slightly

decrease with longer differentiation timepoints. α-1-antitrypsin (AAT), a marker of mature hepatocytes, was not detectable in mouse liver tissue at any age for unknown reasons. AAT should be detectable at all liver tissue ages analyzed since expression initiates in the fetal liver and is therefore present before birth (Dr. S. Strom, personal communciation July 2008, University of Pittsburgh). However, AAT was strongly expressed in ESCs differentiated at all timepoints in FGF. As determined previously, E2 was strongly expressed in all analyzed mouse livers regardless of age. E2 was also strongly expressed in undifferentiated R1-GFP (**Figure 31**) ESCs. BCKDH subunits are normally localized to the mitochondria and are therefore present in all cells containing mitochondria, so this finding was not surprising. Upon initiation of differentiation, E2 protein levels appeared to decrease slightly at all timepoints compared to Day 0. However, GAPDH (loading control) protein also appeared to decrease slightly upon initiation of differentiation (**Figure 31**). Therefore, the observed change is likely due to a difference in protein loading and not an effect of differentiation. Oct4, a specific marker of undifferentiated ESCs, was present at a low level in Day 0 ESCs collected prior to the addition of differentiation media, and was very faintly detectable in subsequent timepoints after differentiation. This suggested that some cells were still undifferentiated even after 12 days of FGF stimulation. Therefore, prior to transplantation of PEPs into a recipient, a cell sorting step to remove undifferentiated cells may be beneficial to reduce the likelihood of teratoma (Damjanov and Solter, 1974; Sherman and Solter, 1975). The observed difference in GAPDH protein level between ESCs and liver tissue was due to the difference in cell type, not due to a mistake in protein loading.

ESC lines were differentiated by two different protocols in an attempt to determine the more efficient method to produce further differentiated hepatocyte-like cells. One differentiation

protocol used was 100μg/mL of FGF supplemented in culture media, which was used previously to differentiate PEPs from mouse ESCs by Fair et al. (2005). FGF is required to initiate early embryonic liver development (Fair et al., 2005; Jung et al., 1999). The second differentiation protocol used consisted of 100μg/mL FGF used in conjunction with 100μg/mL activin A supplemented in culture media. Activins are members of the transforming group beta (TGβ) superfamily of proteins, and activin A, a dimer of two TGβA subunits, is vitally involved in apoptosis, cell growth, and tissue architecture in the healthy liver (Deli et al., 2008). Activin A was used previously to enhance stimulation of ESCs along a hepatocyte lineage (Hay et al., 2008; Kubo et al., 2004). In a recent publication in which several different growth factors to induce hepatic differentiation in human ESCs were compared, ESC culture in the presence of activin A produced the greatest number of AFP-producing cells after treatment on both collagen and matrigel coated plates (Ishii et al., 2008).

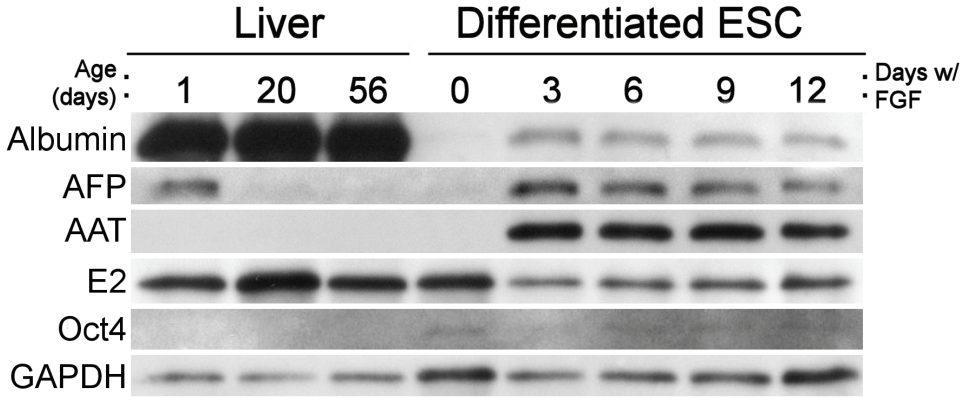

Figure 31. R1-GFP ESC following differentiation with FGF alone.
Western blot analysis of **LEFT**: livers from 1, 20, and 56 day old control mice and, **RIGHT**: R1-GFP ESCs prior to addition of differentiation media (day 0), and R1-GFP ESCs differentiated with 100μg/mL FGF for 3, 6, 9, and 12 days. Samples were analyzed for hepatocyte markers (albumin, AFP, AAT), BCKDH E2, an undifferentiated ESC marker (Oct4), and a loading control (GAPDH). Liver markers in differentiated ESCs were apparent after 3 days in culture. Oct4 expression in differentiated ESCs indicated some cells remained undifferentiated even after 12 days in differentiation media. 25μg of protein was loaded/lane.

Although both mouse ESC lines differentiated equally well by both differentiation methods as determined by Western blot, variations in cell growth was evident, particularly after addition of differentiation media. In standard R1 media, cellular growth of C57BL/6-GFP cells was much slower than R1-GFP cells typically requiring an extra 3-5 days to arrive at confluency. ESCs from either line were able to form healthy colonies and survive standard cell culture manipulations (trypsinization, media washes, plate splitting) without issue. Upon addition of differentiation media, no change in R1-GFP cell growth was detected. However, C57BL/6-GFP cell growth decreased dramatically upon initiation of differentiation; a majority of cells were dead after only 1 day of exposure to differentiation media. Collection of sufficient differentiated C57BL/6-GFP cells for protein and enzymatic analysis for all timepoints was therefore difficult. Also, since >90% of C57BL/6-GFP cells died during the differentiation process, acquiring enough cells for multiple mouse transplantations would require a greater number of cells to start with and thus a much larger volume of differentiation media. This was a problem due to the high economic cost associated with both FGF and activin A. For these reasons, R1-GFP ESCs were used for all subsequent experiments. In addition, due to equal success in producing PEPs using either differentiation protocol, FGF differentiation media without activin A was used for differentiation in all subsequent experiments.

5.3.3 Differentiated ESC BCKDH activity

Expression of hepatocyte protein markers in PEPs suggested that differentiation by FGF was able to stimulate ESCs along a hepatocyte lineage (**Figure 31**). Therefore, R1-GFP derived PEP BCKDH enzyme activity was analyzed and compared to mouse liver tissue and undifferentiated ESCs.

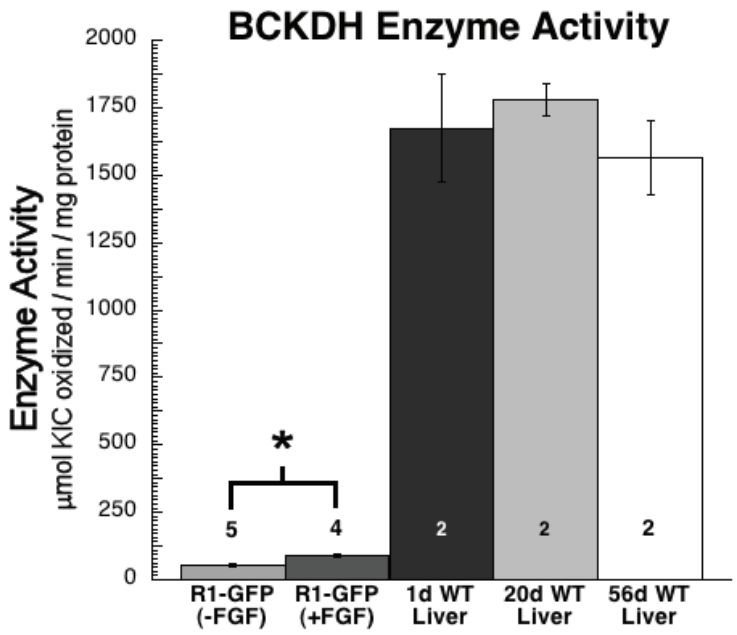

Figure 32. BCKDH enzyme assay of R1-GFP ESC differentiated for 12 days.
BCKDH enzyme activity assay displaying undifferentiated R1-GFP ESC [R1-GFP (-FGF)], differentiated R1-GFP ESC PEPs [R1-GFP (+FGF)], and mouse liver (1, 20, and 56 days of age). R1-GFP (+FGF) was increased significantly over R1-GFP (-PBS); *, $p<0.001$). Control (WT) livers were shown to serve as a reference for normal enzyme activity levels. The numbers either on or above data columns represent the group n value.

BCKDH activity of differentiated R1-GFP (+FGF) ESCs was significantly different compared to undifferentiated R1-GFP (- FGF) controls (**Figure 32**; $p<0.001$). Undifferentiated ESC activity was approximately 3.2% of the mean WT control activity (1, 20, and 56 day livers) while differentiated ESC PEP activity was ~5.4%. There was no significant difference in enzyme activity between 1, 20, and 56 day old mouse livers, and the averaged WT control enzyme activity from all mouse liver ages was similar to values previously determined by this assay. While any additional BCKDH enzyme activity may ameliorate symptoms to some small degree, 5.4% activity is insufficient to correct MSUD in iMSUD mice. iMSUD disease models were determined to have 5-6% of BCKDH activity, which corresponded to the 3-30% range for human iMSUD (Chuang and Shih, 2001; Homanics et al., 2006). An additional 5-6% of activity provided by differentiated ESCs would still be in the enzyme activity range associated with

human iMSUD. However, once engrafted in the mouse liver exposed to endogenous liver-specific cues, PEPs may further differentiate along a hepatocyte lineage (Chinzei et al., 2002) and BCKDH enzyme activity may increase to a more significant level.

5.3.4 PEPs were able to engraft in the wildtype mouse liver

Preliminary experiments transplanting PEPs into WT mice were completed prior to testing the procedure in iMSUD mice. $1x10^5$ PEPs/50μL PBS were injected either directly into the liver parenchyma, as was done previously to correct a FIX deficiency (Fair et al., 2005), or the spleen. The livers were analyzed for the presence of GFP by immunohistochemistry either 3 hours or 1 month post-transplant. Mock-transplanted animals (n=4) injected with 50μL PBS either into the liver or spleen had no DAB positive staining indicating the absence of GFP-positive cells, as expected (**Figure 33A**). This control also confirmed the absence of nonspecific primary or secondary antibody binding to liver tissue. Small colonies of GFP-positive cells directly injected into the spleen (n=3) were detectable 1 month after transplantation (**Figure 33B**, middle and right panels, dark staining). All splenic transplanted mice had small, distinct colonies of engrafted GFP-positive cells, though observed colonies were somewhat rare. Splenic transplantation of hepatocytes in mice was determined through HTx to be a direct avenue to the liver resulting in the migration of ~55% of cells within two minutes of injection (Ponder et al., 1991) and engraftment and survival of cells displayed normal hepatic function for the life-time of the recipient (Gupta et al., 1991; Holzman et al., 1993; Mito et al., 1979; Ponder et al., 1991). As a positive liver transplant control, PEPs were transplanted directly into the liver and analyzed 3 hours later (n=3) to determine whether cells were able to stay in the liver once directly injected as well as test our ability to identify engrafted GFP-positive cells (**Figure 33C**, middle and right

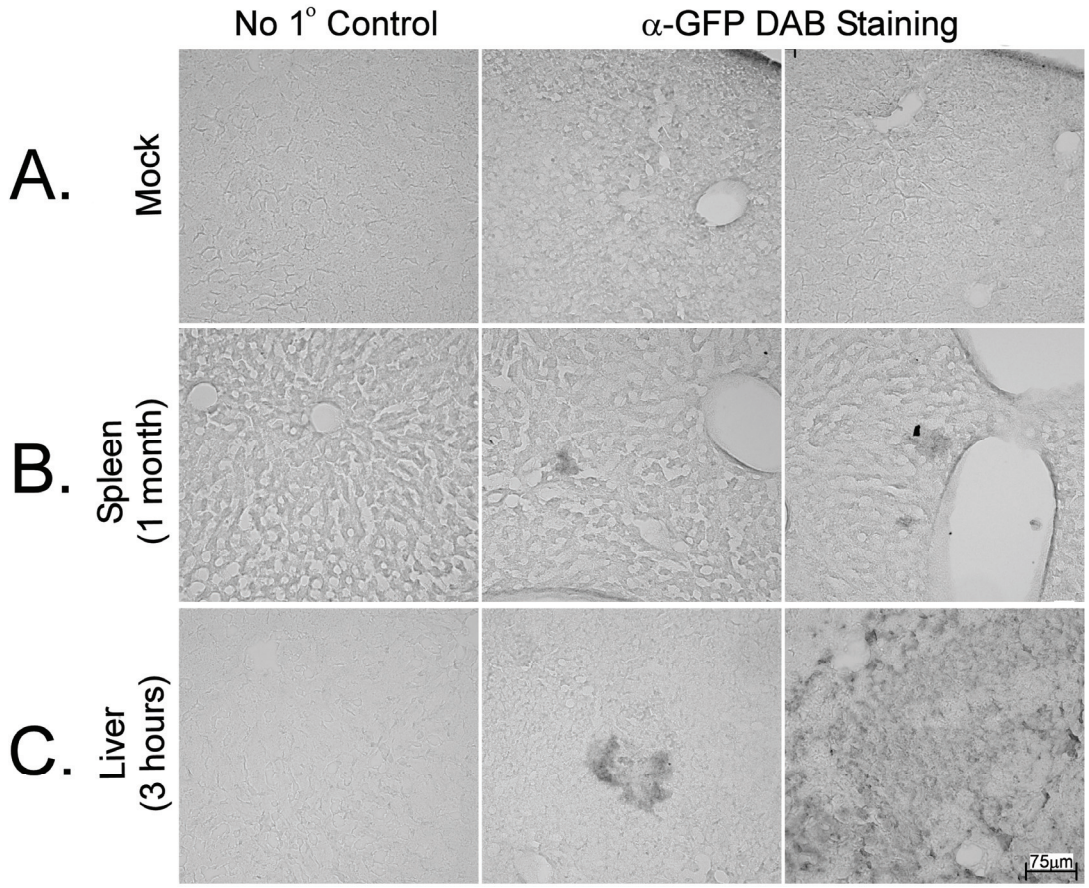

Figure 33. Engrafted differentiated ESC in control mouse liver.
Control (WT) livers either injected with **A.** PBS (mock) or **B.-C.** R1-GPF derived PEPs. PEPs were injected either directly into the **B.** spleen and harvested 1 month later, or **C.** liver and harvested 3 hours later as a positive control. A no primary (1^{o}) antibody control was done for each treatment to assess background staining (left panels, **A-C.**). The dark areas in the middle and right panels in **B-C.** indicate GFP-positive staining by DAB of engrafted PEPs. Tissue section thickness was 30μ, 40x magnification.

panels, dark staining). As expected, large clusters of GPF-positive cells confirmed α-GFP antibody specificity as well as our ability to identify engrafted cells following transplantation.

Experiments to inject PEPs into liver (and analysis at 1 month) and PEP injection into spleen (and analysis at 3 hours) were initiated, but could not be completed due to technical difficulties. Issues encountered involved defective FGF stocks from Sigma-Aldrich, the high cost of replacement FGF, one animal death immediately following PEP injection, and substandard fixation in paraformaldehyde of liver tissue following collection resulting in poor tissue quality for analysis.

5.4 CONCLUSIONS AND FUTURE DIRECTIONS

Based upon previously published data reporting correction of an inherited metabolic liver disorder (Fair et al., 2005), I hypothesized ESCs differentiated into hepatocyte-like PEPs could functionally engraft in the livers of transplanted mice. Using ESCs that expressed a high level of GFP protein, PEP cell liver engraftment was verified up to 1 month later in WT mice (**Figure 33B**) though only small colonies were identified. Injecting a larger volume of cells or multiple injections into the spleen at later timepoints could potentially increase the total number of engrafted cells.

Differentiated ESCs were able to express liver-specific markers after only 3 days in culture (**Figure 31**) and BCKDH activity of R1-GFP derived PEPs was significantly higher than undifferentiated control ESC populations (**Figure 32**). The average activity of PEPs differentiated for 12 days with FGF was just 5.4% of averaged WT mouse liver activity, which was lower than initially hoped. However, it was hypothesized that once engrafted in the liver

and surrounded by endogenous liver cues, average BCKDH activity may increase due to cells further differentiating down a hepatocyte lineage. For example, ESCs differentiated into embryoid bodies *in vitro* formed albumin-producing cells *in vivo* after transplantation into mouse livers (Chinzei et al., 2002).

Fair et al. (2005) noted a small percentage (~6.2%) of teratoma attributed to the use of 129-derived ESC, which were reported to cause a statistically higher incidence of spontaneous teratoma relative to other laboratory mouse lines (Stevens, 1973). Therefore, two ESC mouse lines were used for these experiments, 129 derived R1 cells and C57BL/6 cells. Both R1-GFP and C57BL/6-GFP ESCs were able to express liver markers after only three days in the two tested differentiation mediums (100μg/mL FGF or 100μg/mL FGF + 100μg/mL activin A; **Figure 31**). However, due to increased sentitivity of C57BL/6 ESCs to the differentiation media, which caused high mortality and poor growth, R1-GFP ESCs were used for enzymatic analysis and transplantation studies. Transplantation of undifferentiated ESCs carry the risk of teratoma formation (Chinzei et al., 2002; Choi et al., 2002; Teramoto et al., 2005; Thomson et al., 1998). However, there was no incidence of teratoma in any of the PEP transplanted mice. This was despite Oct4 expression in cells exposed to differentiation media, which suggested not all cells in a PEP population had differentiated even after 12 days in culture (**Figure 32**). If the incidence of teratoma in PEP injected mice becomes an issue in future studies, a previous publication suggested that longer culture times significantly decreased the incidence of teratoma *in vivo* (Chinzei et al., 2002), or a cell sorting step could be completed prior to transplantation to separate out undifferentiated ESCs.

Possible plans for the future involve a larger scale analysis of hepatocyte markers by Real Time qPCR. qRTPCR is a highly sensitive assay which may detect very low levels of liver-

specific gene expression that may otherwise be undetectable by other methods. Also, a larger panel of investigated hepatocyte markers is needed to more specifically establish how well differentiated the ESCs become. In addition to the markers analyzed here (albumin, AAT, AFP), liver markers tested in other publications comparing ESC-to-hepatocyte differentiation methods (Lavon and Benvenisty, 2005) could be analyzed (e.g., FOXA1, FOXA2, CEBPA, CEBPB, HNF4, etc.). Functional hepatocyte-specific assays could also be utilized, such as urea production. Following a more complete characterization of PEP cells, PEP injection into iMSUD mice would also be attempted. PEP therapy would most likely employ the HTx protocol described in Section 4.1 since early treatment of iMSUD disease model mice would be most beneficial. Untreated iMSUD mice are very fragile and unhealthy by weaning age when surgery for CTx would be most feasible, and most do not survive long beyond weaning (**Figure 29B**). CTx at birth, identical to HTx treatment (described in the previous section), has the potential to improve health to the same degree as HTx so that a more aggressive treatment protocol could be established around weaning age. Administration of a larger volume of PEPs could be transplanted into the spleen of iMSUD animals, and multiple injections at later timepoints would be possible. The presence of immune response markers following CTx would also be investigated since they were not examined in the preliminary studies described here. If needed, immunosuppression could be administered to prevent cell rejection. As an alternative to ESCs, human stem cells isolated from human umbilical cord blood could also be transplanted into mice to correct the iMSUD disease phenotype. Cord blood reportedly has less immunogenic issues, less incidence of host rejection, and a reduction in the incidence and severity of graft-vs-host disease (Brown and Boussiotis, 2008).

6.0 DISSERTATION SUMMARY AND CONCLUSIONS

This dissertation focused primarily upon evaluation of new therapeutic approaches to correct MSUD in a novel mouse model of this disease. Although the BCKDH complex, responsible for the permanent degradation of BCKAs in the body, reportedly functions in all tissues containing mitochondria (e.g., heart, skeletal muscle, liver, brain, kidney, etc.), BCKDH in tissues other than liver is largely kept in an inactivated state (Harper et al., 1984). Furthermore, OLT in MSUD patients has demonstrated that restoration of BCKDH in liver alone was sufficient to correct the disease, or at least relieve symptoms to those associated with a very mild phenotype, greatly improving long-term health and survival (Bodner-Leidecker et al., 2000; Netter et al., 1994; Strauss et al., 2006; Wendel et al., 1999). My research focused on alternative liver-directed therapies to provide rationale for their future clinical development and use. The first section of this chapter will address the use of animal models to test novel treatments eventually intended for human use. The similarities between the iMSUD mouse model and the human disease will also be discussed. The second section addresses the problems encountered with AAV gene therapy and the possible causes behind these issues. Intended courses of action to improve gene therapy approaches for MSUD are presented. The third section describes the partial metabolic correction of the iMSUD mouse model observed through HTx therapy. The anticipated potential for differentiated "liver-like" ESCs to provide similar disease protection is also discussed. Possible issues and future studies intended to improve upon these results are

presented. The final section addresses the relevance of this work towards a greater understanding of how MSUD causes disease and the importance of the further development of liver-directed therapies. This may not only benefit MSUD, but other liver-based metabolic diseases as well.

6.1 GENETICALLY ENGINEERED MOUSE MODELS OF DISEASE

Once a patient has succumbed to a disease, the study of cadavers can often be informative. However, cadavers cannot determine what contributed to disease onset or how disease progression occurred. Only the resulting cause of death can be established. Study of a disease in a living being in real time can determine the root cause and thus elucidate a way to prevent or correct the disease. The study of animal models in research has provided invaluable opportunities to determine the pathophysiology associated with a specific disease from animal development *in utero* to death. Once the disease mechanism is identified, novel therapies or drugs can be investigated.

Relevance of an animal model to the human disease is of great concern. Occasionally a spontaneous animal model of a known human disease becomes available, such as the Polled Shorthorn and Polled Hereford bovine models of MSUD (Harper et al., 1989; Harper et al., 1986). However, although homologous genes were affected in both human and cow, the disease pathogenesis and presentation were different. Therefore, bovine MSUD was not an ideal animal model due to dissimilarities between it and the human disease. If a model must be generated, some thought must be put into the genetic design. A genetic knockout may be most ideal in some cases while a knockin may provide additional benefits in others. Genetic knockouts carry

some limitations (i.e., compensation, lethality) since the change is global. Interpretation of data to determine a gene of interest's function may be difficult when results could be due to modification of a gene of interest or compensatory changes in other genes. However, for models of disease, compensation may be an advantage rather than a limitation. Compensatory changes occurring in a disease model are likely also occurring in humans, thereby creating a more relevant model. However, a model's relevance would be lost if compensatory effects are due to genes without a corresponding human ortholog. In cases where early lethality was an issue (i.e., cMSUD), knockin or transgenic animals (i.e., iMSUD) may provide a more useful tool for study.

A specific model's relevance may change depending upon the final objective of the research. Similar genetic disruptions and pathophysiology between an animal model and the corresponding human disease is useful when determination of the mechanism of disease is the goal. However, that same model may not be the most relevant to test novel therapies for later clinical use. For example, mdx mice are one of the most widely used models to mimic Duchenne's muscular dystrophy (DMD) (Bulfield et al., 1984). However, since mouse muscles are so tiny, disease phenotype was very mild despite total dystrophin loss (Vainzof et al., 2008). Therefore mdx mice did not generate reliable data for clinical trials. In this case, a canine model with much larger muscles comprises a better model for therapeutic study. The choice of therapy may also play a role in the relevancy of an animal model. For example, cellular immune responses to AAV capsid proteins were recently acknowledged in clinical trials (Mingozzi and High, 2007). These immune responses did not occur in previous animal studies with the vector (Li et al., 2007). Therefore, due to the presence of a unique cell population in humans not present in mice, mice may not make the most ideal model for AAV pre-clinical trials.

Human and mouse genomes are very similar; over 99% of mouse genes have a corresponding human homologue while 96% of mouse proteins are shared with humans (Okazaki et al., 2002; Venter et al., 2001; Waterston et al., 2002). Mice have been widely accepted to reliably model a multitude of human diseases (Huttenlocher, 2000; Thompson, 2008; Vainzof et al., 2008). Furthermore, a mouse model of iMSUD (Homanics et al., 2006), characterized further in Section 2.0 of this dissertation, has thus far proved to be highly similar compared to the human disease (**Table 9**). iMSUD was modeled through standard genetic engineering techniques to create a deficiency in the BCKDH E2 subunit by mouse E2 knockout and subsequent introduction of transgenic human E2. Mouse and human E2 are 91% homologous at the protein level [NCBI accession # NM 010022 (mouse), # NM 001918 (human)] and were expected to function interchangeably. iMSUD mice exhibited developmental delay, seizures, reduced BCKDH enzyme activity, dysregulated blood and brain amino acids, disrupted brain monoamines, and physical brain abnormalities [KJS dissertation, 2008; (Homanics et al., 2006; Zinnanti et al., 2008)], all consistent with human cases of disease (Chuang and Shih, 2001; Crome et al., 1961; McKenna et al., 1998; Silberman et al., 1961; Wajner et al., 2000; Zinnanti et al., 2008).

Transgenic human E2 resulted in 5-6% of normal BCKDH activity (**Figure 5B**), thus providing a superior disease model of iMSUD. However, despite many similarities to human MSUD, transgenic E2 may be functioning suboptimally *in vivo*. Therefore, testing certain therapies may be restricted in this model due to the possible disruption of proper enzyme formation and function from inclusion of a C-terminal c-myc tag on transgenic E2 [**Figure 4** (Knapp et al., 2000; Mattevi et al., 1992)]. Competition for inclusion into BCKDH could likely occur between transgenic E2 and therapeutic-derived E2 and therefore any complexing of the

two may produce a similar reduction in enzyme activity. In addition, although mouse and human E2 are highly homologous, it is unknown whether human E2 can function optimally with mouse BCKDH subunits. Improper trafficking of E2 to mitochondria may also have been to blame. Therefore, further studies to definitively identify why BCKDH activity was reduced in the iMSUD model are required.

Prior to the creation of the iMSUD model, leucine interaction in the brain was theorized to cause neurological dysfunction; however the precise mechanism was poorly understood (Chuang and Shih, 2001; Wajner et al., 2000). In MSUD, deficient levels of BCKDH increase leucine, isoleucine, and valine in the blood and tissues, including the brain. Leucine was shown to be 30-fold higher in MSUD patients compared to normal levels while isoleucine and valine suffered less dramatic increases (9 and 3 times higher than normal, respectively) (Wajner et al., 2000). In addition, increased leucine concentrations alone corresponded to reduced large neutral amino acids (i.e., BCAAs, tryptophan, tyrosine, phenylalanine, and methionine) in the serum. Recently, through study of the iMSUD animal model, a mechanism for MSUD has been proposed (Zinnanti et al., 2008) based upon these previous publications. Systematic exposure to high and low protein diets and subsequent analysis at various ages confirmed the relationship between high BCAA/BCKA in the brain and neurophysiological abnormalities (Zinnanti et al., 2008). Dietary studies were conducted involving norleucine, an atypical amino acid previously determined to compete at the blood brain barrier for access at the same transporter as leucine (Tews and Harper, 1991). Norleucine was shown to reduce leucine and KIC levels in the brain compared to untreated iMSUD animals (Zinnanti et al., 2008). Disrupted bioenergics were also restored and survival increased in both cMSUD and iMSUD models.

The proposed disease mechanism (**Figure 34**) was originally reported in Zinnanti et al. (2008). Increased leucine competes at the large neutral amino acid transporter with other essential amino acids at the blood brain barrier for entrance into the brain. As a result, reduced large neutral amino acids caused a reduction in neurotransmitters along with related precursors and metabolites (**Figure 34 part 1**). Decreased essential brain amino acids caused a reduction in protein synthesis, dysmyelinization, and growth restriction in the brain. In the astrocytes and neurons, KIC accumulation caused reverse BCAT flux to reproduce leucine (**Figure 34 part 2**). An increase in leucine also increased α-ketoglutarate (αKG), an important intermediate in the Krebs cycle and precursor to glutamate and GABA. As a result, glutamate and aspartate were reduced. Aspartate is metabolized from oxaloacetate, another important intermediate in the Krebs cycle, and is involved in the malate/aspartate shuttle. This shuttle relies on equal exchange of aspartate for glutamate and translocates electrons across the impermeable inner mitochondrial membrane for oxidative phosphorylation and generation of ATP. Increased αKG coupled with decreased glutamate alters ATPase activity inhibiting conversion of lactate to pyruvate, also important to Krebs cycle function (**Figure 34 part 3**). Disrupted energy metabolism was previously suggested to be secondary to BCAA and BCKA accumulation in the brain (Patel et al., 1973; Yudkoff et al., 2005). In support of this, KIC was proposed to inhibit pyruvate dehydrogenase (PDH) and α- ketoglutarate dehydrogenase (αKGDH) in the mitochondria causing Krebs cycle dysfunction and reduced bioenergics. Reduction in required cellular energy and ATPase function caused cell swelling and cerebral edema in iMSUD animals (Zinnanti et al., 2008). Treatment with norleucine competed with leucine at the blood brain barrier, which effectively reduced leucine and KIC levels in the brain and thus diminished

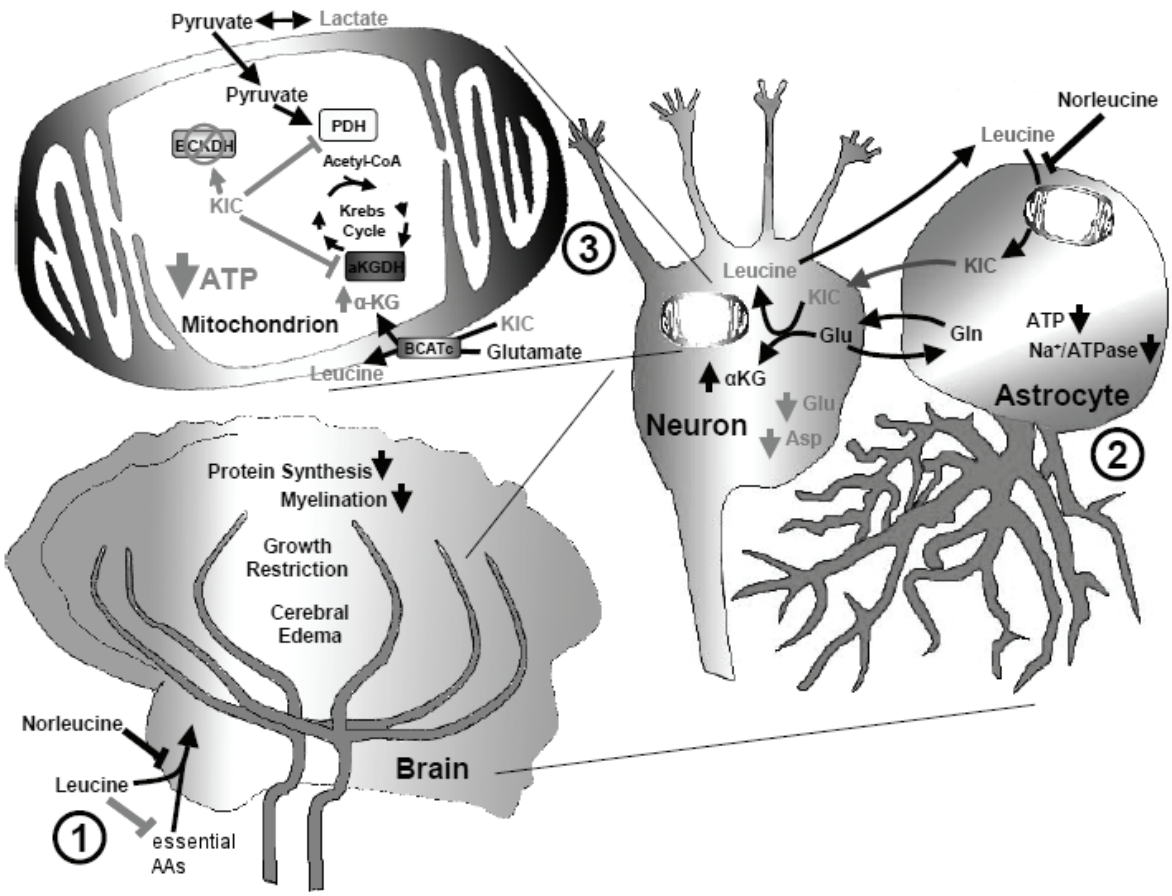

Figure 34. Proposed MSUD disease mechanism causing neuropathology.
Adapted from (Zinnanti et al., 2008). **(1)** During MSUD crisis, increased leucine competes at the blood brain barrier with other essential amino acids, in turn causing reduced precursors for monoamines. Protein synthesis is reduced causing growth restriction of the brain. This, paired with dysregulation of BCAAs, impairs myelination. **(2)** Ketoisocaproate (KIC) accumulation causes reverse BCAT flux to reproduce leucine, which increases α-ketoglutarate (αKG) and reduces glutamate and aspartate. **(3)** Increased αKG and decreased glutamate alters ATPase activity inhibiting conversion of lactate to pyruvate, important to Krebs cycle function. Additionally, KIC inhibits pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (αKGDH) in the mitochondria causing Krebs cycle dysfunction and reduced bioenergics. Reduction in required cellular energy and ATPase function causes cell swelling and cerebral edema. Norleucine competes with leucine at the blood brain barrier reducing leucine and KIC levels in the brain and thus improving dysregulation of bioenergics and amino acids.

dysregulation of amino acids and the Krebs cycle improving body bioenergics. Data reported in Section 4.0 was found to support the proposed model. 5-HT (metabolized from tryptophan), DA (a metabolite of phenylalanine and tyrosine), glutamate, and GABA (synthesized from glutamate) were reduced in iMSUD mice compared to controls (**Figure 28**). Alanine (synthesized from pyruvate), alloisoleucine, ornithine, and aspartate were also reduced (**Figure 27**).

6.2 LIVER-TARGETED AAV GENE THERAPY IN IMSUD MICE

Results reported here suggested that AAV-mediated gene therapy to direct human E2 to the liver was unable to increase BCKDH activity and reduce the MSUD phenotype in a mouse model. This was unexpected considering previous studies targeting liver to cure inherited liver diseases such as hemophilia in humans, mice, and dogs (Manno et al., 2003; Manno et al., 2006; Sarkar et al., 2004; Scallan et al., 2003; Wang et al., 2005a), as well as inborn errors of metabolism in mice (Conlon et al., 2005; Ding et al., 2006; Harding et al., 2006; Mochizuki et al., 2004; Sun et al., 2005a). No discernable difference was determined in survival, E2 protein or BCKDH activity in liver, or BCAA levels in the blood between iMSUD treatment groups (**Figure 15**). At the time of sacrifice, no AAV genomes were identified in any analyzed tissues (**Figure 16**). This may correspond to a loss of AAV concatemers in targeted cells. However, liver cells are largely quiescent with liver turnover occurring on average 1-2 times per year. Therefore, dilution of AAV genomes due to cell division was unlikely. It was more likely our results were due to a low treatment dose, which correlated to approximately 1 v.g./cell.

Administration by IP injection rather than the tail or portal vein may potentially reduce this number even more.

Additional issues with the vector may also have contributed to the failure of these studies. The mouse albumin promoter, although able to direct expression in hepatocytes (Jiang et al., 2001; Koeberl et al., 1999; Su et al., 1996; Xiao et al., 1998a), was determined to be the weakest out of a panel of recently evaluated liver-specific promoters (Al-Dosari et al., 2006). The incorporation of an intron, absent in our therapeutic vectors, was also previously suggested to strengthen AAV expression (Ostedgaard et al., 2005). However, others (Wang et al., 2005b) reported minimal benefits suggesting lack of an intron did not significantly damage our chances for success.

As alluded to above, potential molecular issues concerning the iMSUD model may be interfering with gene therapy function. Competition for inclusion into BCKDH is likely occurring between transgenic E2 and AAV-derived E2. Therefore, if transgenic E2 can disrupt proper enzyme formation and function due to inclusion of a C-terminal c-myc tag (Knapp et al., 2000; Mattevi et al., 1992), transgenic E2 complexed with AAV-derived E2 will likely produce a similar effect. In addition, although mouse and human E2 are highly homologous, it is unknown whether human E2 can function optimally with mouse BCKDH subunits.

With regard to AAV gene therapy approaches in general, recent events may make clinical testing more difficult to approve. A patient in a Phase 1 clinical trial to cure active inflammatory arthritis died on July 24, 2007 (Weiss, 2007). All AAV clinical trials were temporarily halted and a federal investigation was begun (Kaiser, 2007); thus far no link has been found between the viral vector and the recent death. In addition, a known humoral response to AAV exists, which may limit readministration of therapeutic vectors (Hernandez et al., 1999; Moskalenko et

al., 2000). However, this issue was overcome through the use of transient immunosuppression following re-administration of a serotype (Halbert et al., 1997; Hernandez et al., 1999; Manning et al., 1998). More recently, a cellular immune response to AAV capsid proteins reportedly occurred in clinical trials (Mingozzi and High, 2007) which did not exist in previous animal studies (Li et al., 2007). It may be possible to overcome cellular immune responses with immunosuppression use as well.

6.2.1 Future studies

Despite many potential issues, it was likely the therapeutic AAV vectors used here were functional, albeit at a low level. However, before improvements are made to the gene therapy vectors to improve liver targeting and expression, certain questions must first be answered regarding the iMSUD model. Gene therapy, and potentially any therapy that requires the use of endogenous BCKDH cellular components, may not work in this particular model if transgenic c-myc tagged E2 disrupts enzyme function. Therefore, an experiment proposed previously in this dissertation, which would compare human, mouse, and c-myc tagged E2, may establish if the iMSUD model is indeed a relevant choice to test gene therapy. If needed, another model of iMSUD may be made. Engineering a new transgenic mouse model from the current one may take as little as 6 months – 1 year. The cMSUD model may also be used, though establishment of a special diet is needed to keep them alive long enough for the therapy to work. Another mouse model of classic MSUD, in which E1α is disrupted, may also be available shortly (Dr. S. Hutson, Wake Forest University, Winston-Salem, NC; personal communication).

To improve the therapeutic vectors to cure MSUD, a stronger promoter could be used in place of the mouse albumin promoter. For example, the human AAT promoter directed high

expression specifically in hepatocytes (Knapp and Liu, 2004; Nash et al., 2004). Other investigators have incorporated multiple copies of enhancers before a liver-specific promoter to increase expression (Harding et al., 2006; Wang et al., 1999). Pseudotyped AAV2/8 vectors have also demonstrated improved liver targeting and expression over other AAV serotypes (Gao et al., 2002; Harding et al., 2006; Sarkar et al., 2004). Addition of an intron may help strengthen sustained AAV expression *in vivo*; however it must be omitted if space requirements do not allow for its incorporation. A higher dose (10^{12} v.g./animal) paired with hydrodynamic viral administration via the tail vein, which can have a 40% or greater hepatocyte transfection efficiency with a single injection (Liu et al., 1999; Zhu et al., 2006), could also greatly increase transfection efficiency and subsequent gene expression in the iMSUD mouse model.

6.3 LIVER-DIRECTED CELLULAR THERAPIES

Previously discussed molecular issues involving the iMSUD model may limit the therapeutic use of certain genetic cures in this model. However, infusion of whole healthy cells into the liver may bypass these problems. Cellular therapies function independently from dysfunctional endogenous cells and therefore would not interact with the transgenic human E2 present in iMSUD cells. HTx, discussed below, was able to partially correct the metabolic defect in iMSUD mice. ESC therapy may prove to be equally, or perhaps even more, effective.

6.3.1 Hepatocyte transplantation

Data presented here demonstrated that HTx therapy (2x liver injections of $1x10^5$ cells/50μL during the first two weeks of life) was able to improve the MSUD disease phenotype in iMSUD mice. During the course of these experiments, 236 mice were given HTx at birth. Of these animals, 21 (~8%) were found to be iMSUD disease models. Dead pups were very rarely found during daily checks for new litters. However, dead pups may have been removed by animal care or cannibalized by the parents prior to daily checks. In addition, the transgenic A Line of iMSUD mice used for these studies corresponded to a lower survival rate at weaning and beyond compared to the 525A Line used in previous studies (Homanics et al., 2006). The number of live pups was typically recorded between 0-3 days after birth. All pups recorded at that time were still alive at weaning. Untreated iMSUD animals rarely survived any significant length of time beyond weaning. It is unknown why expected Mendelian genetics were skewed. Dead pups or body parts were not genotyped to determine whether those that did not survive were iMSUD animals. It was possible some iMSUD pups died *in utero* and were reabsorbed, though this is unlikely since in the human disease, human babies are protected by the mother until birth (Chuang and Shih, 2001).

Analysis of blood at weaning (~21 days of age) revealed a 75% reduction in BCAA/ala levels compared to untreated and PBS-treated iMSUD controls (**Figure 22**). Body weight at weaning, a sensitive measure of health, was also significantly improved (**Figure 29A**). However, elevated serum and brain BCAAs coupled with reduced alanine at the time of sacrifice (~35 days of age) suggested HTx therapy was not as effective at this later timepoint (**Figures 23 & 27**). Because immunosuppression was not utilized, a loss of donor cell function or death may be the cause. Immunosuppression is typically used to avoid cell rejection in HTx both clinically

(Strom et al., 2006a) and in laboratory animals (Bissiq et al., 2007; Puppi et al., 2008). β-gal expression could also not be identified in HTx livers several weeks after transplant, which may also indicate cell rejection (data not shown). However, ROSA26 liver characterization suggested failure to identify β-gal activity may be due to gradual hepatic LacZ gene silencing as mice aged (**Figure 19**).

Despite potential cell rejection at the time of sacrifice, survival was significantly improved in iMSUD-HTx mice (**Figure 29B**). Therefore, partial metabolic correction provided at earlier timepoints was effectively able to delay more severe MSUD symptoms and lengthen survival. In addition, mouse E2 RNA was present at a level of 2.8% of control and BCKDH enzyme activity in iMSUD-HTx animals was increased ~8% over untreated and iMSUD-PBS animals (**Figures 24A** & **25**). BCKDH enzyme activity, when considered with the level of mouse RNA present, suggested each donor cell was more active than usual. A similar occurrence was documented in a case of human HTx (Muraca et al., 2002b).

Alterations in neurotransmitters and their related precursors/metabolites in iMSUD mice reported here and in an upcoming manuscript (Zinnanti et al., 2008) mirrored imbalances in the human disorder (McKenna et al., 1998; Wajner et al., 2000). Correction of DA, 5-HT, and DOPAC in iMSUD-HTx brains (**Figure 28**), the site of injury for MSUD, was very encouraging from a clinical perspective. In addition, a significant reduction in alloisoleucine, a sensitive diagnostic marker for MSUD (Schadewaldt et al., 1999), and ornithine was observed in HTx brains compared to untreated iMSUD mice (**Figure 27**). However, many disruptions remained unchanged following HTx compared to untreated disease models [(i.e., asp, gln, GABA, ser, gly, tau, and phosphoethanolamine); **Figure 27** & **28**]. Perhaps at weaning, correction of brain amino

acids and monoamines would have been enhanced if rejection was prevented (assuming rejection was indeed occurring at the time of sacrifice).

Disrupted energy metabolism was previously suggested to be secondary to BCAA and BCKA accumulation in the brain (Patel et al., 1973; Yudkoff et al., 2005). Data presented here and by Zinnanti et al. (2008) support the occurrence of severely altered bioenergics and Krebs cycle dysfunction in the iMSUD model. A mechanism illustrating how this occurs and also explaining how MSUD causes brain injury was discussed previously (**Figure 34**).

6.3.2 Differentiated "liver-like" ESCs

Donor livers for OLT, and potentially HTx, are in very high demand (Strom et al., 2006a; Strom et al., 1999). Therefore, ESCs are viewed as a promising alternate source of cells for CTx. Similar to studies with HTx, ESCs differentiated into endodermal "liver-like" cells have successfully corrected liver failure (Hu et al., 2006; Ishii et al., 2007; Soto-Gutiérrez et al., 2006; Tabei et al., 2005) and two inherited liver disorders (Fair et al., 2005; Sharma et al., 2008) in animal models. Also similar to HTx, the presence of both a humoral and cellular immune response to transplanted ESC cells in mice suggested a need for immunosuppressive therapy (Kofidis et al., 2005). Alternately, the recent creation of iPS cells, which could be made from a patient's own cells, may solve immunogenic issues associated with traditional ESCs (Takahashi et al., 2007).

Data presented in Section 5.0 of this dissertation demonstrated that differentiation with FGF alone or FGF + activin A produced cells able to express high levels of liver-specific markers [(albumin, AAT, AFP); **Figure 31**]. This was consistent with other studies using these differentiation agents (Hu et al., 2004; Imamura et al., 2004; Ishizaka et al., 2002; Kubo et al.,

2004). BCKDH activity was also significantly increased from ~3.2% +/- 0.2% SEM of wildtype control liver activity (undifferentiated ESCs) to ~5.4% +/- 0.3% SEM in FGF differentiated PEPs (**Figure 32**). However, persistent, albeit low, Oct4 expression in cells differentiated *in vivo* for 12 days suggested not all cells were able to form PEPs (**Figure 31**). Transplantation of pluripotent stem cells could potentially result in teratoma formation (Chinzei et al., 2002; Choi et al., 2002; Teramoto et al., 2005; Thomson et al., 1998). Despite this concern, no evidence of teratoma was observed in adult PEP-injected mice analyzed here. However, such a small group of animals (n=3 were followed for 1 month post-transplant) does not likely represent what may occur in all mice. Finally, GFP-positive cells could be identified in the livers of all three animals analyzed 1-month post-transplant (**Figure 33B**) suggesting at least some engrafted cells were still present. Markers of transplant rejection were not assessed; therefore the possibility of rejection cannot be eliminated.

6.3.3 Future studies

With regard to differentiated ESC populations, longer culture times may reduce the incidence of possible teratoma formation (Chinzei et al., 2002). Alternately, a cell sorting step may be added to remove undifferentiated ESCs from those intended for animal transplantation. For further and more complete characterization of PEP cells, a more comprehensive panel of liver-specific markers and functional assays may also prove useful.

Partial metabolic correction of MSUD by HTx served as an exciting proof-of-concept study both for clinical HTx and differentiated ESC cellular transplant despite evidence suggesting cell rejection was occurring. In addition to hepatocytes and differentiated ESCs, transplantation of human placental cord blood cells (McGuckin and Forraz, 2008; Prasad et al.,

2008) are also proposed for the future. Plans to improve liver engraftment largely involve a more aggressive treatment of iMSUD mice. Cell injections would begin at birth as described for HTx. However, since human clinical cases of HTx have determined that a much larger percentage of liver mass (up to 5%) can be tolerated without incident (Dhawan et al., 2004), cells per injection could be increased. In addition, supplementary splenic injections (Gupta et al., 1991; Holzman et al., 1993; Mito et al., 1979; Ponder et al., 1991) could be given at weaning age since significant improvement in body weight and BCAA/ala blood levels were observed at that time (**Figures 22** & **29A**). In order to further enhance liver repopulation, selective regeneration pressure, such as preconditioning with retrorsine and CCl_4 (Gupta et al., 1999; Laconi et al., 1998) and/or partial hepatectomy, may be used to give transplanted hepatocytes a greater growth advantage over endogenous cells. Finally, the presence of immune response markers following transplant could be investigated since some HTx results suggested cell rejection may be occurring. Immunosuppression could be administered if needed.

6.4 RELEVANCE TO HUMAN METABOLIC DISEASES

This work has potential importance to human MSUD and possibly other human metabolic diseases. MSUD and other inborn errors of metabolism are often very serious with few options for treatment. Further characterization of the iMSUD mouse model reported here supports its value as an ideal tool to identify the specific role of genes/gene products in the biological action and pathophysiology of human MSUD. Stemming from recent work with the cMSUD and iMSUD models, a disease mechanism has already been proposed (Zinnanti et al.,

2008). A better understanding of the causative mechanism behind the human disease may lead to new or improved therapies to treat or potentially cure the disorder.

In addition to the iMSUD model accurately mimicking human MSUD, it may also prove useful as a model of other disorders. For example, MSUD dysfunction occurs in mitochondria, which results in Krebs cycle dysregulation and decreased bioenergics (Patel et al., 1973; Yudkoff et al., 2005). Therefore, the iMSUD model may be a viable model for other mitochondrial diseases with impaired bioenergics. iMSUD animals are also prone to seizures when stressed or exposed to loud noise, which often leads to death (unpublished observations). Therefore, iMSUD mice may be valuable as a seizure model as well. In addition, a dysfunction in fatty acid metabolism occurs in MSUD, which results in cuticular delamination (Jones et al., 1996; Smith and Swift, 2005). Therefore, the iMSUD model may also be useful in the dermatology field.

Results presented in Section 4.0 of this dissertation of partial metabolic correction in iMSUD mice by HTx were very encouraging. Although HTx has already been used clinically to treat other metabolic diseases as an alternate treatment to OLT (Bodner-Leidecker et al., 2000; Netter et al., 1994; Strauss et al., 2006; Wendel et al., 1999), these studies provided further rationale to pursue cellular therapies for the clinical treatment of MSUD. Furthermore, other liver-based diseases or inborn errors of metabolism may also be benefited by cellular therapy approaches. In addition, many advantages of CTx exist over OLT, such as cost, it's a non-invasive procedure, and the possibililty of multiple manipulations to further enhance liver engraftment and repopulation, among others (Fisher and Strom, 2006; Strom et al., 2006a). Therefore, CTx may represent a promising therapeutic intervention for human MSUD and other liver-based metabolic disorders which should be investigated further.

Despite AAV's failed ability to demonstrate any improvement in iMSUD mice, AAV gene therapy may still represent a promising therapeutic alternative to a low BCAA diet or OLT. Aside from other reported AAV-mediated animal studies with metabolic disease, recently a mouse model of phenylketonuria, a liver-based inborn error of metabolism that is also controlled by diet much like MSUD, was subjected to AAV gene therapy. Both liver-directed (Ding et al., 2006; Mochizuki et al., 2004) and muscle-directed (Ding et al., 2008) approaches achieved complete correction. Because BCKDH also has substantial activity in the skeletal muscles of a normal person (Tessari et al., 1996), muscle-directed approaches may also be viable to correct MSUD if enough cells can be transfected. However, the iMSUD model must be further evaluated before AAV studies can be continued.

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