GENE THERAPY FOR THE TREATMENT OF PROPIONIC ACIDEMIA

A THESIS
SUBMITTED TO THE FACULTY OF
MAYO CLINIC
MAYO GRADUATE SCHOOL
COLLEGE OF MEDICINE

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES –
VIROLOGY AND GENE THERAPY

December 2014

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ACKNOWLEDGEMENTS

I would first like to acknowledge my thesis advisor and mentor, Dr. Michael Barry. Thank you for giving me this project and allowing me to run with it. I feel I was given the ability to make many decisions in the course of my research that aren't necessarily allowed by other mentors. He focused not just on the details of science and laboratory techniques but also on the bigger picture of experimental and study design. Beyond that he helped me maintain the perspective that what we are really trying to do is help people with a terrible disease and our decisions should always be focused on that endpoint. His ability to tackle multiple forms of scientific research is impressive and I feel that has influenced me as well. I am also grateful for my thesis advisory committee – Drs. Steve Russell, Yasuhiro Ikeda, Gregory Gores, and Kah Whye Peng. They always offered sage advice and provided many valuable insights into my work.

I would also like to acknowledge all of the current Barry lab members, thanks for creating a positive atmosphere where we could freely share ideas and help each other on a daily basis – Mary Barry, Catie Crosby, Mallory Turner, Dr. Tien Nguyen, Clara Castillejo-Becerra. A big thanks to former Barry lab members, - Drs. Chris Chen, Reeti Khare and especially to Shannon May, Drs. Matt Hillestad, Eric Weaver, and Sean Hofherr who were directly involved in the work presented here. Additionally Dieter Matern and all of the folks in the Biochemical Genetics lab deserve a huge thanks for analyzing hundreds of blood spot cards that I relentlessly provided.

Finally, I would like to acknowledge the people closest to me. I have many wonderful friends who have become like family over the years. Of course my actual family as well, thanks to my parents John and Karen; they put me on the right track and always

encouraged me to succeed. Most of all, a final thanks to my wonderful wife Rachael. She has supported me in every way possible and has more to do with the successful completion of my time as a PhD student than she may ever know. To Allison… you provide distractions when sometimes a few minutes away from work and a smile is the best way to stay on task.

THESIS ABSTRACT

Propionic acidemia is an organic acidemia that results from mutations in the PCCA or PCCB genes responsible for the two protein subunits of the propionyl-CoA carboxylase enzyme. Patients with PA have several metabolic abnormalities including elevated levels of glycine, propionylcarnitine, and methyl citrate. They also experience growth delay, developmental delay, and pathologies involving the brain, heart, pancreas, eyes, and muscles. The only viable treatment options for PA are protein restriction via a formula diet or liver transplantation, but neither of these treatments result in cures. To study the possible benefit of gene therapy for the treatment of PA we generated a mouse model of PA by introducing a hypomorphic human transgene with an A138T mutation onto a *Pcca* null mouse background. The resulting *Pcca*-/-(A138T) mice recapitulated many characteristics of PA in humans, and showed similar growth delay and biochemical perturbations. These mice were then used to study the utility of adeno-associated virus (AAV) serotype 8 and adenovirus serotype 5 expressing human PCCA. Both vectors mediated significant reductions in PA metabolite levels. Efficacy lasted for approximately 2 months in adenoviral treated mice but persisted for 1.5 years in male mice treated with AAV vector with expression remaining in the liver, heart, and skeletal muscle. Further studies examined the effect of tissue-specific treatments using alternate AAV serotypes and transcriptional regulation. When PCCA expression was restricted to the liver or muscle of treated mice metabolite levels were significantly lower in both indicating that there was likely a significant amount of these metabolites being produced within the muscle. Together these data provide evidence that PA disease is amenable to treatment with gene therapy and AAV vectors are able to mediate a significant degree of

correction over long periods of time in mice. Additionally, the optimal treatment for individuals with PA will include correction of PCC activity in liver and muscle at a minimum to decrease the amount of PA metabolites such as methyl citrate being produced in these tissues. These studies also provide previously unknown insight into the molecular basis of the disease.

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ABBREVIATIONS

AAV	Adeno-Associated Virus
Ad	Adenovirus
C3	**Propionylcarnitine**
CMV	Cytomegalovirus Immediate Early Promoter
Cre	Cre Recombinase
dsRed	Red Fluorescent Protein from Discosoma sp.
GL	EGFP/Luciferase Fused Dual Reporter
GFP	Green Fluorescent Protein
HD	Help Dependent or "Gutless" Adenovirus Vector
ITR	Inverted Terminal Repeat
i.v. or IV	Intravenous
kg	Kilogram
Luc	Firefly Luciferase
MeCit	**Methylcitrate**
mRNA	Messenger RNA
PCC	Propionyl-CoA Carboxylase
qPCR	Quantitative Real Time Polymerase Chain Reaction
sc	Self-complementary
SIN	Self-Inactivating
ss	Single-stranded
vg	Viral Genomes (or) Vector Genomes

A note on genetics abbreviations:
Standard genetic nomenclature was used throughout this thesis to denote human and murine genes and proteins. For example:
PCCA denotes a **human gene**
PCCA denotes a **human protein**
Pcca denotes a **murine gene**
Pcca denotes a **murine protein**

Chapter I: Introduction

Background

Propionic acidemia (PA, MIM #606054) was first identified in 1961 as idiopathic hyperglycinemia [1, 2]. It was later observed that patients had high levels of ketones in response to challenge with amino acids, particularly branch-chain amino acids [3]. Although the first biochemical findings of PA manifested as increases in urine glycine concentration, it was later observed that elevated levels of both glycine and hydroxypropionate were present in the urine of PA patients as well as propionate in serum [4]. In 1971 Hsia *et al* demonstrated that the previous observations were due to an inherited deficiency in the enzyme propionyl-CoA carboxylase [5].

The worldwide incidence of PA is approximately 1 in 100,000 births, but founder mutations in populations in Greenland, Amish, and Saudi Arabian sects may increase incidence to a level as high as 1 in 3,000 births. In the United States, PA is generally detected before symptoms manifest by newborn screening although late-onset forms have been described and can present as developmental regression, hypotonia, and movement disorders [6].

Biochemistry and Molecular Biology

Propionyl-CoA is not directly synthesized in the human body, but results from the consumption of food and production by gut bacteria (**Figure 1**). The primary source of propionyl-CoA is the result of catabolism of the amino acids valine, isoleucine,

threonine, and methionine that accounts for approximately 50% of the pool of total propionyl-CoA [7, 8]. β–oxidation of odd chain fatty acids accounts for an additional 25% of the total propionyl-CoA in the body [7-9]. Even-chain fatty acids do not contribute to the propionyl-CoA pool because as fatty acids are shortened with each complete cycle of β–oxidation two carbons are removed as acetyl-CoA molecules, however, at the end of odd-numbered chains 3 carbons remain and form propionyl-CoA [10]. The final 25% of the propionyl-CoA pool is the result of propionate production by bacteria in the gut [7, 8, 11].

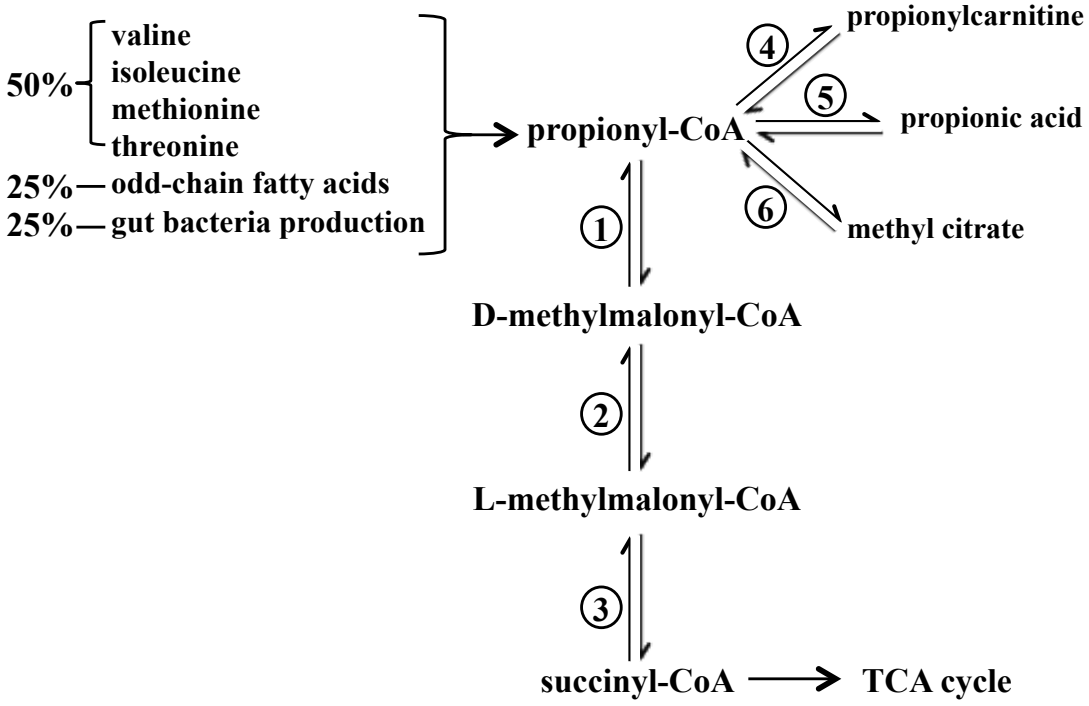

Figure 1.1. PA-Relevant Metabolism

Amino acids, odd-chain fatty acids, and production from gut bacteria form the total pool of propionyl-CoA that is normally processed by propionyl-CoA carboxylase (1), DL-methylmalonyl-CoA racemase (2), and methylmalonyl-CoA mutase (3) to eventually form succinyl-CoA which feeds into the TCA cycle. When PCC (1) is inhibited as in PA, additional pathways are emphasized in response to high propionyl-CoA levels. Carnitine acyltransferase (4) enables transport into mitochondria, propionate-CoA transferase (5) yields propionic acid, and citrate synthase (6) combines propionyl-CoA with oxaloacetate to form methyl citrate.

The primary pathway of propionyl-CoA metabolism results in the conversion of propionyl-CoA to methylmalonyl-CoA and eventually to succinyl-CoA; a substrate in the TCA cycle. Propionyl-CoA carboxylase (PCC) is the first enzyme in this pathway, it is a large enzymatic complex consisting of six α subunits (72 kDa each) and six β subunits (56 kDa each) [12]. The α subunit requires a biotin cofactor and is covalently biotinylated by holocarboxylase synthetase [13, 14] at a 1:1 molar ratio. Carboxylation of propionyl-CoA by PCC is a two-step process where bicarbonate is first attached to nitrogen in biotin, this step requires ATP and Mg^{2+}. In the second step the biotin complex interacts with propionyl-CoA to transfer the carboxyl group of the biotin-bicarbonate complex to the second carbon of propionyl-CoA [9]. The actions of PCC form D-methylmalonyl-CoA which is then converted to L-methylmalonyl-CoA by DL-methylmalonyl-CoA racemase [15] because only the L isoform can be utilized by methylmalonyl-CoA mutase to form succinyl-CoA (**Figure 1** and [16, 17]). The downstream fate of propionyl-CoA is relevant because it is the only significant metabolic source of methylmalonyl-CoA, and succinyl-CoA contributes to energy production as it enters the Kreb's cycle and is used to generate a large amount of the nicotinamide adenine dinucleotide (NADH) used in the mitochondrial electron transport system.

In normal humans, the pathway outlined above metabolizes the vast majority of available propionyl-CoA. However, in the absence of PCC activity in PA patients, alternate pathways attempt to mitigate the products of aberrant metabolism. A high level of propionyl-CoA leads to a host of abnormal compounds accumulating which can be detected in the blood and urine of PA patients (**Table 1**). Of particular importance for PA

is the condensation of propionyl-CoA with oxaloacetate by citrate synthase to form methylcitrate (**Figure 1** and [18, 19]). Additionally, PA patients may have increased levels of odd-chain fatty acids because propionyl-CoA can be used as a primer of fatty acid synthesis instead of acetyl-CoA which results in an odd-chain fatty acid product [20].

Detection and Diagnosis

Newborn screening of blood spots collected from infants 24 hours after birth is used in the United States to detect patients with PA. Newborns are not usually thought to be symptomatic at the time of sample collection. However, in one study by Grünert *et. al*. 74% of patients actually did show symptoms by the time screening results were available [21]. In supplemental newborn screening, tandem mass spectrometry detects elevations in propionylcarnitine as a hallmark of PA. However, elevated propionylcarnitine alone is not sufficient to provide a definitive diagnosis of PA. Therefore, second tier testing is performed to detected elevated methylcitrate in the blood [22]. In addition, PCC enzyme activity in cultured fibroblasts is used to differentiate between PA and other disorders. The utility of neonatal screening has been questioned since early detection does not appear to have any impact on intelligence quotient (IQ), metabolic crises, and other long-term symptoms observed in PA patients. However, long-term survival is increased in patients detected early by newborn screening, reinforcing the need for early detection [21, 23].

Table 1.1. Summary of Abnormal Biochemical Findings in Patients With Propionic Acidemia.

amino acid species		**Source(s)**
glycine	elevated	[1, 24]
glutamine	decreased	[25]
lysine	elevated in young	[26]
alanine	elevated	[27]
BCAA	decreased in treated	[27]
tiglylglycine	elevated	[28]
propionylglycine	elevated	[29]
organic acids in urine		[30]
2-methyl-3-oxovaleric	elevated	
methyl citric	elevated	
lactic	elevated	
3-hydroxypropionic	elevated	
3-hydroxy-2-methylbutyric	elevated	
3-oxovaleric	elevated	
3-hydroxy-n-valeric	elevated	
2-methyl-3-oxobutyric	elevated	
propionyl-CoA and derivatives		
Propionyl-CoA	elevated	
propionylcarnitine	elevated	
odd-number long-chain fatty acids	elevated	[31]

Pathology

Presentation of PA is heterogeneous in nature and can manifest in different organs including the cardiovascular, nervous, gastrointestinal [32, 33], renal [34, 35], and immune systems (**Figure 2** and [36-38]). Symptoms in these organ systems are all important targets for treatment and monitoring of PA patients. However, we will focus here on cardiovascular and neurological pathology in PA because these two organ systems are associated with high percentage of the morbidity and mortality associated with PA.

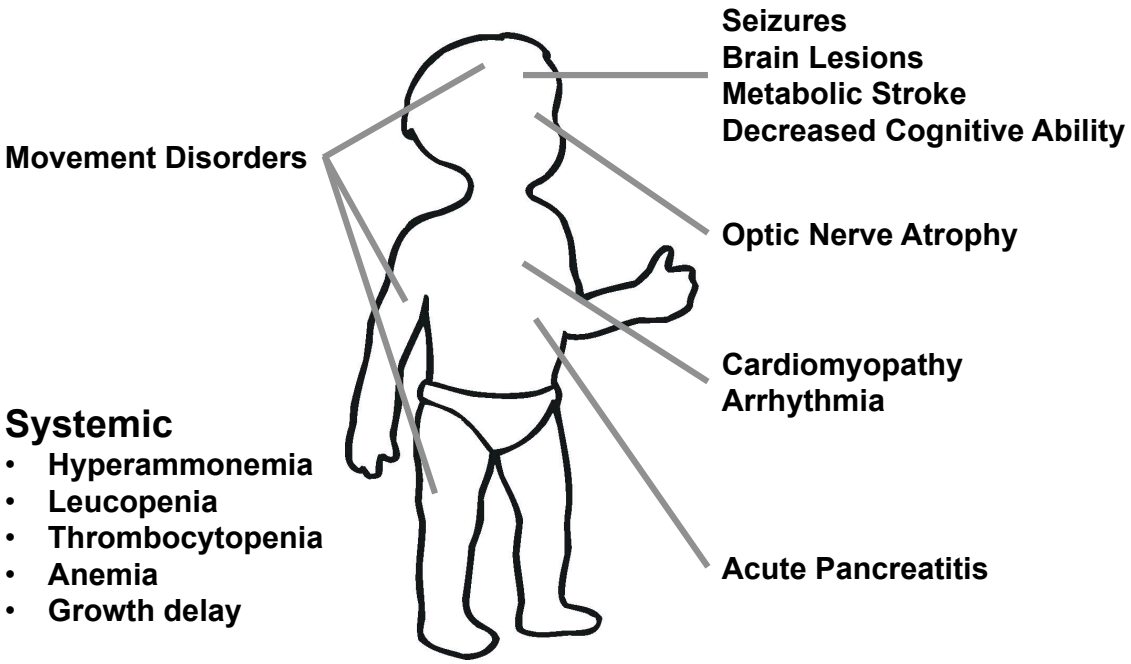

Figure 1.2. Common Symptoms in Patients With Propionic Acidemia

Cardiac Pathology

Cardiac pathology in PA most often manifests as cardiomyopathy or cardiac arrhythmias [39, 40]. Arrythmia incidence is reported to be as high as 70% [41]. Cardiac symptoms often manifest in young patients, but can also occur for the first time in the teenage years and beyond [42, 43]. PA-related cardiomyopathy can begin suddenly and rapidly progress to death of the patient [42-44]. Case reports highlight the need for regular cardiac screening and rapid cardiac assessment during hospitalization of PA patients. Studies have been undertaken attempting to identify PA-related markers that may correlate with decreased cardiac function in PA patients. For example, Romano *et al.* (2010) analyzed PCC enzyme activity and measured methylcitrate, propionylglycine, 3-hydroxypropionate, and tiglylglycine in 26 PA patients over several years and found no significant change of these parameters in patients that developed cardiomyopathy [39]. To date, development of cardiomyopathy in PA patients has not been associated with any single specific risk factor such as the previously mentioned biochemical markers, PA disease severity, or age-of-onset [39, 45]. Significant carnitine abnormalities are seen in individuals with PA and since cardiomyopathy has often been observed in individuals with primary carnitine deficiency [46], carnitine represented another possible parameter that could relate to cardiomyopathy in PA disease, however. However, the 26 patient study by Romano *et al*. (2010) and other case studies do not support a relationship between plasma carnitine and cardiac dysfunction [39, 41, 42, 44].

Premature deaths of patients with PA due to cardiac failure are unfortunate, but provide insights into the pathology of PA. A post mortem study of a PA patient with fatal

cardiomyopathy from Mardach *et al.* (2005) reported a possible mechanism for cardiac complications in PA [44]. This study highlighted findings from an 8-year-old girl with PA who succumbed to heart failure. Post-mortem evaluation revealed that she had cardiac hypertrophy with low tissue carnitine levels despite normal plasma carnitine values. Microscopic evaluation showed increased myofiber size. Biochemical analysis revealed a complete absence of NADH cytochrome *c* reductase activity, indicating a deficiency in electron transport chain complex I [44]. Subsequent studies have analyzed electron transport chain activity in multiple tissue types and found significant reductions in activity levels of the skeletal muscle, heart, and liver in multiple PA patients experiencing severe cardiac symptoms (**Table 2**). More recently a coenzyme Q_{10} (CoQ_{10}) defect has been observed in a small number of PA patients suffering from cardiomyopathy [47]. While these results were not available early enough to assist this patient a more recent observation of CoQ_{10} deficiency in PA-related cardiomyopathy enabled CoQ_{10} supplementation that was able to reverse the cardiac defects observed in one patient [45].

Table 1.2. Biochemical Findings in PA-Associated Cardiomyopathy

Age	Respiratory Chain Findings	Tissue(s)	Source
8 years	Complex I deficiency	Muscle, Heart	[44]
6 years	Complex III and IV deficiency	Muscle, Liver, Heart	[48]
	Complex IV deficiency	Muscle, Liver	
8 years	Normal	Heart	[48]
15 years	Complex IV deficiency	Heart	[45]
11 years	Complex II, III, IV deficiency	Muscle	[47]
	Complex I, I+III, II+III deficiency	Liver	

The ultimate mechanism for PA-associated cardiomyopathy remains unclear and is likely multifactorial. Now that biochemical analysis of tissue collected from PA patients suffering from cardiomyopathy has become more common it seems likely that inhibition of the respiratory complexes in mitochondria are at least part of the problem in many individuals with PA. These results have prompted several hypotheses regarding the source of respiratory chain inhibition that dovetail with previously proposed mechanisms [41]. First, the perturbed metabolism of individuals with PA may result in decreased levels of a necessary metabolic substrate, resulting in an energy deficiency. This seems likely in the case mentioned above of decreased CoQ_{10} levels where cardiomyopathy was resolved with the addition of CoQ_{10} [45]. The known biochemistry of propionate metabolism would also suggest a possible decrease in the levels of succinate, which may ultimately cause a decrease in the amount of available NAD+ for use in the respiratory chain. Second, inhibition of the respiratory chain could be the result of direct inhibition by propionyl-CoA and its derivatives such as methylcitrate. This has been observed by *in vitro* experiments showing that propionyl-CoA inhibits pyruvate dehydrogenase complex [49] and may act synergistically with methylcitrate to inhibit respiratory complex II [50]. Methylcitrate itself also inhibits the activity of several enzymatic complexes including citrate synthase, aconitase, and isocitrate dehydrogenase in the Kreb's cycle which may contribute to energy deficiency and ketogenesis [51].

We further hypothesize two possible sources for the pathology caused by respiratory complex inhibition. 1) propionate and its metabolic derivatives are circulating throughout the body in the bloodstream and accumulate to a higher degree in specific tissues and

cause localized pathology, or 2) local accumulation of propionate and its metabolic derivatives acts locally in the tissue where it was produced i.e. toxic metabolites produced in the heart accumulate and act locally on the heart.

Neurological Pathology

Complications in the nervous system are common in PA and can manifest as encephalopathy, metabolic stroke, delayed development, intellectual disability, seizures, hypotonia, lethargy, myoclonus, coma, and hyperreflexia [52]. The most common neurological symptoms (**Table 3**) reduce the quality of life and create significant financial strains on families with PA patients. An excellent review of neurological findings in PA is already available [52] so I will focus on specific aspects of the molecular mechanisms relating to pathophysiology of neurological symptoms.

Table 1.3. Most Frequent Neurological Symptoms in PA patients Reported in Several Sources

Symptoms	total n	sources	total %
intellectual disability	127	[53-56]	74
lethargy	145	[30, 55-57]	63
hypotonia	190	[30, 53, 55-57]	57
seizures	220	[30, 54-58]	42

Neurological symptoms are often tied to periods of metabolic stress as illustrated by a study in which Grünert *et al* observed a negative correlation of IQ with the number of metabolic crises experienced by individuals with PA [21]. However, patients with stable

PA disease still develop neurological symptoms including hypotonia and intellectual disability while being maintained under "optimal" metabolic control [59]. While the pathology observed in the hearts of PA patients is complicated by the cell-autonomous nature of the disease coupled with circulating metabolites that may damage tissues, the blood brain barrier adds another level of complexity to potential neurological pathology. The compounds that accumulate in PA (**Table 1**) are not known to be directly neurotoxic in humans, but administration of propionic acid to rats has been shown to cause cognitive defects that can be normalized by administration of ascorbic acid [60]. It is likely that propionic acid and methylcitrate present at extremely high concentrations in PA patients reaches a level that becomes harmful to the brain. There is precedence for intracerebral accumulation of normally benign compounds causing neurotoxicity in glutaric aciduria type I (MIM 231670). In this case, the accumulation of glutarate and 3-hydroxyglutarate have been linked to endothelial cell damage [61], vascular dysfunction [62], and neuronal cell damage [63]. In another study, a 3D model of rat brains was used show that glutarate and 3-hydroxyglutarate trigger accumulation of ammonium which would likely cause intracerebral hyperammonemia *in vivo* [64]. Accumulation of these compounds occurs because there is limited influx and efflux of dicarboxylic acids across the blood-brain-barrier [65]. The methylcitrate produced in PA is a tricarboxylic acid, which likely remains trapped by the blood-brain-barrier as well. In another study by Jafari *et al*. using a 3D rat brain model methylmalonic acid, propionic acid, and methylcitrate were applied to rat brain cell cultures at increasing concentrations [66]. At 0.01 mM of methylcitrate neuronal and glial cells exhibited morphological changes, as methylcitrate concentration increased to 0.1 mM ammonium concentration increased significantly and glutamine

concentration decreased which is consistent with findings in PA patients [27]. At the higher methylcitrate concentration (0.1 mM), an increase in apoptosis was also observed by activation of caspase-3 [66]. These findings coupled with case reports of PA patients experiencing neurological dysfunction in the absence of metabolic crises or high circulating ammonia levels [59] provide strong evidence for a tissue-specific response to locally produced methylcitrate in the brain independent of circulating metabolites. This does not preclude further damage as a result of metabolic crises, which likely damage the brain with high ammonia concentrations [21].

Current Treatment of Propionic Acidemia

Dietary Modification

The primary long-term treatment for PA is dietary restriction of threonine, methionine, isoleucine, valine, and odd-chain fatty acids. This is usually achieved with medical foods designed to restrict these compounds but supplement appropriate levels of other essential amino acids and provide necessary vitamins, minerals, and calories through fats [67]. The efficacy of modified diets for PA is difficult to assess since urinary and plasma metabolites as well as *in vitro* enzyme activity in fibroblasts often do not correlate with disease severity [68]. Elevated levels of serum propionic acid [69] and branch-chain amino acids as well as urinary methylcitrate [70] correlate with hyperammonemia, but these elevations and even elevations in serum ammonia levels do not always correlate with problematic clinical findings and metabolic decompensation. The percentage of *in vivo* propionate oxidation (as measured by expiration of $^{13}CO_2$ after infusion of [1-

^{13}C]propionate) correlates with disease severity [68]. However, this test is not widely utilized so treatment efficacy is generally assessed by growth assessment including length, height, weight, and head circumference. By these criteria, patients treated with modified protein diets perform better [71-73]. Treatment with a formula diet seems relatively straightforward, but feeding disorders coupled with lack of good metabolic markers of treatment efficacy result in frequent incidence of hospitalization due to metabolic decompensation even when good dietary control is maintained [73]. It has been suggested that acute metabolic decompensation actually occurs more often as a result of infection or other illness that causes decreased caloric intake. This implicates catabolism and not dietary protein intake as a driver of acute metabolic decompensation [67, 74]. When patients are admitted to the hospital during acute metabolic episodes, treatment is usually focused on rehydration and toxin removal, sometimes involving dialysis or hemofiltration. Infections also cause metabolic decompensation in individuals with PA. A high percentage (>80% in multiple studies) of hospital admissions of PA patients undergoing acute decompensation actually also present with sepsis [36, 75, 76]. Therefore antibiotic use is recommended and initiated rapidly after evaluation for bacterial infection [77]. Care is also focused on halting and reversing catabolism by providing non-protein calorie sources and then reintroducing small amounts of protein and normalization of nutrition [77, 78].

Liver transplantation

Despite the improvements in dietary therapy, the outcome for many individuals with PA remains disappointing [78, 79]. There is some difficulty in complying with these severely protein-restricted diets. However, even when compliance is good, potentially fatal metabolic decompensation still occurs and patients suffer continuing neurological and cardiac defects. It has been hypothesized that correction of PCC deficiency in the liver alone would mitigate some metabolic sequela by normalizing transamination of the amino acid precursors of propionic acid [80]. In 1995 Schlenzig *et al*. reported the first case of liver transplantation (LT) for PA [81], which has been followed by additional reports of liver transplantation [82, 83]. Results of these early transplant cases were mixed. Concentrations of propionylglycine and tiglyglycine normalized and instances of hyperammonemia decreased. After transplantation, patients experienced correction of anemia, improvement of growth rate and mental development, there were few episodes of metabolic decompensation, and protein intake could be increased [82]. However, episodes of severe metabolic events have still been reported as long as 3 years after transplantation, highlighting the need for continued monitoring and perhaps dietary therapy [84]. In addition, concentrations of methylcitrate, propionylcarnitine, and 3-hydroxypropionate remained elevated after transplantation and several patients have developed fatty liver. Of the initial 4 cases, 50% had severe graft rejection with one death within 15 months after transplantation due to lymphoproliferative disorder [81-83]. It is important to note transplantation was only utilized in these initial cases for patients with very severe and advanced PA disease including many prior instances of metabolic decompensation. Studies relating to tissue-specific pathology have shown that liver

transplantation has reversed cardiomyopathy in some cases [39], but in others it was unaffected [82]. Liver transplants seem to halt neurocognitive decline in many patients, although prior neurological injury is not reversed [84-86]. As more case studies have been compiled the paradigm has shifted towards earlier, more preventative transplant procedures in less severely affected patients.

Even with relatively few case reports to analyze it becomes clear that liver transplantation provides a benefit to individuals with PA by correcting hyperammonemia and decreasing the number of metabolic emergencies they experience. The low incidence of PA and even lower rate of liver transplants in PA patients makes it difficult to draw conclusions about the efficacy of liver transplants and their effect on cardiac and neurological dysfunction. Although techniques have improved greatly, transplantation of any kind is still associated with a risk of morbidity and mortality from the operation. Indeed, some PA patients cannot even consider transplantation as they would be unlikely to survive anesthesia. Beyond anesthesia and surgery, transplantation of an organ from one person to another necessitates life-long immunosuppression. This increases risks of infection and lymphoproliferative disorders. Increasing the risk of infection also increases metabolic events due to infection. While liver transplantation may currently provide the best treatment that affects the cause of the disease (at least in one organ) instead of the symptoms, additional treatment options are needed. This thesis explores the utility of gene therapy as an alternate therapy for PA.

Gene Therapy

Gene therapy attempts to treat disease by introducing exogenous genetic material into host cells. There has been interest in performing gene therapy for a variety of diseases since it was realized in the 1960's that simian virus 40 was able to integrate its DNA into the mammalian genome of tissue culture cell lines [87, 88]. The first approved gene therapy clinical trial occurred in 1990 to treat adenosine deaminase (ADA) deficiency in a group of four children [89]. Including this initial study there have been a total of 1,973 clinical gene therapy trials between 1990 and 2014 aimed at treating some form of human disease [90]. A majority of gene therapy trials (64.1%) have been for various forms of cancer (**Figure 3**). Only 9.1% of the clinical gene therapy trials over this period were for monogenic genetic diseases such as PA [90]. The most basic form of gene therapy would be to deliver simple DNA into cells. However, this is generally not efficient, so many different strategies have been employed to increase transduction of cells. Using viruses to carry genes is currently the most efficient approach for gene therapy *in vitro* or *in vivo.* To date retroviruses, lentivirus, adenoviruses, adeno-associated viruses, and many others have been used as viral gene therapy vectors [91]. A brief summary of the properties of the most common, retroviruses, adenoviruses, and adeno-associated viruses, is shown in **Table 4**. More in-depth information is provided in the following sections with discussion focusing on tissue transduction, transgene expression, and safety in previous clinical trials.

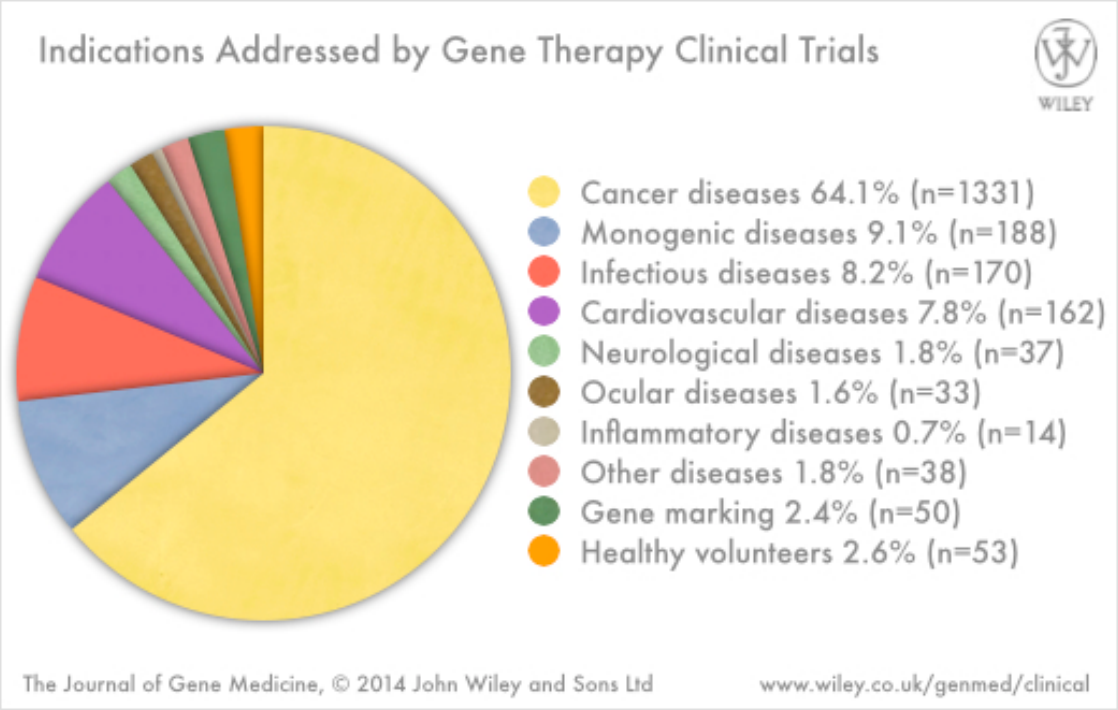

Figure 1.3. Primary Indication for Gene Therapy Clinical Trials Between 1989 and 2014.

Retroviral Vectors

Retroviruses are enveloped viruses with an ssRNA genome that is reverse transcribed into DNA and integrated into the host cell genome. Retroviruses are classified as simple (such as murine leukemia virus) which are capable of only transducing dividing cells [92], or complex (such as human immunodeficiency virus (HIV)) retroviruses that are capable of transducing both dividing and non-dividing cells [93]. Most wild type retroviruses have a fairly narrow cellular tropism, which limits the types of tissue that can be treated, however, *Env* glycoproteins from other viruses can be incorporated into retroviral envelopes and allow expansion of their tropism to other cell types and species [94]. For example, HIV requires CD4 receptor and CCR5 coreceptor on the surface of

cells for transduction, but Kafri *et al* demonstrated that HIV pseudotyped with vesicular stomatitis virus glycoprotein is able to enhance transduction of both liver and muscle, although less than 5% of hepatocytes expressed transgene after injection of 1-3x10^7 infectious units [95]. Both forms of retrovirus have been utilized to transduce patient stem cells *ex vivo*, which are then expanded in tissue culture and reintroduced into the patient [96]. This technique is particularly effective when treating disorders of hematopoietic cell lineages because CD34+ stem cells are easily isolated and have been extensively studied. In one of the first gene therapy clinical trials Hacein-Bey-Abina *et al* (2002) reported the use of a Moloney murine leukemia virus vector carrying cDNA for the cytokine receptor γ-chain to transduce patient $CD34^+$ cells *ex vivo* for treatment of X-linked severe combined immune deficiency (SCID). Results were initially positive and upon reintroduction of modified $CD34^+$ cells 8 of 9 patients experienced a significant reconstitution of their adaptive immune system [97]. However, half of these patients later developed a leukemia-like expansion of clonal T cells that occurred because of insertion of the transgene near the LMO2 proto-oncogene [98]. Fortunately, many of these patients responded positively to chemotherapy and ten years later 7 of 9 patients were leading relatively normal lives [99]. These results led to the development of self-inactivating vectors (SIN) in which the promoter and enhancer regions in the downstream LTR are deleted to prevent overexpression of nearby endogenous genes in transduced cells and greatly improve safety profile [100]. SIN vectors maintain their efficacy as well, follow up trials in patients with SCID have been promising with no lymphoproliferative disorders to date [101].

Table 1.4. Properties of Common Gene Therapy Vectors.

		adenovirus		
	retrovirus	**recombinant**	**helper-dependent**	**AAV**
viral genome	ssRNA	dsDNA	dsDNA	ssDNA
transgene capacity (kb)	**8.8-9.6**	**3.0-8**	**36**	**4.5**
integration	**yes**	no	no	no
adaptive immune response	**no**	yes	**no**	**no**
duration of expression	**long**	short	**long**	**long**

Adenoviral Vectors

Adenoviruses (Ads) are icosehedral (80-120 nm diameter) non-enveloped viruses that carry 36 kb of dsDNA. Ad has been the most commonly used viral gene therapy vector accounting for 22.8% of gene therapy clinical trials as of June 2014 [102]. Adenoviral gene therapy studies are increasingly being directed towards cancer treatment, but many initial studies were focused on treatment of monogenic diseases similar to PA. One example of a gene therapy clinical trial using an adenoviral vector was to treat ornithine transcarbamylase (OTC) deficiency. Minor increases in OTC activity were observed, however these were not statistically significant and patient immune responses were robust enough to inhibit therapy [103]. More importantly was the adverse event that resulted in the death of one study participant due to a systemic inflammatory response [104]. Early Ad gene therapy vectors such as the one used in the OTC trial had deletions of early gene transcription regions, primarily E1 and E3, which renders the virus unable

to replicate and allows introduction of transgene DNA up to approximately 8 kb. These vectors provide rapid and strong expression of transgene, but also elicit an immune response that will clear transduced cells. Additional deletions to the viral genome can be made, but the viral gene regions must be provided *in trans* for production of viral vectors. Helper-dependent or "gutless" Ad vectors represent the most recent generation of Ads and completely lack viral genes, which are then provided by a helper virus for production [105]. Elimination of all viral genes not only increases transgene capacity from approximately 8 kb to as high as 36 kb and also greatly reduces immune response and enhances the duration of transgene expression. Most adenoviral studies have utilized Ad2 or Ad5, which primarily transduce the liver in animal studies, but there are 57 known human adenovirus serotypes [106] that have varying tissue tropisms.

Table 1.5. Summary of AAV Vectors With Likely Clinical Targets.

Organ	Primary Serotype(s) for Systemic Treatment	Disease Target(s) [107].
Liver	AAV8	Hemophilia, OTC deficiency, α-1 antitrypsin deficiency
Heart	AAV1	cardiomyopathy, heart failure
Skeletal Muscle	AAV1, AAV6, AAV9	lipoprotein lipase deficiency, muscular dystrophies, lysosomal storage disorders, α-1 antitrypsin deficiency
Lung	AAV5	cystic fibrosis, α-1 antitrypsin deficiency
CNS	AAV9	Alzheimer's, Parkinson's, Batten's, Canaan's, epilepsy, ALS, spinal muscular atrophy
Eye	AAV4, AAV8	macular degeneration, Liber's congenital amaurosis

Adeno-Associated Viruses (AAV) Vectors

AAV vectors are icosahedral non-enveloped viruses that carry a single stranded DNA genome enclosed in capsid proteins. AAV's are unique in that they are dependoviruses, which require the actions of genes from other viruses, generally adenovirus or herpesvirus, to complete their life cycle and make new viral particles [108, 109]. AAV particles are only 22 nm in diameter allowing them to readily navigate out of the circulation and transduce a wide variety of tissues. The most extensively studied AAV serotypes (AAV1-9) show detectable expression of luciferase transgene in heart, lung, liver, kidney, brain, testes, gastrocnemius, and hamstring [110]. However, the study by Zincarelli *et al* revealed inherent biases in the amount of each vector transducing these tissue types, for example AAV4 exhibited the strongest transgene expression in lung tissue, but was one of the weakest vectors in the liver. AAV9 exhibited the strongest expression in multiple tissue types including the heart and brain [110]. The widespread tissue transduction comes with a cost as AAV's small size leaves only enough space to package approximately 4.7 kb of ssDNA. In order for transcription of vector transgene to take place the single-stranded genome must be converted into double-stranded DNA [111]. Second-strand-synthesis is the rate-limiting process for AAV transgene expression [112] and is believed to be carried out by DNA repair machinery in the host cells. The need for second-strand-synthesis can be circumvented by mutating one of the inverted terminal repeats (ITR) at the end of the vector genome which causes the entire genome to fold back on itself and form a self-complementary (sc) vector [113]. When they were developed these scAAV vectors exhibited increased transgene expression kinetics, but decreased the transgene capacity to only 2.2 kb [113]. The effect of the DNA genome on

gene therapy studies could be striking as expression from dsDNA vectors is 10 – 100 fold higher than that of ssDNA vectors in hepatocytes and muscle [114-116]. AAVs have been utilized in clinical trials to treat many types of diseases and have shown particular promise for the treatment of metabolic, cardiac, neurological, and ophthalmological diseases (**table 5**). The earliest clinical trials to utilize AAV were for the treatment of monogenic disorders such as cystic fibrosis [117] and hemophilia B [118] with AAV2 vectors. Results of these initial trials and subsequent trials totaling hundreds of patients treated with AAV vectors have been extremely positive from a safety perspective. Low transduction efficiency coupled with immune response to the treatment limited early efficacy [117, 118], but it is now known that alternate AAV serotypes promote more robust expression [110]. In 2012 Glybera became the first gene therapy treatment platform granted approval for use in humans [119, 120]. Glybera is an AAV1 vector carrying the cDNA for lipoprotein lipase for use in patients with lipoprotein lipase deficiency.

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CHAPTER II: GENERATION OF A HYPOMORPHIC MODEL OF PROPIONIC ACIDEMIA AMENABLE TO GENE THERAPY TESTING

A selection of this work is published as the manuscript: Guenzel, A., Hofherr, S., Hillestad, M., Barry, M.E., Weaver, E., Venezia, S., Kraus, J.P., Matern, D., and Barry, M.A. Generation of a Hypomorphic Model of Propionic Acidemia Amenable to Gene Therapy Testing. *Molecular Therapy*. July 2013.

Abstract

Propionic acidemia (PA) is a recessive genetic disease that results in an inability to metabolize certain amino acids and odd-chain fatty acids. Current treatment involves restricting consumption of these substrates or liver transplantation. Deletion of the *Pcca* gene in mice mimics the most severe forms of the human disease. *Pcca*- mice die within 36 hours of birth, making it difficult to test intravenous systemic therapies in them. We generated an adult hypomorphic model of PA in *Pcca*- mice using a transgene bearing an A138T mutant of the human PCCA protein. *Pcca-/-(*A138T) mice have 2% of wild-type PCC activity, survive to adulthood, and have elevations in propionyl-carnitine, methylcitrate, glycine, alanine, lysine, ammonia, and markers associated with cardiomyopathy similar to those in PA patients. This adult model allowed gene therapy testing by intravenous injection with adenovirus serotype 5 (Ad5) and adeno-associated virus 2/8 (AAV8) vectors. Ad5 mediated more rapid increases in PCCA protein and PCC activity in the liver than AAV8 and both vectors reduced propionyl-carnitine and methylcitrate levels. Phenotypic correction was transient with first generation Ad whereas AAV8 mediated long-lasting effects. These data suggest that this PA model may be a useful platform for optimizing systemic intravenous therapies for PA.

Introduction

Propionic acidemia (PA) is an inborn error of metabolism affecting approximately 1 in 100,000 live births in the United States and up to 1 in 1,000 births in high-risk populations [1]. PA results from defects in the mitochondrial enzyme propionyl-CoA carboxylase (PCC). The alpha and beta subunits of the PCC enzyme are encoded by the nuclear *PCCA* and *PCCB* genes, respectively [2-4]. Missense, nonsense, or splicing mutations can occur in either gene [5-8]. Approximately 49% of all observed mutations are missense mutations that can produce hypomorphic proteins with reduced, but not completely ablated enzyme activity (http://cbs.lf1.cuni.cz/pcc/pccmain.htm [9]). Loss of PCC function leads to an inability to metabolize odd chain fatty acids and the amino acids valine, isoleucine, methionine, and threonine (reviewed in [10]). These metabolic effects produce elevated levels of propionyl carnitine (C3), methyl citrate (MeCit), and glycine in the blood, allowing PA to be detected during newborn screening [11-14].

Clinical presentation of PA usually occurs within the first week of life and includes poor feeding, vomiting, lethargy, and metabolic acidosis [15, 16]. Because only certain amino acids and odd-chain fatty acids are substrates for PCC, standard treatment focuses on restricting consumption of protein [17]. Patients under active protein restriction may still undergo a high number of metabolic crises, explained in part by difficulty adhering to these life-long dietary interventions [17]. Even when careful dietary control is maintained, patients can still undergo metabolic decompensation, particularly in response to infection, trauma, or stress [18]. Recurrence of these metabolic crises can cause cognitive damage and sometimes death [19]. Grunert *et al.* showed a significant

correlation between reduced Intelligence Quotient (IQ) and the number of metabolic crises experienced by patients [20]. While the frequency of metabolic decompensation generally decreases with age, long-term survival is still reduced to 67% [19]. Additionally, PA can lead to cardiac abnormalities including cardiomyopathy and arrhythmia [21-25].

There is no cure for PA. Elective liver transplantation is being increasingly used to reduce metabolic crises and temper some of the most severe symptoms of the disease, including hyperammonemia [26, 27]. While liver transplantation has positive effects, it is invasive, has high morbidity, requires long-term immunosuppression, and rejection of the transplant is possible. Because of these drawbacks, it is important to develop alternative and safer therapies for PA.

A mouse model of PA was generated previously by disrupting the murine *Pcca* gene [28]. These *Pcca-/-* mice lack Pcca protein, have drastic increases in C3 and MeCit and die within 36 hours of birth [28]. Proof of principle for liver transplantation and liver gene therapy in this model was demonstrated by transgenesis with a liver-specific *PCCA* transgene. *Pcca-/-* mice with this covering transgene maintained 50% of wild type enzyme activity and survived for many months. In contrast, mice with less than 20% of wild-type activity died within 3 weeks.

Pcca-/- mice have been used to test the feasibility of gene therapy for PA. In a first study, treatment of newborn mice with helper-dependent adenovirus serotype 5 (HD-Ad5) or adeno-associated virus 2/8 (AAV8) vectors expressing the human PCCA cDNA were able to statistically extend lifespan, however the increases were modest [29].

Subsequently, AAV8-*hPCCA* treatment of newborn *Pcca-/-* mice extended the lifespan of 50% of animals up to 150 days [1].

Although *Pcca-/-* mice have provided proof of principle for gene therapy of PA, death of Pcca null animals within 36 hours makes treatment of this model challenging. In particular, the need to inject newborn mice within hours of birth makes intravenous injections strategies difficult to test. Given the need to test systemic therapies for application to humans, in this work, we have developed a hypomorphic model of PA in mice that allows mice to survive to adulthood. These mice express a mutant human PCCA protein that to partially replace deletion in the original *Pcca-* mice. . We show that this model accurately recapitulates many aspects of PA in humans, particularly in the 50% of PA patients who have hypomorphic enzyme activity. We also show that this adult model now allows testing of gene therapy approaches by intravenous administration.

Results

A hypomorphic mouse model of PA.

To avoid the difficulties in testing systemic gene therapy in newborn *Pcca-/-* mice used in previous studies, a hypomorphic model of PA was engineered using a cDNA for human PCCA harboring a nucleotide mutation that results in an A138T mutation. This mutation was previously identified in PA patients with mild to moderate symptoms [6, 7, 30]. This mutation in exon 5 results in reduced PCCA protein levels in patient cells. Transfection of PCCA-deficient human fibroblasts with A138T cDNA produces a protein with reduced mitochondrial stability and only 9.4% of wild-type PCC activity [9].

The A138T human cDNA was generated and cloned into a construct under control of a ubiquitously active CAG promoter followed by an IRES-EGFP to aid in phenotyping (**Figure. 1**). This cassette was microinjected into mouse embryos for random insertion into the genome. Six A138T founder mice were identified by PCR and three of them had detectable GFP fluorescence. A138T founders were crossed to *Pcca*+/- mice to identify *Pcca-/-* mice with covering transgenes. *Pcca-/-*(A138T) mice were rescued and ultimately bred from one GFP-positive founder and used for subsequent studies.

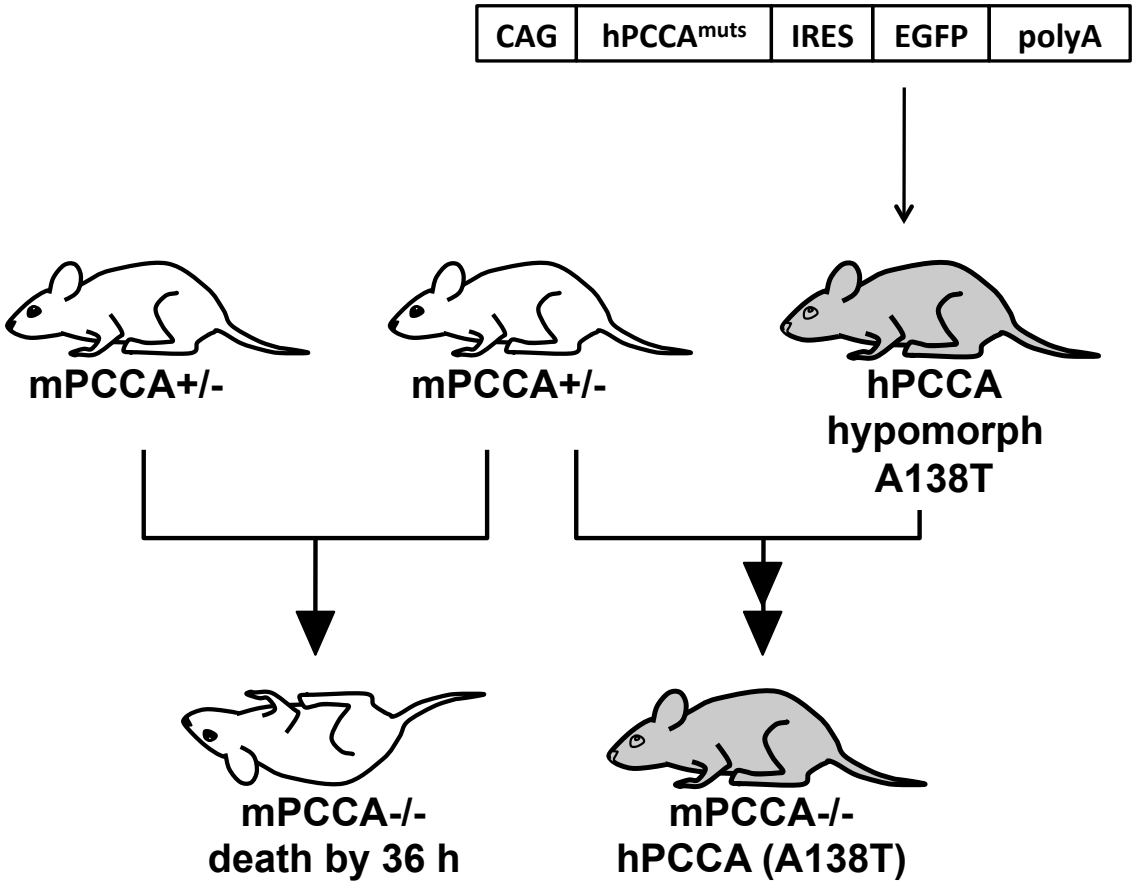

Figure 2.1. Strategy for Generating PCCA Hypomorphic Mice.

Depiction of the insertion cassette microinjected into FVB mouse fertilized eggs (Top). Two separate hypomorphic constructs were injected, one with an A138T mutation and another with an A75P mutation. IRES-GFP DNA sequence was included to aid in genotyping founder mice. (Bottom) Breeding strategies of *Pcca-/-* and *Pcca-/-*(A138T) mice. The A138T transcript increases survival well beyond the 36 hours observed in full knockout mice. The following abbreviations are used: CAG, Cytomegalovirus early enhancer coupled with chicken beta-actin promoter; IRES, Internal Ribosome Entry Site; EGFP, Enhanced Green Fluorescent Protein.

Copy number analysis of *Pcca-/-*(A138T) mice by Taqman assay demonstrated a single copy of transgene, which was transmitted in a Mendelian pattern of inheritance (data not shown). *Pcca+/-*(A138T) mice were phenotypically normal. Crossing *Pcca+/-*(A138T) to *Pcca+/-*(A138T) to produce *Pcca-/-*(A138T) mice was feasible, but produced only 2 to 3 *Pcca-/-*(A138T) pups per litter. *Pcca-/-*(A138T) mice produced from *Pcca+/-*(A138T) dams were generally indistinguishable from wild type littermates until after weaning at which point their phenotype became more evident as they exhibited significantly delayed growth (**Figure. 2a**). *Pcca-/-*(A138T) mice with two copies of the A138T transgene could also be bred to each other, generating litters with 5 to 9 *Pcca-/-*(A138T) pups. Unlike the phenotypically normal *Pcca-/-*(A138T) pups produced from *Pcca+/-*(A138T) dams, mice born to *Pcca-/-*(A138T) mothers were drastically smaller and experienced significantly delayed growth throughout their neonatal development (**Figure 2b,c**). These data suggested that low PCC activity in the dams affected the growth and development of their mutant pups. Since these mice survived and were produced in larger numbers per litter, subsequent studies were performed with mice from *Pcca-/-*(A138T) parents. These *Pcca-/-*(A138T) mice had marginally decreased survival over three months after birth (**Figure. 2d**). While survival was reduced, it was still markedly longer than the 36-hour survival of the *Pcca-/-* mice [31].

Liver PCC activity in *Pcca-/-*(A138T) mice.

The A138T human cDNA produced 9.4% activity in transfected fibroblasts [9]. To test PCC activity in *Pcca-/-*(A138T) mice, livers from wild type and *Pcca-/-*(A138T) mice were analyzed for PCC activity by incorporation of labeled CO_2 (**Figure 3a**). This assay demonstrated that PA mouse livers had 2.2% of wild type PCC activity.

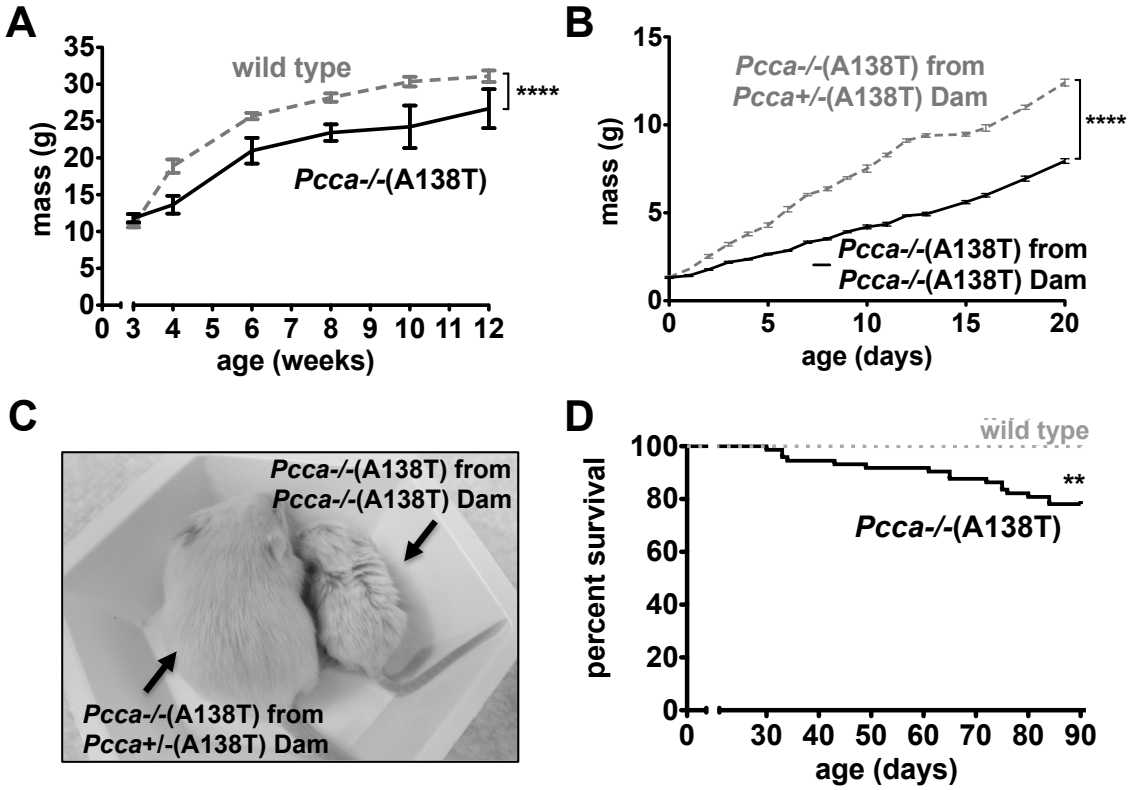

Figure 2.2. Growth of *Pcca-/-*(A138T) Hypomorphic Mice.

(A) Growth curve of *Pcca-/-*(A138T) mice versus wild type animals with intact endogenous mouse *Pcca* ($n \geq 7$ for all data points). (B) Growth curve of pups born from indicated dams. Pups from litters with ≥5 pups were analyzed (n=14). (C) Photo of *Pcca-/-*(A138T) mice birthed by a *Pcca+/-* dam (left) alongside a mouse birthed by a *Pcca-/-*(A138T) dam (right), picture was taken when both mice were weaned at three weeks of age. (D) Kaplan-Meier survival curve depicting death rate of *Pcca-/-*(A138T) mice (n = 73) versus wild type (n = 30). Error bars in the line graph depict standard error of mean (SEM). **** indicates $p<0.0001$, ** indicates $p < 0.01$.

Elevated PA-associated biomarkers in *Pcca-/-*(A138T) mice.

Elevated propionylcarnitine (C3) and MeCit are observed in PA patients during newborn screening. Blood spots were collected on filter paper from the animals in a manner similar to the method used for newborn screening to assess these biomarkers. C3 levels were normalized to acetylcarnitine (C2) to control for variations in sample recovery from blotted blood. Measurements of *Pcca-/-*(A138T) mice beginning at 4 weeks of age showed significantly increased levels of C3/C2 and MeCit (**Figure 3b,c**). On average, there was a 18-fold increase in both C3/C2 ratios and in MeCit levels when compared to wild type mice ($p < 0.0001$ for both by one way ANOVA). Analysis of amino acid levels also demonstrated significant increases in glycine, alanine, and lysine in the *Pcca-/-*(A138T) mice (**Figure. 3d**).

Hyperammonemia and cardiac perturbations in *Pcca-/-*(A138T) mice. Hyperammonemia and cardiomyopathy are significant life-threatening symptoms in PA patients. Ammonia levels in humans are below 35 μM in humans [32, 33] and are similar in wild-type mice (**Figure. 4a**). In PA patients, ammonia levels are usually elevated from 50 to 150 μM with some being higher than 400 μM [32]. Significant elevations in plasma ammonia levels similar to those in humans were observed in 8-month-old *Pcca-/-*(A138T) mice when compared to age-matched wild type animals (**Figure. 4a**). Hearts from these 8-month-old mice were also analyzed and mass measurements revealed that those from *Pcca-/-*(A138T) mice were significantly larger than those in wild type mice (**Figure. 4b**). Elevations in Brain Natriuretic Peptide (BNP) has been proposed as a potential marker for cardiomyopathy and cardiac dysfunction [34]. When BNP mRNA levels in the hearts of these animals were compared, message levels were significantly

higher in the *Pcca-/-*(A138T) mice (**Figure. 4c**).

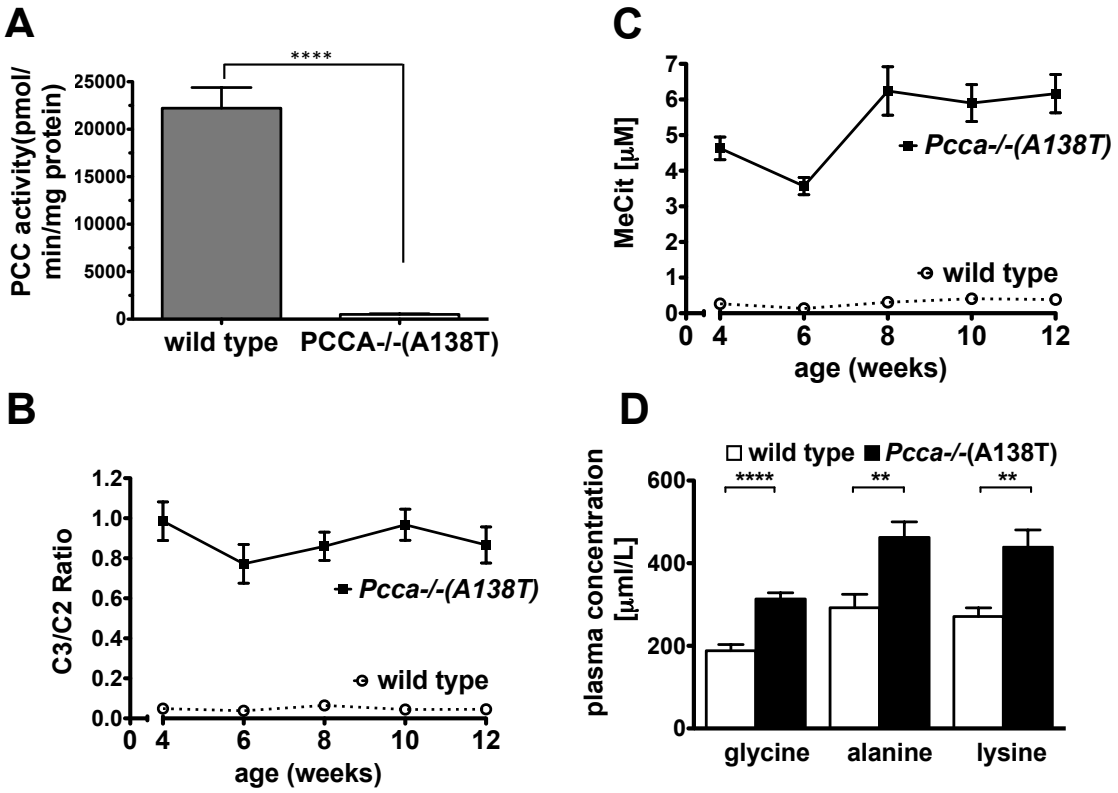

Figure 2.3. Biomarker Concentrations in PCCA Hypomorphs.

(A) Determination of PCC enzyme activity in liver of *Pcca-/-*(A138T) mice (n = 10) as compared to wild type mice (n = 3). (B) Ratio of propionylcarnitine (C3) to acetylcarnitine (C2) in dried blood spots. *Pcca-/-*(A138T) mice exhibit approximately 18-fold higher C3/C2 than wild type animals ($n \geq 12$ for all data points). (C) Levels of methyl citrate (MeCit) in the same samples ($n \geq 12$ for all data points). (D) Comparison of indicated amino acid levels in plasma of wild type or *Pcca-/-*(A138T) mice. Error bars in line and bar graphs depict standard error of mean (SEM). **** indicates $p < 0.0001$, ** indicates $p < 0.01$.

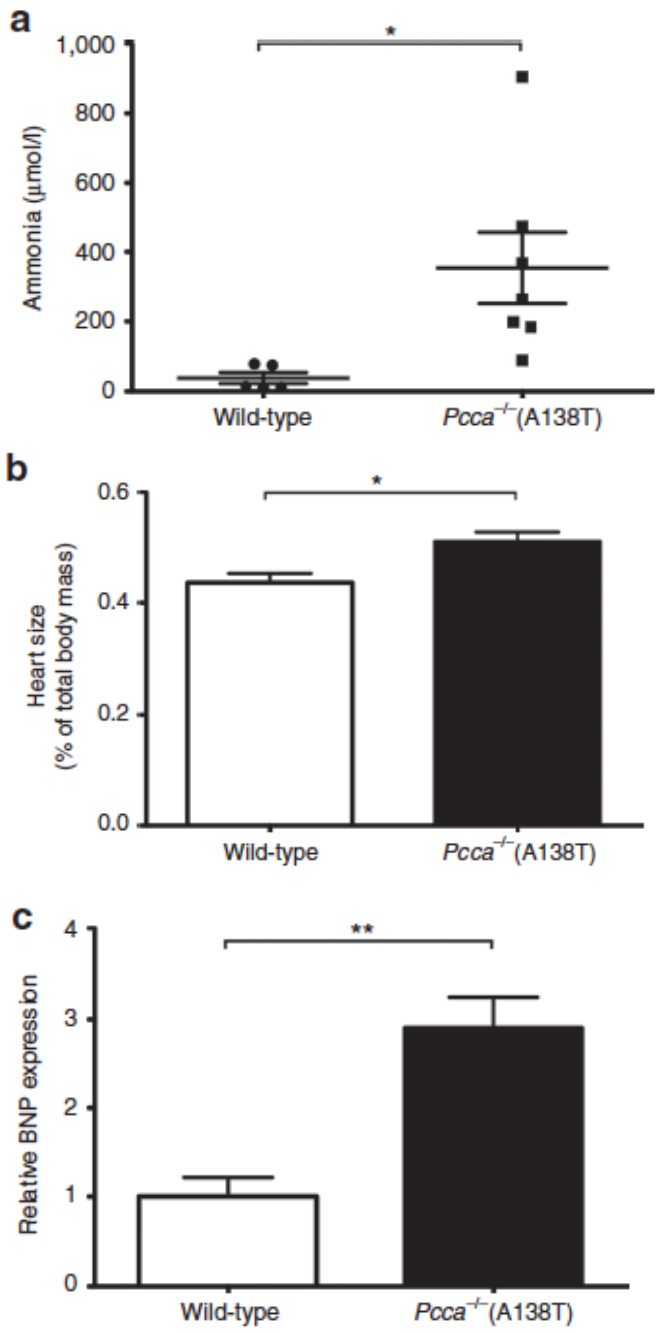

Figure 2.4. Phenotype Effector Markers.

At 8 months of age mice were sacrificed and their body mass was recorded. (A) Blood was drawn to determine concentration of ammonia in plasma samples. (B) Hearts were removed from the animals and their mass recorded and was normalized to total body mass. (C) Level of BNP mRNA obtained from mouse heart tissue was also determined. ** indicates $p<0.01$, * indicates $p<0.05$.

Gene therapy in adult *Pcca-/-*(A138T) mice.

To test gene therapy in the new mouse model, a codon-optimized human *PCCA* cDNA (hPCCAco) was inserted into a first generation Ad5 (FG-Ad5) vector and into a single-stranded AAV2/8 vector. 10-Week-old adult *Pcca-/-*(A138T) mice were injected with optimal doses of either vector: 5 x 10^{11} vg of AAV2/8 CMV-hPCCAco or 5 x 10^{10} vp of Ad5 CMV-hPCCAco by tail vein and C3/C2 ratios and MeCit levels were assayed after one week (**Figure. 5**). Treatment with either vector drove C3/C2 and MeCit below the levels observed in untreated mice, but the Ad5 vector mediated more significant reductions one week after injection (**Figure. 5**, $p < 0.0001$ by one way ANOVA). When metabolite levels were tracked over several weeks, phenotypic correction by the two vectors was quite different (**Figure. 6a and b**). FG-Ad5 treated mice had a rapid reduction in C3/C2 ratios within 1 week of treatment, but this effect was transient and rebounded within 8 weeks (**Figure. 6a**). In contrast, AAV8 induced gradual reductions in C3/C2 ratios over 8 weeks and these remained low throughout the experiment. MeCit levels generally mimicked changes in C3/C2 ratios, but with somewhat different kinetics (**Figure. 6b**).

Gene therapy in young *Pcca-/-*(A138T) mice.

FG-Ad5 and AAV8 treatment of adult mice (age 10 weeks) had detectable therapeutic effects. Given that FG-Ad5 effects were short-lived presumably due to anti-Ad immune responses, both vectors were tested again in younger 5-week-old mice (**Figure. 5 and 6**). AAV8 and FG-Ad5 treatment of 5-week-old mice drastically reduced C3/C2 ratios and MeCit levels within one weak of treatment when compared to untreated *Pcca-/-*(A138T) mice ($p < 0.0001$ for both vectors for both metabolite levels by one way ANOVA).

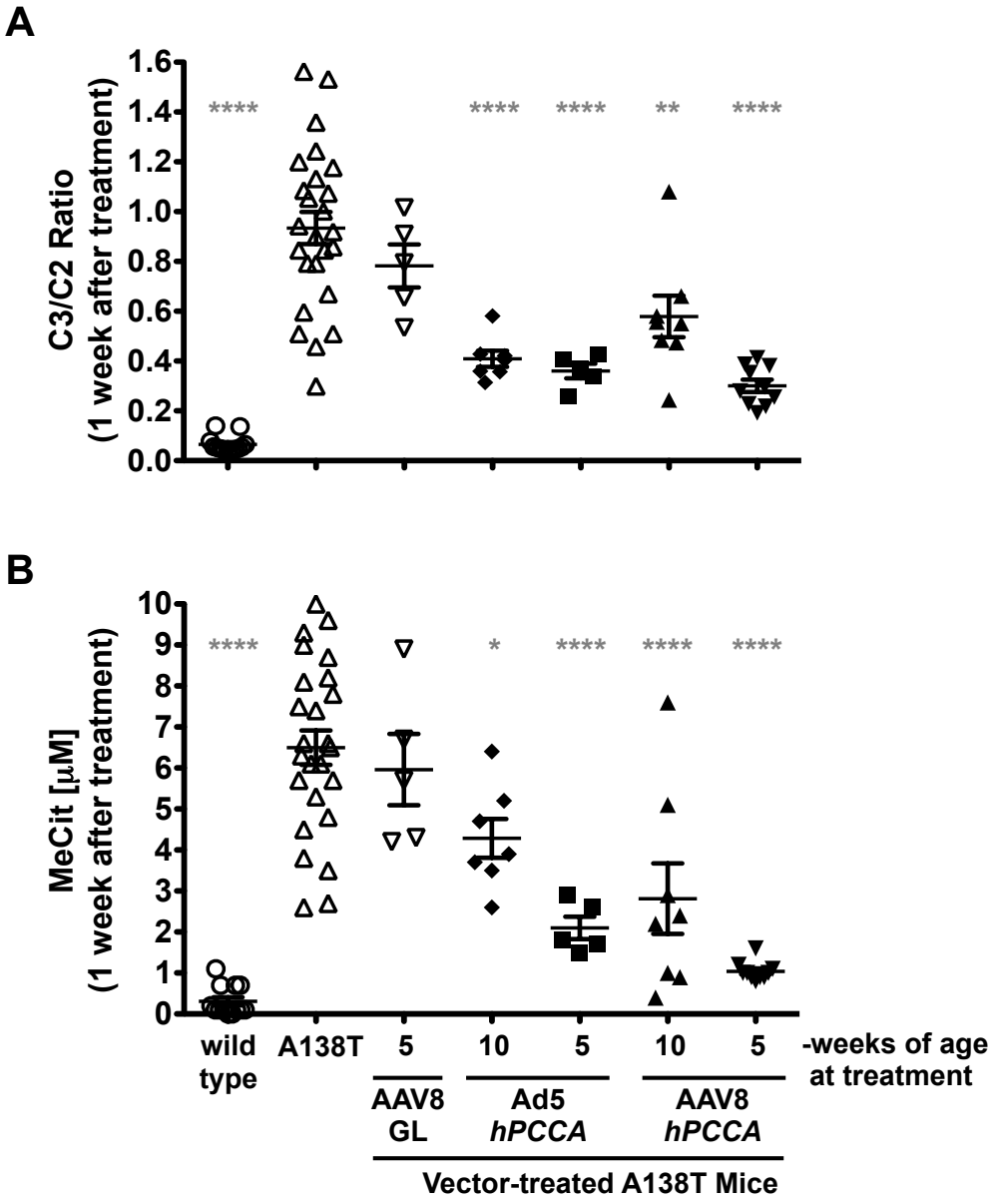

Figure 2.5. Biomarker Response to Treatment.

(A) C3/C2 ratio assayed 1 week after administration of 5 x 10^{11} vg AAV2/8-*hPCCAco* at either 5 or 10 weeks of age along with 5 x 10^{10} vp Ad-*hPCCAco* at either 5 or 10 weeks of age. (B) The same samples were analyzed for MeCit levels. **** indicates $p < 0.0001$, ** indicates $p < 0.01$, * indicates $p < 0.05$ and represent statistical significance compared to wild type. Error bars in graphs depict standard error of mean (SEM).

These metabolite levels were not significantly different than wild-type animals by one-way ANOVA. C3/C2 ratios declined over time in mice treated with AAV8 at 5 weeks, but increased over time when mice were treated with Ad at 5 weeks. MeCit levels remained low in both groups at least through 8 weeks after treatment. These results indicate that earlier administration of both AAV and FG-Ad vector greatly increased their efficacy in this mouse model. Ad and AAV treatment also significantly increased body mass in the young animals (**Figure. 6c**). Mice injected with Ad-hPCCAco and AAV2/8-hPCCAco gained 34% and 15% body mass respectively within just the first week as compared to a 7% increase observed in mice injected with control AAV2/8-GFPLuc and a 6% increase in mice injected with control Ad.

Effects of gene therapy on PCCA protein and activity levels.

To determine whether the two gene therapy vectors produced different protein levels, we assayed liver PCCA protein levels in SDS-PAGE gels using Neutravidin and PCCA antibody. PCCA is one of four proteins in mammalian cells that are covalently biotinylated by holocarboxylase synthetase [35]. Therefore, Neutravidin can detect all four. Therefore, whereas PCCA antibody is specific for our protein of interest, Neutravidin not only detects PCCA, but also detects three other proteins that serve as an internal loading control.

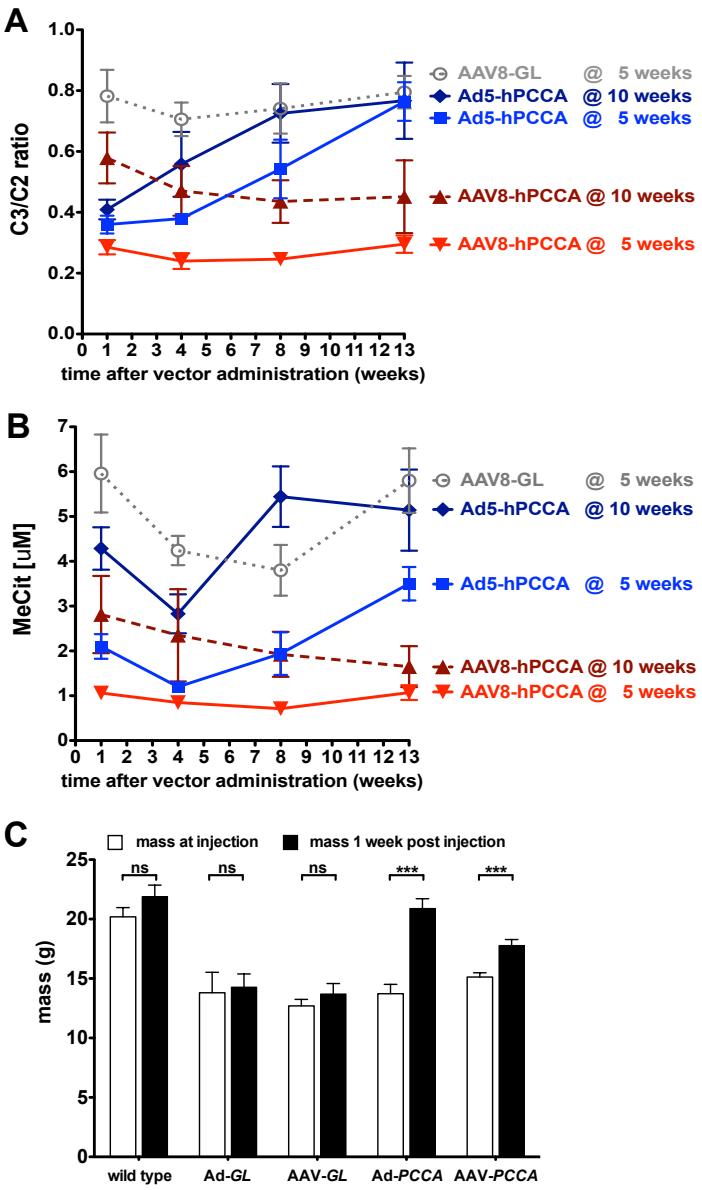

Figure 2.6. Therapeutic Response to Viral Vector Treatment.

(A) C3/C2 ratio assayed at time points up to 13 weeks after administration of control AAV2/8-*GFPLuc* at 5 weeks old (n = 5), AAV2/8-*hPCCAco* (n = 10 at 5 weeks old, n = 9 at 10 weeks old) or Ad-*hPCCAco* (n = 5 at 5 weeks old, n = 7 at 10 weeks old) as in Figure 4. (B) The same samples were analyzed for MeCit levels. (C) Body mass response one week after vector administration. Mass was recorded at 5 and 6 weeks of age for wild type and *Pcca-/-*(A138T) mice born from a *Pcca-/-*(A138T) dam. At the time of the 5-week measurement *Pcca-/-(*A138T) mice were injected with control AAV2/8-*GFPLuc* (n = 5), Ad5-*GFPLuc* (n = 5), Ad5-*hPCCAco* (n = 5), or AAV2/8-*hPCCAco* (n = 10). 5-Week mass measurements are represented in the left column and

6-week measurements are represented in the right column for each treatment data set. Error bars in line and bar graphs depict standard error of mean (SEM).

Using this system we probed PCCA protein levels in the livers of wild type mice and *Pcca-/-*(A138T) mice by western blot (**Figure. 7a**). PCCA band intensities from the livers of wild type mice were visually stronger than that from *Pcca-/-*(A138T) livers. The signal observed in the *Pcca-/-*(A138T) lane may be from residual mouse Pcca, human PCCA(A138T), or overlapping signal with methylcrotonoyl CoA carboxylase (MCC) when probed with Neutravidin. When blots were probed with PCCA antibody that recognizes both human and murine PCCA, no PCCA protein band was detected in untreated *Pcca-/-*(A138T) mice (**Figure. 7a**) consistent with the reduced protein stability of the A138T protein [9].

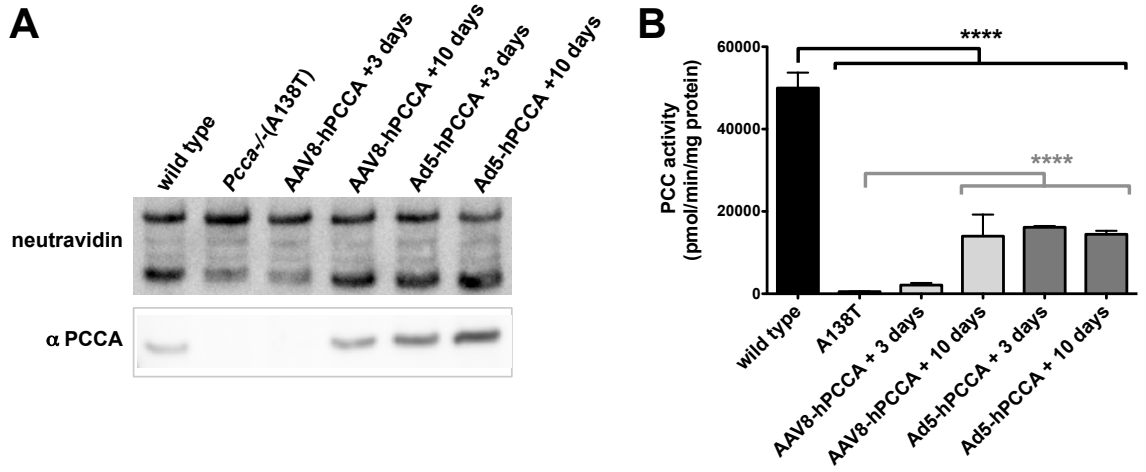

Figure 2.7. Endogenous and Treatment Protein Levels.

Pcca-/-(A138T) mice were treated with 5 x 10^{11} vg AAV2/8-*hPCCAco* or 5 x 10^{10} vp Ad-*hPCCAco* at 5 weeks of age. Livers were removed either 3 or 10 days after vector administration. (A) 25 μg of total liver protein was run on SDS-PAGE gels and blotted onto PVDF membrane. Blots were probed with either Neutravidin or anti-PCCA antibody. The upper band is consistent with pyruvate carboxylase and served as a loading control. (B) Pieces of the same livers were analyzed for PCC enzyme activity.

PCCA protein levels increased drastically in both AAV and Ad-treated mice as assessed by both Neutravidin and anti-PCCA antibody, reaching levels much higher than that seen in wild type mice (**Figure. 7a**).

When PCC enzymatic activity was measured from the livers of the same animals, activity trended in a similar manner to that for protein levels (**Figure. 7a,b**). Ad5-*hPCCAco*-induced PCC activity reached maximal levels within 3 days of treatment whereas AAV8-*hPCCAco*-induced levels were higher at 10 days post-treatment. However, although the vectors produced PCCA protein levels higher than were observed wild type mice (**Figure. 7a**), PCC enzyme activity reached only 30% of wild-type levels (**Figure. 7b**).

Discussion

This work was motivated by a need for an adult small animal model of PA where systemic therapies could readily be tested by intravenous injection. Full knock-out of mouse Pcca generated a model wherein mice die within 36 hours of birth [28] making this a challenging system with which to test intravenous delivery methods and vectors.

In the original mouse model study by Miyazaki's group, Pcca knockout was rescued by transgenesis with the mouse *Pcca* cDNA that was expressed in a largely liver-specific fashion using the serum amyloid protein (SAP) promoter. This transgene's ability to rescue Pcca deficiency provided excellent proof of principle for liver-directed gene therapy or stem cell therapy [28], but did not recapitulate human disease where PCC deficiency is cell autonomous and affects every cell in the body.

To recapitulate systemic disease and generate a less stringent mouse model, we introduced human PCCA cDNA containing a well-described hypomorphic A138T mutation under control of the promiscuous CAG promoter. Under these conditions we were able to obtain A138T transgenic mice on the *Pcca-/-* background. In these mice, the A138T human transgene partially rescued the mouse Pcca deficiency resulting in 2.2% of wild-type liver PCC activity in the mice. This enzymatic activity is interestingly similar to the 4% activity that has been directly observed in fibroblasts of a human patient homozygous for the A138T mutation [9, 30]_ENREF_8.

Numerous aspects of the *Pcca-/-*(A138T) mice were probed to determine how closely their phenotype mirrors that of human patients. The 2.2% level of PCC activity

translated to 18-fold increases in C3/C2 ratios and MeCit levels that mimicked levels observed in humans with PA. Unlike *Pcca-/-* mice that die within 36 hours, *Pcca-/-*(A138T) mice survive through the neonatal period with greater than 75% survival beyond 90 days. *Pcca-/-*(A138T) mice were sufficiently healthy to allow breeding as homozygote mutants. However, PCC deficiency in the *Pcca-/-*(A138T) dams clearly affected the health of their progeny as demonstrated by the markedly reduced size of their pups when compared to genetically identical pups that were produced from *Pcca+/-*(A138T) mothers.

Analysis of plasma amino acid levels indicated that the *Pcca-/-*(A138T) animals exhibit elevations in glycine similar to that seen in humans [36]. Additionally, increases in alanine are a consistent correlate of lactic acidosis, which is often observed in patient samples [10, 37]. Elevations in lysine are also frequently observed in PA patients and positively correlate with hyperammonemia [10, 38, 39]. We observed hyperammonemia in the mice similar to the levels observed in PA patients [32, 33]. Hyperammonemia likely causes some degree of neurological perturbation in addition to contributing to premature death in some of the animals. The relative increases in heart mass and in BNP mRNA in the hearts of *Pcca-/-*(A138T) mice also suggest some degree of cardiac involvement. While these data are suggestion, additional studies will be needed to determine how well this mouse model recapitulates cardiac involvement observed in PA patients.

These data indicate that the A138T model mimics many of the phenotypes observed in PA patients. To investigate usefulness of this model for testing systemic therapies, Ad

and AAV vectors were injected intravenously into 5- and 10-week old *Pcca-/-*(A138T) mice. Adenoviral vectors expressing codon-optimized human *PCCA* produced significant increases in growth of animals and partial correction of C3/C2 and MeCit levels. However, these effects were transient and were only observed for 4 weeks after vector administration. These results were not surprising, since first generation adenoviral vectors are known to be attenuated by immune-mediated clearance of Ad-transduced cells [40]. Future work will test if helper-dependent Ad vectors lacking all Ad genes mediate a more persistent phenotypic correction in this model.

AAV8 vectors expressing codon-optimized human *PCCA* produced more persistent and robust correction of disease marker levels. In contrast to the Ad vector, mice treated with AAV8 underwent a rapid drop in C3/C2 and MeCit that was maintained for the duration of the 13-week study. Of particular interest is the differential effect of both vectors when they were administered to mice that were 5 weeks of age rather than 10 weeks. 5 week-old animals receiving either vector had markedly stronger effects on metabolite levels as exhibited by lower C3/C2 and MeCit levels. This could be due to modification of a higher fraction of the liver by the vectors in the slightly smaller mice or be due to reduced therapeutic effects in older animals that have suffered longer from metabolic damage.

Comparison of PCCA protein levels and PCC enzyme activity levels also revealed an interesting discordance in gene therapy efficacy. Although both vectors produced PCCA protein levels that actually exceeded normal levels, this high level expression did not translate into PCC enzyme activities at or above wild type activities. This discrepancy could be due to two intrinsic features of the PCC enzyme. First, PCC consists of a

dodecamer of two protein subunits: PCCA as well as PCCB. Therefore, the failure to reconstitute 100% PCC activity may be due to imbalanced PCCA and PCCB protein expression. If so, better results may occur if both cDNAs are delivered at the same time. Dual cDNA delivery would also be appealing given that approximately 50% of PA patients have mutations in PCCB rather than PCCA. An alternate explanation for the discrepancy could be due to the fact that PCC is a mitochondrial enzyme. Therefore, both PCCA and PCCB need to be coordinately targeted to the mitochondria for activity. It is therefore possible that imbalanced expression might disrupt mitochondrial targeting. Finally, our use of human PCCA protein may not optimally combine with endogenous mouse PCCB protein and limit full PCC enzymatic activity.

Previous testing in the *Pcca-/-* mice indicated that the liver-tropic AAV8 vector has utility for treating PA [1, 29]. Our study confirms this observation, and reinforces the promise of AAV8 or other liver-tropic vectors as possible treatments for PA. Currently, the most promising form of treatment is liver transplantation. Liver transplantation relieves most of the symptoms experienced by PA patients and aids in normal development. However, liver transplantation itself poses significant risks to the patient and requires life-long administration of immunosuppressant drugs, which can be associated with an array of side effects themselves. Gene therapy with AAV or other gene therapy vectors may potentially be equally efficacious, but may have better safety margins, since they express one or a few genes rather than a whole organ of non-self antigens.

Materials and Methods

Generation of hPCCA Hypomorphic Mice. Segments of human PCCA cDNA with mutations leading to A75P or A138T defects were synthesized by GenScript USA Inc. (Piscataway, NJ). These were used to replace wild-type *Pcca* in plasmid pShuttleCMV-FL-hPCCA-IRES-hrGFP. These mutant *PCCA* cDNAs were transferred to pCALL2-D-LoxP to generate plasmids pCALL2-D-LoxP-*hPCCA*-A75P and pCALL2-D-LoxP-*hPCCA*-A138T in which hPCCA is followed by an IRES-enhanced green fluorescent protein (EGFP) element to allow screening for transgenics (**Figure. 1**). The pCALL2-D-LoxP plasmids were digested with BamHI and BsaWI and this transgene fragment was microinjected into the fertilized eggs of FVB mice by the Mayo Clinic Transgenic and Gene Targeted Mouse Shared Resource (TGTMSR). Founder mice were screened for GFP expression and by polymerase chain reaction (PCR) using primers specific for the transgene cassette (F: CGGATTACGCGTAGCATGGTGAGCAA R: GCCTAAACGCGTTTACTTGTACAGCT). Positive mice were then crossed to *Pcca*+/- mice. All resulting progeny were screened using primers specific for the endogenous mPCCA gene, neomycin resistance gene (*neo*) and the transgene cassette described previously by Hofherr *et al.* [29].

Animals. All mice were housed at Mayo Clinic under the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) guidelines. The Mayo Clinic Animal Care and Use Committee approved all animal use protocols. All animal experiments were carried out according to the provisions of the PHS Animal Welfare Policy, Animal Welfare Act, the principles of the NIH Guide for the Care and Use of

Laboratory Animals, and the policies and procedures of Mayo Clinic. Mice were fed standard PicoLab 5053 rodent chow (LabDiet, Brentwood, MO) with no substitutions throughout all experimental time points.

PCC Enzyme Activity Assay. The mouse livers were each weighed and a volume of lysate buffer (50mM Tris pH 8.0, 1mM glutathione, 1mM EDTA, protease inhibitor cocktail) equal to 3-times the weight was added. The mouse tissues were homogenized using a handheld glass tissue homogenizer. The homogenized lysate was then spun at 15,000 rpm at 4°C for 30 minutes. The supernatant was then used for the PCC radiometric activity assay performed as previously described [41]. Protein concentration was determined by the Lowry method. The PCC assay used 75 µg of liver lysate in a total of 50 µL for 15 minutes at 37°C.

Metabolite Analysis in Blood. Blood was obtained via submandibular puncture with a lancet and was spotted on Whatman 903 Protein Saver blood collection cards (GE Healthcare, Westborough, MA). Acylcarnitines and methylcitric acid (MeCit) were analyzed in the dried blood spots (DBS) by tandem mass spectrometry following protocols established for newborn screening [11, 14]. Analysis of amino acid concentrations were also performed on plasma samples using tandem mass spectrometry [42].

Plasma Ammonia Assay. Blood was obtained from anesthetized mice via cardiac puncture. Plasma was separated by spinning for 10 minutes at 1400xg and snap frozen in liquid nitrogen. Ammonia analysis was performed using the Sigma Ammonia Assay Kit

(St. Louis, MO).

Cardiac BNP Assay. Assay for brain natriuretic peptide (BNP) was performed as described by Peche *et al.* [43]. SYBR® Green Master Mix (Life Technologies, Grand Island, NY) was used with the previously published BNP primers and GAPDH reference primers (GAPDH Forward: AGCTGAACGGGA AGCTCACT GAPDH Reverse: GCTTCACCACCTTCTTGATGTC).

Adenovirus Vector Production. The human PCCA cDNA was codon optimized for mammalian expression and synthesized by GenScript USA Inc. (Piscataway, NJ). This hPCCAco cDNA was cloned into the pShuttle-CMV plasmid (Q-Biogene) and was recombined with pAd-Easy, then virus was rescued in 293A cells as described in Mok *et al* [44]. All adenoviruses were purified using two sequential ultracentrifugation CsCl density gradients and quantitated by OD260.

AAV Vector Production. Codon-optimized human *PCCA* DNA (*hPCCAco*) was synthesized by GenScript USA Inc. (Piscataway, NJ) and cloned into a CMV expression cassette (Figure 1) containing AAV2 inverted terminal repeats (ITR) and packaged with an AAV2/8 packaging system. AAV particles were produced by a procedure similar to that of Lock *et al*. with some modifications [45]. Briefly, Polyethylenimine (PEI)- based transfection of HEK 293 cells was performed in Corning Inc. (Corning, NY) 10 chamber CellSTACKs®. Plasmids were transfected at a molar ratio of 1:1:1 (Adenoviral helper plasmid: *cis* AAV Rep 2/Cap 8 plasmid: *trans* hPCCAco plasmid) and a PEI/DNA ratio of 3:1 (w/w) was utilized for transfection. The cell portion was subjected to three freeze-thaw cycles to form a lysate fraction. The media portion was clarified through 0.45 μm

Nalgene (Thermo Fischer Scientific, Waltham, MA) bottle-top filters and then concentrated by tangential flow filtration (TFF) through a disposable 0.01 m^2 Sius-LS HyStream Hydrophilic membrane with a 100 kDa Molecular Weight Cutoff (TangenX Technology, Shrewsbury, MA) housed in a NovaSet-LS LHV holder (TangenX Technology, Shrewsbury, MA) to a volume of approximately 50 mL. At this point both the cell lysate and media fractions received DNAse and were incubated for 30 min at 37ºC. Discontinuous iodixanol gradients were formed as stated in Lock *et al*. and the tubes were sealed and centrifuged at 350,000 x g in a Beckman type 70.1 Ti rotor for 120 min. Cell and media fractions were pooled separately and concentrated by centrifugation using Amicon 100 kDa molecular weight cutoff filter units (EMD Millipore Corporation, Billerica, MA).

Vector Titer. Real time PCR was performed using Sybr Green (Life Technologies, Grand Island, NY) with primers specific for the CMV promoter region (Forward: CAA GTG TAT CAT ATG CCA AG TAC GCC CC, Reverse: CCC CGT GAG TCA AAC CGC TAT CCA CGC C).

Vector Administration. AAV vectors were diluted in PBS to yield a final concentration injectable in a volume up to 100 µL. $5x10^{11}$ viral genomes (vg) per mouse of AAV8-hPCCAco were injected intravenously via the tail vein. Adenoviral vector was diluted in the same manner to yield a concentration injectable in a volume of 100 µL. $5x10^{10}$ viral particles were injected per mouse intravenously via the tail vein.

PCCA Protein Analysis. Protein blots were obtained by homogenizing sections of mouse liver in T-Per Reagent (Fischer Scientific, Rockford, IL). 25 μg of total protein was loaded onto an SDS-PAGE gel from each sample. The subsequent blot was probed with Neutravidin-HRP, and imaged for luminescence. 50 μg of total protein was loaded onto an SDS-PAGE gel from each sample for Western analysis using anti-PCCA antibody (ProteinTech, Chicago, IL). Quantitation of bands was performed using Kodak MI Software.

Data Analysis. Graphing and statistical analysis was performed using GraphPad Prism software. Statistical significance was determined using the Student's two-tailed t test for the bar graph in Figure 3, one-way ANOVA with Bonferroni post test for the line graphs and scatter plot in Figure 2-5, or log-rank tests for the Kaplan-Meier curve in Figure 2. Data are expressed as mean value ± standard error of mean in bar, line, and scatter graphs in Figures 2-5.

Acknowledgements. This work was supported by grants to M.A.B. and J.P.K. from the Propionic Acidemia Foundation (PAF). It was also supported by a grant from the Department of Laboratory Medicine and Pathology (DLMP) at Mayo Clinic. M.L.H. was supported by the Kidney Disease Research Training Program T32-DK007013.

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CHAPTER III: LONG-TERM SEX-BIASED CORRECTION OF CIRCULATING PROPIONIC ACIDEMIA DISEASE MARKERS BY ADENO-ASSOCIATED VIRUS VECTORS

A selection of this work is submitted as the manuscript: Adam J. Guenzel, Jan P. Kraus, Dietrich Matern, and Michael A. Barry. Long-Term Sex-Biased Correction of Circulating Propionic Acidemia Disease Markers by Adeno-Associated Virus Vectors. ***Human Gene Therapy*****.**

Abstract

Propionic acidemia occurs due to mutations in the PCCA or PCCB genes encoding the two subunits of propionyl-CoA carboxylase, a pivotal enzyme in the breakdown of certain amino acids and odd-chain fatty acids. There is no cure for PA, but dietary protein restriction and liver transplantation can attenuate its symptoms. We show here that a single intravenous injection of AAV8 or AAVrh10 expressing PCCA into PA hypomorphic mice decreased systemic propionylcarnitine and methyl citrate for up to 1.5 years. However, long-term phenotypic correction was always better in male mice. AAV-mediated PCCA expression was similar in most tissues in males and females at early time points and differed only in the liver. Over 1.5 years, luciferase and PCCA expression remained elevated in cardiac tissue for both sexes. In contrast, transgene expression in the liver and skeletal muscles of female, but not male, mice waned - suggesting that these tissues were major sinks for systemic phenotypic correction. These data indicate that single systemic intravenous therapy by AAV vectors can mediate long-term phenotype correction for PA. However, tissue-specific loss of expression in females reduces efficacy when compared to males. Whether similar sex-biased AAV effects occur in human gene therapy remains to be determined.

Introduction

Propionic acidemia (PA) is an inborn error of metabolism that results from mutations in the a or b subunit of propionyl-CoA carboxylase (PCC) [1]. There are early- and late-onset forms of disease, but symptoms generally manifest shortly following birth. Early symptoms include hyperammonemia, failure to thrive, poor muscle tone, vomiting, and ketoacidosis [2]. In many countries, PA is detected during newborn screening by elevations in byproducts of disrupted PCC activity. In particular, elevations in propionylcarnitine (C3) and methyl citrate (MeCit) are diagnostic for PA [3, 4].

While it is unclear exactly how the symptoms of PA manifest, they may arise from accumulation of potentially toxic metabolites in affected tissues. These may include elevations in propionic acid, propionyl-CoA, and methyl citrate (**Figure 1**). Propionic acid by itself can cause neurocognitive deficits like those observed in PA when this metabolite is injected alone into normal rats [5]. Propionyl-CoA may also cause symptoms, since it competes with acetyl-CoA [6, 7]. This competition can form N-propionylglutamate rather than N-acetylglutamate, a compound that is implicated in hyperammonemia. Propionyl-CoA may also cause elevated levels of odd-number long-chain fatty acids [8]. Finally, methyl citrate may inhibit citrate synthase, aconitase, isocitrate dehydrogenase, and phosphofructokinase activity; enzymes that are involved in glycolysis and the citric acid cycle [9].

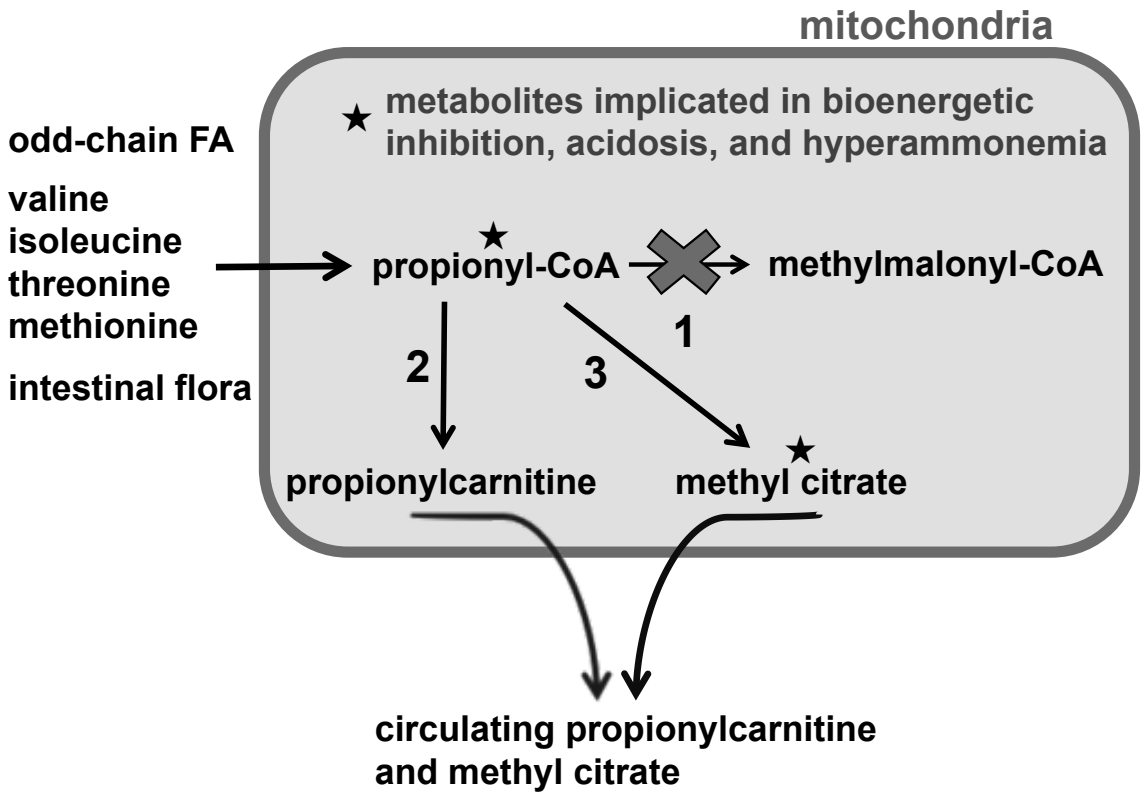

Figure 3.1. PA-Relevant Metabolic Pathways.

Several relevant disease metabolites are discussed in this publication and summarized here. The three main sources of propionyl-CoA are odd-chain fatty acids, the amino acids valine, isoleucine, methionine and threonine, and production from gut bacteria. When deficiencies of the PCC enzyme (**1**) exist propionyl-CoA concentration in the mitochondria increases and is dealt with in two ways. Addition of carnitine by carnitine acyltransferase I (**2**) to form propionylcarnitine allows export across the mitochondrial membrane and eventually into the circulation. Alternatively, propionyl-CoA is combined with oxaloacetate by the actions of citrate synthase (**3**). Both propionyl-CoA and methyl citrate have been shown to cause inhibition of necessary energy-generating processes on the mitochondria.

If PA is detected early, disease can be mediated by adopting a stringent protein-restricted diet sometimes coupled with carnitine supplementation and antibiotics. While this has been successful at mitigating the worst symptoms of the disease in many patients and significantly increasing growth at young ages [10-12], no current treatments are capable of curing PA and patients often continue to experience serious symptoms. Even with good dietary adherence acute metabolic decompensation occurs in response to infections or other illnesses due to protein catabolism [13, 14]. The result of repeated episodes of metabolic decompensation can result in neurological dysfunction, cardiomyopathy, arrhythmia, pancreatitis, and in some cases death.

More recently, elective liver transplantation has been performed in response to repeated episodes of metabolic decompensation. While this invasive intervention can blunt the worst metabolic issues in patients PCCA deficiency is corrected only the liver, and patients still have high levels of propionic acid, propionyl-CoA, and methyl citrate [15-17], likely because liver transplantation does not account for the cell autonomous nature of PA where every cell in the body is deficient for PCC and could be producing these compounds. For example, PA patients still experience neurological and cardiac complications after liver transplantation and their long-term survival is 60-70% with transplantation [18]. At this point it is unclear if these symptoms can be attributed to buildup of circulating compounds such as propionyl-CoA and methyl citrate, or if local production is the primary cause of pathology.

To explore other therapeutic options, we developed a hypomorphic model of PA in mice [19]. These mice were generated on a *PCCA* null (*Pcca-/-*) mouse background which normally die within 36 hours of birth [20]. They were rescued by transgenesis with a human cDNA encoding PCCA with an A138T mutation identified in PA patients [21]. These A138T mice survive to adulthood, have 2% of normal PCCA activity, and manifest many systemic and tissue-specific aspects of the disease that are observed in PA patients [19].

We previously showed that single intravenous injection of a first generation adenovirus 5 or adeno-associated virus 2/8 (AAV8) vectors into the A138T mice mediated dramatic reductions in propionylcarnitine and methyl citrate while improving weight gain on normal protein diets [19]. However, phenotypic correction was lost as expected over 10 weeks after adenovirus therapy, but was stable after AAV8 therapy.

Given the efficacy of AAV8, we recently compared the ability of this liver-biased vector with muscle-biased AAV1 and more broadly tropic AAVrh10 vectors [22]. Single intravenous injection resulted in significant corrections of circulating propionylcarnitine and methyl citrate by all vectors. When the muscle bias of AAV1 and the liver bias of AAV8 were made more specific by the use of tissue-specific promoters, liver-restricted AAV8-TTR-PCCA mediated better correction than muscle-restricted AAV1-MCK6-PCCA. This study helped elucidate two main points: 1) like liver transplantation, liver-restricted AAV8 therapy blunted, but did not ablate elevations in systemic PA metabolites, suggesting that liver targeted gene therapy may be a viable and perhaps safer

alternative to liver transplantation for liver-specific PA therapy. 2) The study also demonstrated that tissue-specific and tissue-biased gene therapy by AAV vectors could only blunt certain phenotypes when the disease is cell autonomous and not all cells are corrected. This finding suggests that treatment of non-hepatic tissues, particularly muscle, may provide a potential benefit in relieving some tissue-specific pathology in the disease.

In this work, we have explored the ability of AAV vectors to express transgene in multiple tissues over time and to correct systemic PA symptoms over 1.5 years after single injection. In addition, we document the effects of host sex on the ability to mediate short- and long-term systemic metabolite reduction and maintenance of transgene expression in various tissues. These findings will have implication in the design of tissue-specific gene therapy for PA and possibly other metabolic diseases.

Results

AAV treatment mediates sex-biased decreases in systemic PA metabolites.

In order to remain consistent with previous studies, *Pcca-/-(*A138T) mice were treated with a single intravenous injection by tail vein at 5 weeks of age. AAV8-PCCA was administered at 5 different doses from $5x10^{10}$ to $1x10^{12}$ viral genomes (vg) into male or female mice. AAVrh10-PCCA was also administered at the two highest doses of $5x10^{11}$ and $1x10^{12}$ vg per animal. Four weeks after injection blood was collected and analyzed by tandem mass spectrometry for elevations in the two primary diagnostic metabolites for PA: propionylcarnitine (C3) and methyl citrate (MeCit) (**Figure 1**). At this time point all doses of both AAV serotypes mediated significant decreases in both propionylcarnitine and methyl citrate when compared to untreated *Pcca-/-(*A138T) mice (**Figure 2a,b**). However, there was a notable difference in the levels of PA metabolites in male and female mice. In most cases, female mice were outliers with the highest levels of propionylcarnitine and methyl citrate.

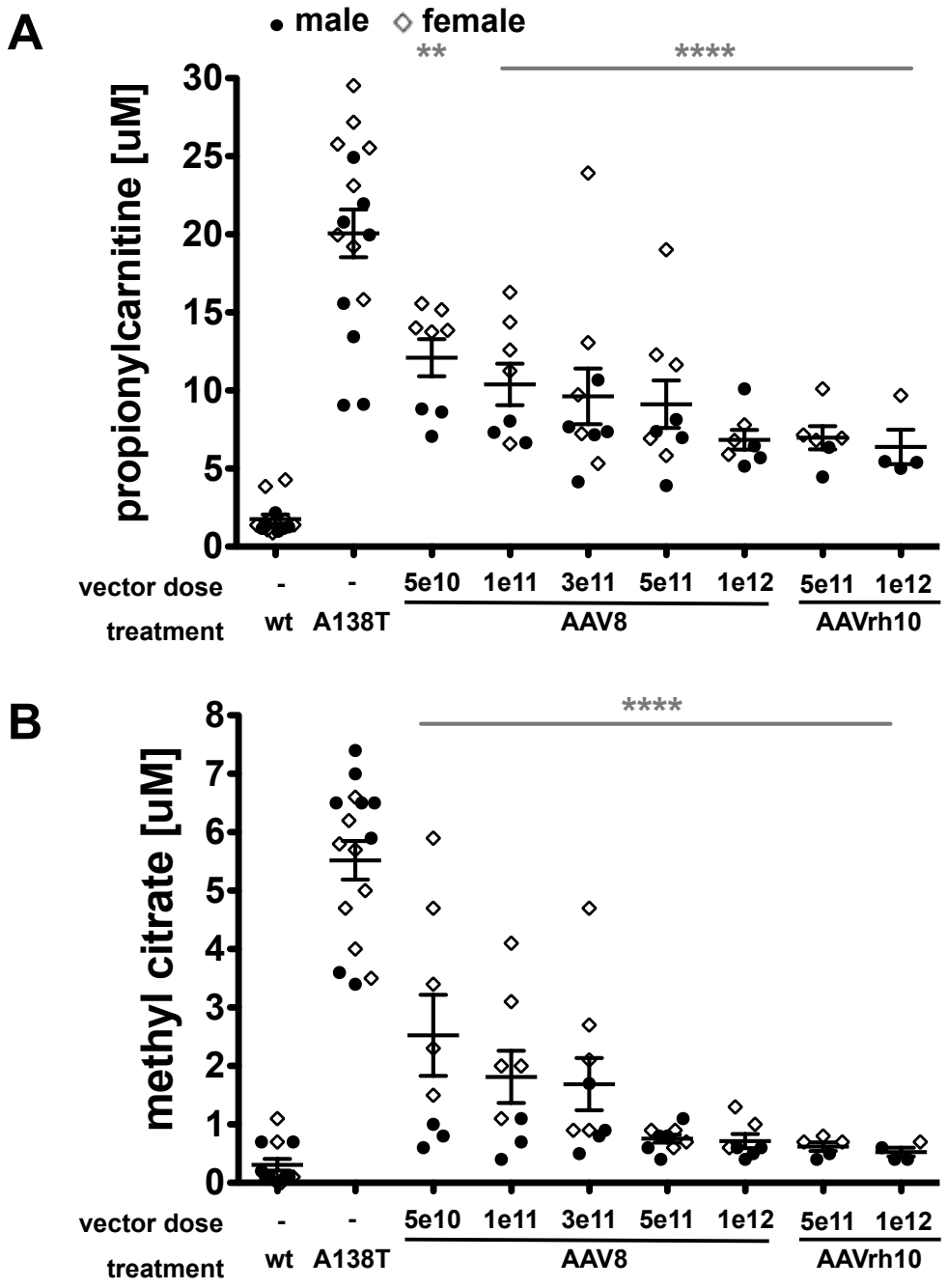

Figure 3.2. Dose-Specific Biomarker Response to Treatments.

5-week-old *Pcca-/-*(A138T) were administered indicated amounts of CMV-PCCA expression vector pseudotyped with either the AAV8 or AAVrh10 capsid. 4 weeks after IV administration of indicated vector Propionylcarnitine (a) and methyl citrate (b) levels were assayed in the blood. Mouse gender was also indicated by a closed circle for males or an open diamond for females. Asterisks indicate statistical significance of the entire treatment group compared to untreated *Pcca-/-*(A138T) mice. **** $p<0.0001$; ** $p<0.01$. Error bars depict SEM.

AAV enhances PCCA expression in multiple tissue types.

Transplantation or gene therapy for PA has focused on correcting PCC deficiency in the liver. However, essentially every cell in the body is affected by PCC deficiency, so the disease manifests in many tissues beyond the liver. To determine the degree to which AAV vectors can affect PCCA expression in different tissues, male and female mice were injected intravenously with $5x10^{11}$ vg of AAV1, AAV8, or AAVrh10 carrying PCCA cDNA and were euthanized four weeks later. Western blots with PCCA antibody revealed increased PCCA in the liver, heart, and skeletal muscle as a result of all AAV treatments, but little detectable change in kidney, pancreas, or brain (**Figure 3**). At this four-week time point, PCCA levels differed between male and females only in the liver. In contrast, cardiac and skeletal muscle PCCA expression appeared similar in male and female mice.

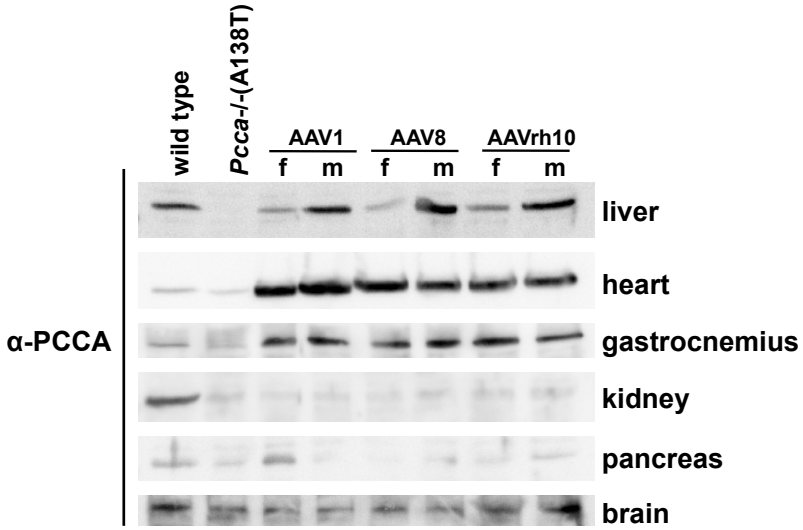

Figure 3.3. Tissue Expression Pattern of AAV Vectors.

5-week-old mice of both gender were administered an IV dose of $5x10^{11}$ vg of AAV1-, AAV8-, or AAVrh10-PCCA. 25 days after injection mice were euthanized along with age-matched wild type and untreated *Pcca-/-*(A138T) male controls. 50 μg of protein lysate for each indicated tissue was run on SDS-PAGE gel and blotted to PVDF membrane. Blots were then probed with anti-PCCA antibody and imaged.

Long-term biochemical benefits of AAV8-PCCA.

Previous studies of AAV gene therapy for PA have revealed significant reductions in PA markers in mice at early time points [19, 23, 24]. To determine if single intravenous AAV treatment could mediate long-lasting metabolite correction, propionylcarnitine and methyl citrate levels were monitored in male and female mice over 1.5 years after injection (**Figure 4**). In untreated male or female *Pcca-/-*(A138T) mice propionylcarnitine levels peaked at 13 weeks and then declined over the 1.5-year period (**Figure 4a** dashed lines). Although AAV8-PCCA therapy in males maintained low propionylcarnitine levels over time, the natural decrease in untreated males resulted in statistical significance only to 45 weeks. MeCit levels in untreated males also decreased over time (**Figure 4b** dashed black line) but in this case the single AAV8-PCCA treatment still resulted in significantly lower MeCit levels when compared to untreated PA males. AAV8 also reduced propionylcarnitine and methyl citrate levels in the female mice through 13 weeks, but the level of correction decreased by week 45 and was lost entirely by the 1.5 year time point (**Figure 4a,b**).

Long-term expression of PCCA by AAV8 varies by tissue type and gender.

At the 1.5-year post-treatment time point the study was terminated and animals were euthanized. PCCA protein expression levels in the heart, liver and gastrocnemius were measured by western blot in three treated male and female mice (**Figure 5a**). PCCA expression remained high in the heart of both male and female animals. Expression in the liver and gastrocnemius muscle remained high in male mice. In contrast, expression in liver and skeletal muscle was barely detectable in treated female mice.

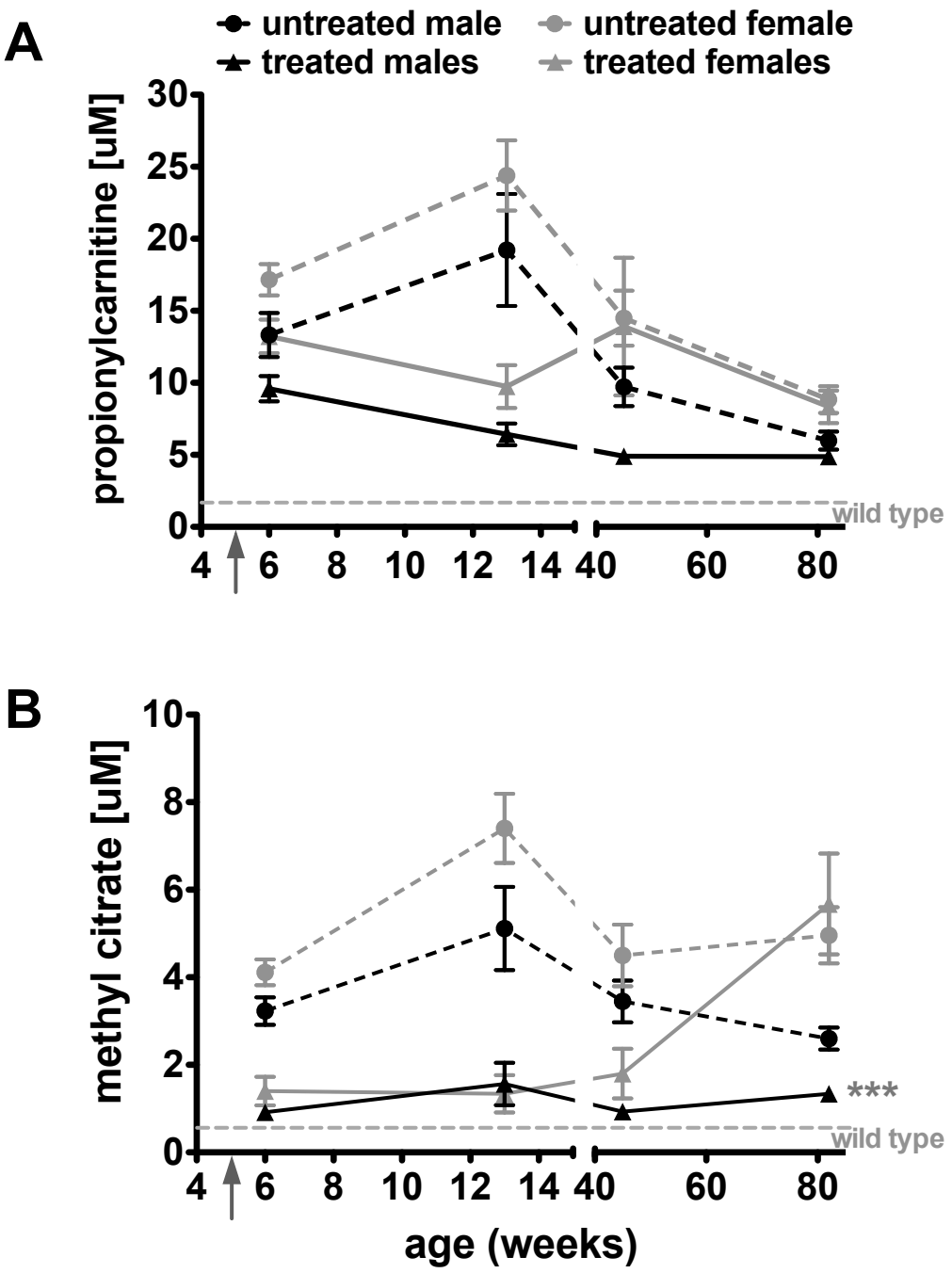

Figure 3.4. Long-Term PA Metabolite Monitoring.

C3 (a) and MeCit (b) were monitored in *Pcca-/-*(A138T) mice for 1.5 years after injection of $5x10^{11}$ vg of AAV8-PCCA. Age-matched untreated animals are denoted by dashed lines while treated animals are denoted by solid lines. N=5 for each untreated and treated group. Error bars depict SEM.

AAV8-PCCA elicits long-term increases in liver PCC activity of male *Pcca-/-(*A138T) mice.

In addition to PCCA protein levels, PCC enzyme activity was also assayed in the AAV8-PCCA treated male mice 1.5 years after vector administration (insufficient numbers of females were available for these tests). Whereas untreated A138T mice had only 2% of wild-type PCC activity, single treatment with AAV8-PCCA increased PCC activity to 15% of wild-type levels even at this late time point (**Figure 5b**).

***In vivo* luciferase expression mimics PCCA expression.**

To confirm the tropism effect observed in AAV8-PCCA treated mice, $5x10^{11}$ vg of AAV8 expressing a green fluorescent-luciferase fusion protein (AAV8-GFPLuc) were injected intravenously into male and female *Pcca-/-(*A138T) mice. 25 days later, luciferase imaging revealed transgene expression in the skeletal muscle and heart of both genders, but strong activity was only observed in the livers of the male mice (**Figure 6a**). This was consistent with differential expression of PCCA in the livers of male and female mice observed in Western blots (**Figure 3**). Imaging 1.5 years after single AAV8-GFPLuc injection revealed lower, but persistent luciferase activity primarily in the heart and skeletal muscle of both male and female mice with little detectable activity in the livers (**Figure 6b**).

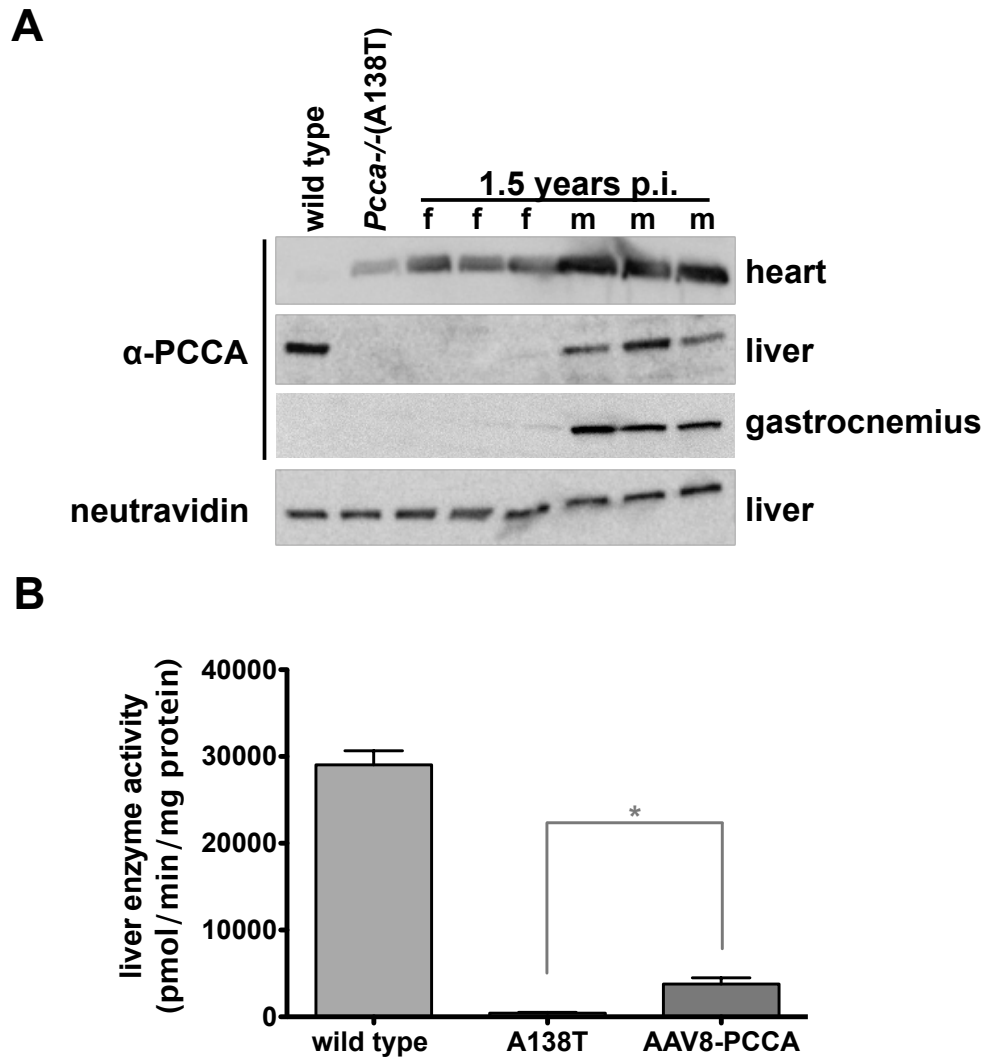

Figure 3.5. Long-Term Protein and Enzyme Activity Response to AAV.

Pcca-/-(A138T) mice treated by IV injection of $5x10^{11}$ vg of AAV8-PCCA along with age-matched wild type and untreated *Pcca-/-*(A138T) male controls were euthanized at 1.5 years after injection of the treatment animals. (a) 50 μg of protein lysate for each indicated tissue was run on SDS-PAGE gel and blotted to PVDF membrane. Blots were then probed with anti-PCCA antibody and or Neutravidin and imaged for protein expression. (b) Liver PCC activity was measured in male mice and compared to male wild type and untreated *Pcca-/-*(A138T) controls. *$p<0.05$ indicates statistical significance compared to untreated *Pcca-/-*(A138T) controls.

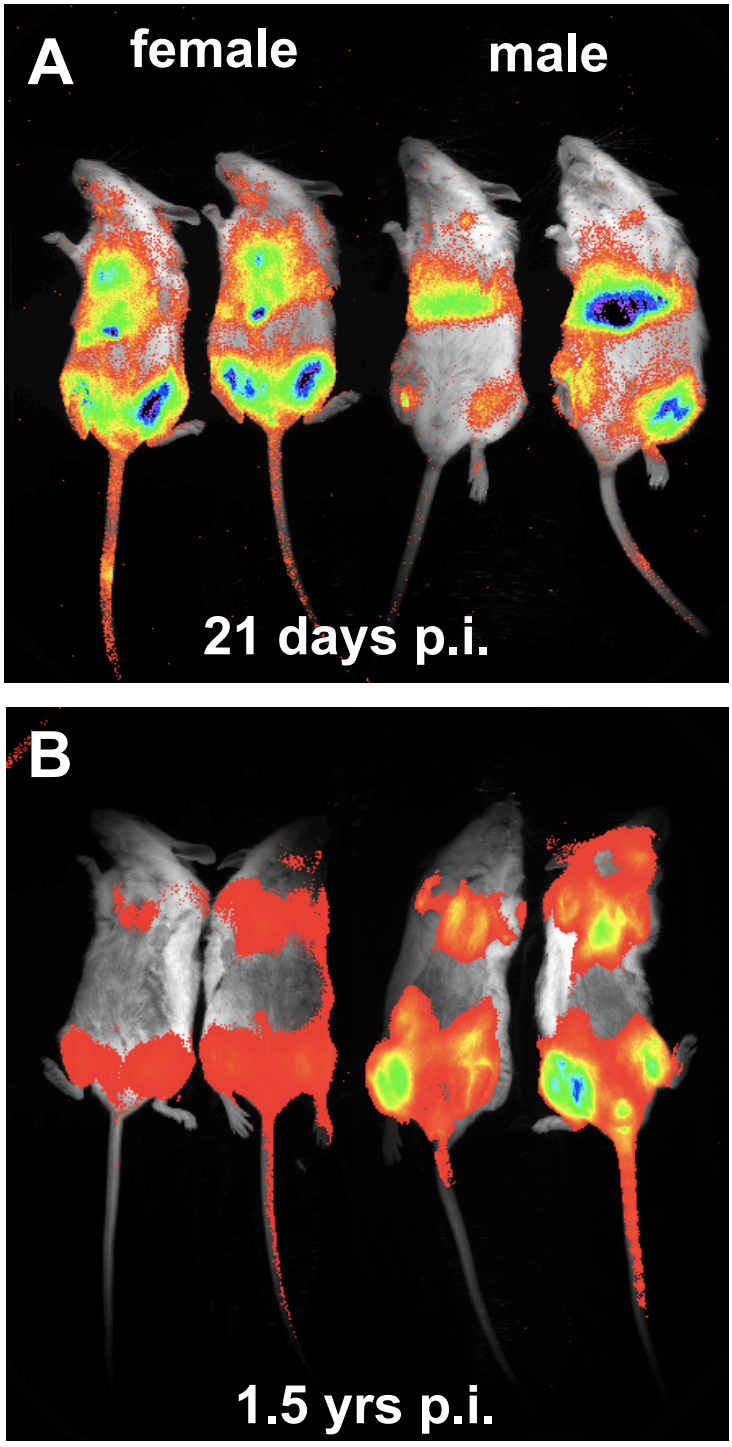

Figure 3.6. *In vivo* Luciferase Imaging of Vector Expression.

5-week-old *Pcca-/-*(A138T) mice of both gender were administered an IV dose of $5x10^{11}$ vg of AAV8-GFPLuc. The mice were injected with luciferin and imaged for 10 minutes at 21 days (a) and 1.5 years (b) after injection.

Discussion

This study demonstrates that adeno-associated virus vectors are able to provide sustained long-term expression of PCCA protein in *Pcca-/-*(A138T) mice over 1.5 years after single intravenous treatment. This expression resulted in levels of PCC activity that we believe are therapeutically relevant as determined by reductions in the levels of circulating propionylcarnitine and methyl citrate metabolites. Previous studies have provided evidence that AAV-mediated transfer of PCCA cDNA to *Pcca* deficient mice mediates significant correction of circulating metabolite levels. However, these studies only demonstrated efficacy at early time points [19, 23, 24]. This work supports the ability of single AAV treatment to mitigate some PA phenotypes over the lifespan of mice.

Previous studies demonstrated that AAV vectors mediate markedly different levels of liver transgene expression in male and female mice that were attributed to reduced second strand DNA synthesis of the AAV vector genome in the absence of androgens [25]. These studies focused on relatively short-term expression of human factor IX and did not examine expression in muscle tissue. Our findings at the four-week time point are in agreement with these earlier studies where we see higher luciferase reporter gene expression and document higher therapeutic PCCA protein expression in the livers of male mice. The present study extends this observation of differential expression between the sexes of mice by examining expression in muscle tissue and liver over long periods of time. While there was a large difference in luciferase and PCCA expression in the livers of male and female mice at four weeks, this effect was not observed to the same degree in

muscle tissues at this early time point indicating that transgene expression in muscle of female mice is not attenuated in the same way the liver is. In contrast, 18 months after treatment, the sex bias for expression was striking in both the liver and skeletal muscle as females had very little PCCA or luciferase remaining in both tissues. Interestingly, cardiac tissue appeared less affected by host sex than the other tissues at both the early and late time point. There are several possible explanations why cardiac tissue is able to maintain higher levels of expression: 1) increased co-transduction with positive and negative stranded genomes for hybridization and expression, 2) better ability to preserve single-stranded AAV vector genomes over time, or 3) more efficient conversion of single-stranded genomes to expressible double-stranded versions. The exact cause of this phenomenon in cardiac tissue remains to be determined.

The present study also builds on earlier work to explore how sex-biased transgene expression influences gene therapy efficacy specifically in PA. Four weeks after therapy, we document reduced expression in the livers, but not other tissues in female mice as compared to males. Higher propionylcarnitine and methyl citrate levels parallel less PCCA expression in the liver suggesting that the liver is indeed an important source or metabolic sink for PA metabolites. This observation is consistent with recent comparisons of liver-specific versus muscle-specific AAV therapy in these mice where liver-directed therapy worked markedly better than targeted expression in the muscle [22]. While PCCA correction in the liver was worse in females than in males, the female mice still derived a therapeutic benefit similar to that observed in males from 1 to 45 weeks after injection despite lower expression in the liver.

It is important to note that regardless of the results in female mice, male *Pcca-/-*(A138T) mice retained significant levels of PCCA expression in the heart, liver, and skeletal muscle throughout the entire 1.5-year duration of the experiment that represented a majority of the life span of a mouse. Natural decreases in C3 and MeCit in untreated PA animals can likely be explained by a decrease in the overall basal metabolic rate of the mice over time as they age coupled with decreased caloric intake indicated by lower body mass compared to wild type animals (data not shown). Increased PCC protein levels resulted in increased liver PCC enzyme activity and decreased levels of circulating propionylcarnitine and methyl citrate. The decreased levels of methyl citrate in male mice was particularly encouraging as this metabolite appears to be a more direct representation of pathological abnormalities than C3. Both propionyl-CoA and methyl citrate have been directly linked to inhibition of several biochemical processes thought to cause pathology in PA patients [9, 26].

It is notable that AAV-mediated PCCA expression persisted in the heart of male and female mice even though it waned in other tissues. PA patients frequently suffer cardiac symptoms including cardiomyopathy and arrhythmias [2]. In addition, certain hypomorphic PCC mutations do not have neonatal onset, but instead manifest as cardiac events in adults [27]. For example, a founder A1606G mutation in *Pccb* is observed in Amish and Mennonite populations (mutation identified in [28]). PCC enzyme with *Pccb* A1606G retains significant enzyme activity, so neonatal presentation is infrequent but approximately 25% of patients with this mutation develop heart failure, usually

secondary to respiratory infections [29].

These data indicate that a single systemic injection by AAV vectors can mediate long-term phenotype correction for PA. However, tissue-specific loss of expression in female mice reduces efficacy when compared to males. Whether AAV vectors mediate similar sex-biased effects in humans remains to be determined. This supports translation of AAV vectors for systemic therapy of PA in humans. The observation that AAV can mediate persistent PCCA expression in the hearts of both male and female mice suggests that this gene therapy may also be a viable option to prevent or treat cardiac phenotypes associated with early-onset or late-onset PA.

Materials and Methods

Animals. All mice were housed in animal facilities at Mayo Clinic and cared for by the department of comparative medicine according to Assessment and Accreditation of Laboratory Animal Care (AALAC) guidelines. All animal experiments were carried out according to the provisions of the PHS Animal Welfare Policy, Animal Welfare Act, and the principles of the NIH Guide for the Care and Use of Laboratory Animals. The Mayo Clinic Institutional Animal Care and Use Committee approved all procedures.

AAV Vector Production. All AAV vectors were produced by triple transfection of HEK 293 cells as published previously [19, 30]. Briefly, 293 cells were transfected with pHelper, pR/C(Rep/Cap), and PCCA or GFPLuc transgene plasmids. Cells were grown for five days in serum free DMEM containing antibiotics after which time NaCl was added to a final concentration of 0.5 M. After pelleting solid material the lysate was concentrated by tangential flow filtration, purified by ultracentrifugation through an Iodixanol gradient, and further concentrated using Amicon 100 kDa MWCO filter units (Millipore). Vector titers were calculated by quantitative real time PCR using SYBR green master mix and primers for the CMV or PCCA region of the vector DNA.

Vector Administration. AAV vectors were diluted in PBS buffer to a concentration that was injectable in a 100 µl volume. $5x10^{10}$ to $1x10^{12}$ vg were injected intravenously via the tail vein.

Blood Analyte Assays. Blood was obtained from mice via submandibular puncture with a Goldenrod Lancet (MEDIpoint Inc., Mineola, NY) and applied to Whatman 903 Protein Saver cards (GE Healthcare, Westborough, MA). Punches of blood-containing filter paper were then taken and assayed by tandem mass spectrometry as previously published [3, 4].

PCCA Protein Analysis. Tissues were removed from mice, rinsed thoroughly in PBS, and flash frozen after euthanasia by exsanguination. Tissue pieces were then homogenized in T-PER buffer and quantitated using a BCA protein quantitation kit (Pierce). 50 μg of lysate was loaded into Mini-PROTEAN® TGX™ gels (Bio-Rad), electrophoresed, and blotted onto PVDF membrane.

PCC Enzyme Activity Assay. Mouse livers were homogenized in lysis buffer (50 mmol/l Tris pH 8.0, 1 mmol/l glutathione, 1 mmol/l EDTA, protease inhibitor cocktail) then spun at 15,000 rpm for 30 minutes. Protein concentration was determined using the Lowry method and 75 μg was used in the assay described previously for radiometric determination of PCC activity [31].

Luciferase Imaging. Mice were imaged at indicated time points after injection of $5x10^{11}$ vg of AAV8-GFPLuc. Prior to imaging animals were anesthetized with a ketamine/xylezine injection, then given an intraperitoneal injection of D Luciferin. Imaging was performed using a Kodak In Vivo F imaging system (Rochester, NY) for a

period of 10 minutes. Accumulation of total luminescence was then overlaid on top of a bright field image.

Acknowledgements. AAV pseudotype 2/1, 2/8, and 2/rh10 constructs were obtained from the University of Pennsylvania Vector Core (Philadelphia, PA). We thank the *Clinical Core* of the Mayo Clinic Center for Cell Signaling in Gastroenterology (P30DK084567), Optical Microscopy Core, the Gene Expression Core, and the Advanced Genomics Technology Core at Mayo Clinic for assistance with the work. This work was supported by funding to M.A.B. from the Propionic Acidemia Foundation (PAF) and the Organic Acidemia Association (OAA). This work was also supported by the Mayo Clinic Department of Laboratory Medicine and Pathology (DLMP), the Liver Regeneration Program in the Center for Regenerative Medicine, and the Department of Molecular Medicine (DMM).

Conflict of Interest

The authors declare no conflict of interest exists regarding the work in this publication.

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CHAPTER IV: EFFECTS OF ADENO-ASSOCIATED VIRUS SEROTYPE AND TISSUE-SPECIFIC EXPRESSION ON CIRCULATING BIOMARKERS OF PROPIONIC ACIDEMIA

A selection of this work is published as the manuscript: Guenzel, A., Hillestad, M., Matern, D., and Barry, M.A. Effects of Adeno-associated Virus Serotype and Tissue-specific Expression on Circulating Biomarkers of Propionic Acidemia.

***Human Gene Therapy*. September 2014.**

Abstract

Propionic Acidemia (PA) is an autosomal recessive inborn error of metabolism caused by deficiency of propionyl CoA carboxylase (PCC). This enzyme is composed of six PCCA and six PCCB subunits and mediates a critical step in catabolism of odd chain fatty acids and certain amino acids. Current treatment options for PA are limited to stringent dietary restriction of protein consumption and some patients undergo elective liver transplantation. We previously generated a hypomorphic model of PA, designated *Pcca*-/-(A138T), with 2% of wild type enzyme activity that mimics many aspects of the human disease. In this study, we used the differing tissue tropisms of adeno-associated virus (AAV) to probe the ability of liver or muscle-directed gene therapy to treat systemic aspects of this disease that affects many cell types. Systemic therapy with muscle-biased AAV1, liver-biased AAV8, and broadly tropic AAVrh10 mediated significant biochemical corrections in circulating propionylcarnitine (C3) and methyl citrate (MeCit) by all vectors. The innate tissue bias of AAV1 and AAV8 gene expression was made more specific by the use of muscle-specific muscle creatine kinase (specifically MCK6) and hepatocyte-specific transthyretin (TTR) promoters, respectively. Under these targeted conditions, both vectors mediated significant long-term correction of circulating metabolites, demonstrating that correction of muscle and likely other tissue types in addition to liver is necessary to fully correct pathology caused by propionic acidemia. Liver-specific AAV8-TTR-PCCA mediated better correction than AAV1-MCK-PCCA. These data suggest that targeted gene therapy may be a viable alternative to liver transplantation for PA. They also demonstrate the effects of tissue-specific and broad gene therapy on a cell autonomous systemic genetic disease.

Introduction

Propionic acidemia (PA) is an autosomal recessively inherited organic acidemia that occurs as a result of mutations in either the *PCCA* or *PCCB* gene. The protein products of these genes are the α and β subunits of the mitochondrial propionyl-CoA carboxylase (PCC) enzyme [1]. The propionyl-CoA substrate of PCC is generated by several mechanisms: (1) catabolism of the amino acids isoleucine, valine, methionine, and threonine (2) beta oxidation of odd chain fatty acids [2] and (3) production by gut bacteria [3, 4]. In patients with reduced PCC activity levels propionyl-CoA accumulates in the body, eventually causing downstream elevations in several compounds including propionylcarnitine (C3), methyl citrate (MeCit), and glycine.

The incidence of PA is approximately 1 in 100,000 live births in the United States, but can be as high as 1 in 1,000 births within select populations [5]. Patients with PA are usually identified within the first few days of life when newborn screening reveals high levels of C3 and MeCit [6, 7]. Clinical signs of PA generally manifest within the first few weeks of life with hypotonia, metabolic acidosis, lethargy, vomiting, poor feeding, and failure to thrive [8]. Additional organ-specific pathologies present later in life and include cardiomyopathy, cardiac arrhythmias, neurological abnormalities, and acute pancreatitis [9].

There is no cure for PA. Treatment options are limited to reducing the production of propionyl-CoA from the previously mentioned sources of amino acids, odd-chain fatty acids, and gut bacteria. This is accomplished mainly through dietary restriction, particularly of the protein substrates. Even when strict dietary control is maintained,

patients may still undergo metabolic decompensation in response to stresses like infections [10]. Elective liver transplantation is being utilized to reduce the rate of serious complications, but transplantation itself is an invasive procedure with associated risks and doesn't actually cure the disease, as circulating C3, MeCit, and glycine remain elevated [11, 12]. Additionally, patients receiving a donor liver will require long-term immunosuppression and risk a relatively high rate of post-transplant death and graft rejection [13]. The pathological involvement of multiple organ systems and lack of complete correction with liver transplantation indicates that systemic therapy treating additional tissues will provide a benefit and result in more complete treatment response than liver transplantation alone.

Gene therapy offers a possible alternative to current approaches. Until recently, the study of PA in animals and validation of gene therapy approaches was limited by the lack of a good animal model. Knockout mice lacking functional PCCA were developed, but these animals die within 36 hours of birth, making intravenous gene therapy very difficult to administer and monitor [14, 15].

We generated a hypomorphic model of PA by introducing a human PCCA transgene harboring an A138T mutation [16]. This A138T PCCA protein with reduced activity was identified in PA patients with a mild form of the disease [17-19]. *Pcca-/-*(A138T) mice survive to adulthood, but have significant elevations of circulating C3, MeCit, glycine, and ammonia along with cardiac defects consistent with PA [16]. With this model, we were able to show that systemic gene therapy with either adenoviral (Ad5) and adeno-associated virus (AAV8) vectors expressing human PCCA mediated significant

reductions in C3 and MeCit levels. Single tail vein injection of $5x10^{11}$ vector genomes (vg) of AAV8-PCCA resulted in significant correction throughout the 13-week duration of the study [16].

Mutations in *Pcca* or *Pccb* have the potential to affect every cell in the body. Therefore transplantation or gene therapy that targets correction to only one organ, will not correct the cell autonomous disease in all affected tissues. Our goal in this study is to determine the degree to which correction of liver or muscle tissue is able to mediate the circulating metabolites associated with PA and determine whether genetic correction of large metabolic organs may serve as sites to eliminate some of the systemic byproducts of the deficiency to temper the worst aspects of the disease.

In this work, we have used the known differences in AAV serotype tropism to probe how genetic correction in the liver and muscle influence PA therapy. We tested a muscle-biased AAV1 vector, a liver-biased AAV8 vector, and a broadly tropic AAVrh10 vector for their ability to reduce systemic metabolite profiles in the A138T mice. We next made these vectors more specific, by adding tissue-specific transcriptional regulation to further restrict protein expression to liver and muscle tissue.

Results

Tissue Specificity of AAV Serotypes.

To evaluate the transduction of multiple tissue types, we used a sensitive Cre recombinase-based reporter system [20]. In this model, all cells of the mouse express the gene for membrane-targeted red fluorescent "tomato" protein (mT) flanked by *LoxP* sites followed by a gene encoding membrane-targeted green fluorescence protein (mG). When Cre is expressed in the cell, the red fluorescence gene is excised and the green fluorescence gene is activated. If AAV successfully transduces a cell, this system gives two signals: 1) Loss of red fluorescence combined with (2) activation of membrane-targeted GFP.

For this study, the Cre transgene was packaged with AAV serotype 1, 8, and rh10 capsid and $1x10^{12}$ vg of each vector were administered intravenously to mice via tail vein injection. 21 days after injection, the liver, heart, and gastrocnemius muscle were harvested to examine the tropism of each AAV serotype (Figure. 1). Examination at this dose revealed that all of the serotypes transduced all of the tissues, but with inherent biases. AAV serotype 8 produced transduction at all sites, but the highest levels of modification were observed in the liver. In contrast, the strongest signal in AAV1-Cre treated mice occurred in skeletal and cardiac muscle, with reduced, but not eliminated transduction in the liver. AAV-rh10 was more broadly tropic than either AAV1 or 8. Some cells are observed that appear to have both red and green fluorescence. It is believed that these instances represent transduction of the main cell type (i.e. myofibers or hepatocytes) but lack of transduction of endothelium lining the blood vessels. In the

heart and skeletal muscle images it may also be due to the multinucleated nature of fused myofibers where some nuclei may have been genetically altered by *cre* recombinase and other nuclei in the same myofiber have not been altered.

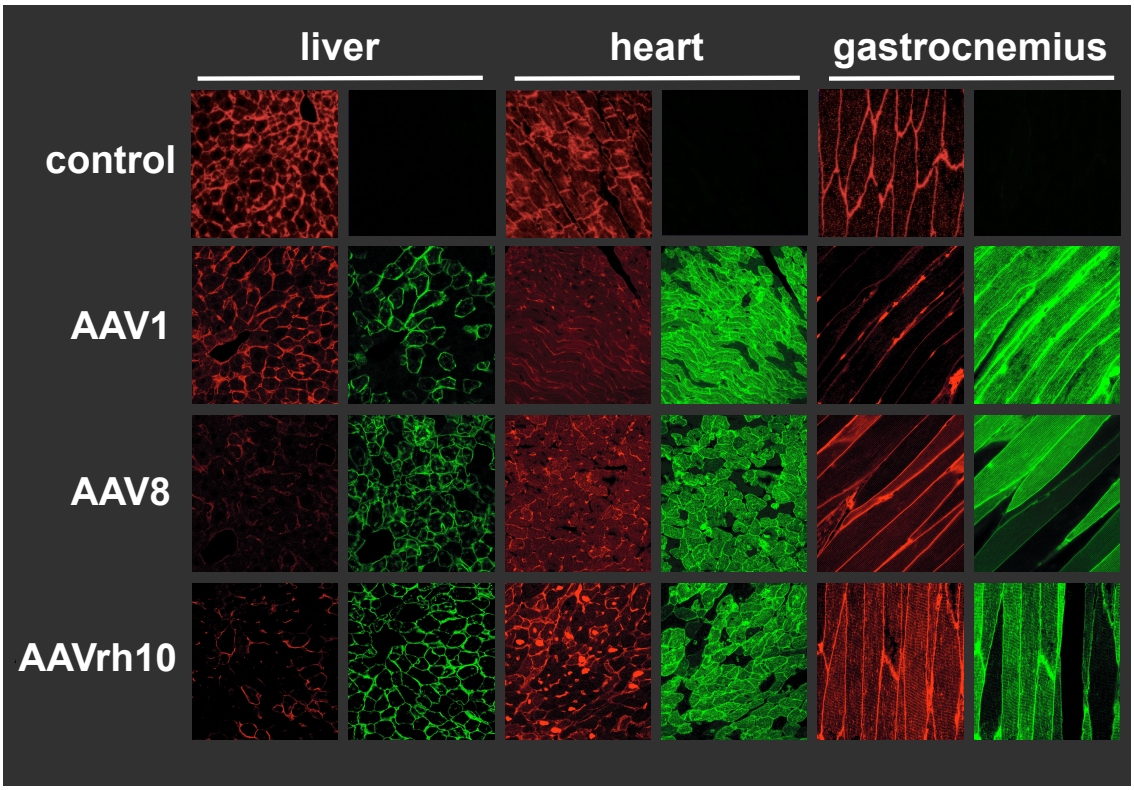

Figure 4-1. Reporter Gene Monitoring in Liver, Heart, and Gastrocnemius.

Pcca-/-(A138T) mice were intravenously injected with $1x10^{12}$ vg of AAV-cre vectors of indicated serotypes. A red to green color change in a cell of these mice indicates transduction of that cell by a cre recombinase expression vector. 25 days after injection tissues were fixed and imaged by confocal microscopy. Images shown are 400x magnification.

Endogenous PCCA Expression in Wild Type Animals.

To clarify whether or not regulation of PCCA levels in non-hepatic tissues may provide benefit for the treatment of PA, PCCA levels were analyzed by Western blot in multiple tissue types of a *Pcca*+/+ mouse (Figure. 2a). Definitive PCCA expression was observed in liver, heart, kidney, and pancreas. These tissues all represent possible treatment targets to restore normal PCC activity.

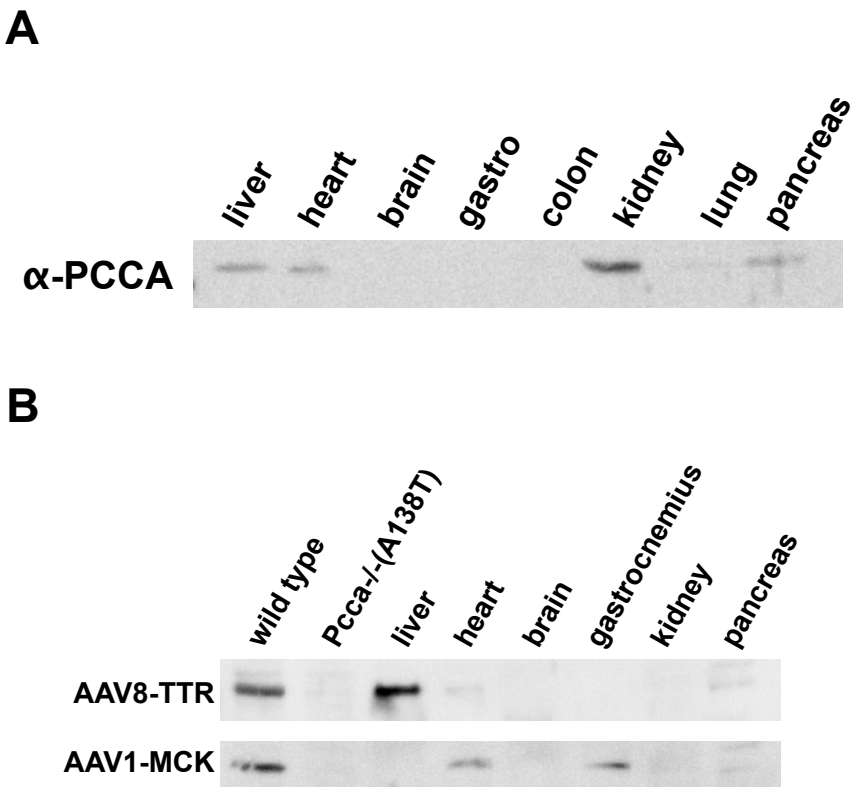

Figure 4.2. Tissue PCCA Expression Levels.

(A) Indicated tissues were removed from an untreated *Pcca*+/+ mouse. Protein content was analyzed by BCA assay and 50 μg of protein from each tissue was run on SDS-PAGE gels and blotted onto PVDF membrane, then probed with anti-PCCA antibody. (B) Indicated tissues were removed from *Pcca-/-*(A138T) mice 25 days after IV injection of $5x10^{11}$ vg of AAV1-MCK-PCCA or AAV8-TTR-PCCA. Protein content was analyzed by BCA assay and 50 μg of protein from each tissue was run on SDS-PAGE gels and blotted onto PVDF membrane, then probed with anti-PCCA antibody. Wild type and *Pcca-/-*(A138T) control lanes are 50 μg of protein from untreated liver tissue.

Systemic Gene Therapy with AAV1, 8, and rh10.

We previously demonstrated that AAV8 vectors expressing codon optimized human *PCCA* under control of the CMV promoter could attenuate disease-associated metabolite profiles in A138T mice [16]. To test alternate AAV serotypes, the same vector genome was packaged with AAV1, 8, and rh10 capsid serotypes. $5x10^{11}$ vector genomes (vg) of each were injected intravenously via tail vein into 5-week-old *Pcca-/-*(A138T) mice. One week after administration blood was collected on filter paper and assayed by tandem mass spectrometry for the PA biomarkers C3 and MeCit (Figure. 3a,b). All three of the AAV vectors mediated significant reductions in both C3 and MeCit when compared to untreated mice ($p < 0.001$ or 0.0001). While the degree of statistical significance in relation to untreated mice varied, there was no statistically significant difference between the AAV1, 8, and rh10 vectors expressing PCCA.

Transcriptional Targeting of PCCA Expression.

To further restrict the expression of PCCA protein, tissue-specific transcriptional regulatory elements were utilized in place of the promiscuous CMV enhancer/promoter. To obtain liver-specific expression we utilized the transthyretin (TTR) promoter in combination with an intron from the small minute virus of mice [21]. This TTR vector was used in the context of the liver-biased AAV8 vector to encourage the highest liver PCCA expression levels possible. To obtain muscle-specific expression the CK6 regulatory element of the muscle creatine kinase (MCK) promoter was used to drive muscle-specific expression when delivered by the muscle-biased AAV1 vector. This MCK6 promoter was previously characterized and found to have 12% of the activity of a

CMV promoter in muscle, but mediates a very muscle-restricted pattern of expression [22].

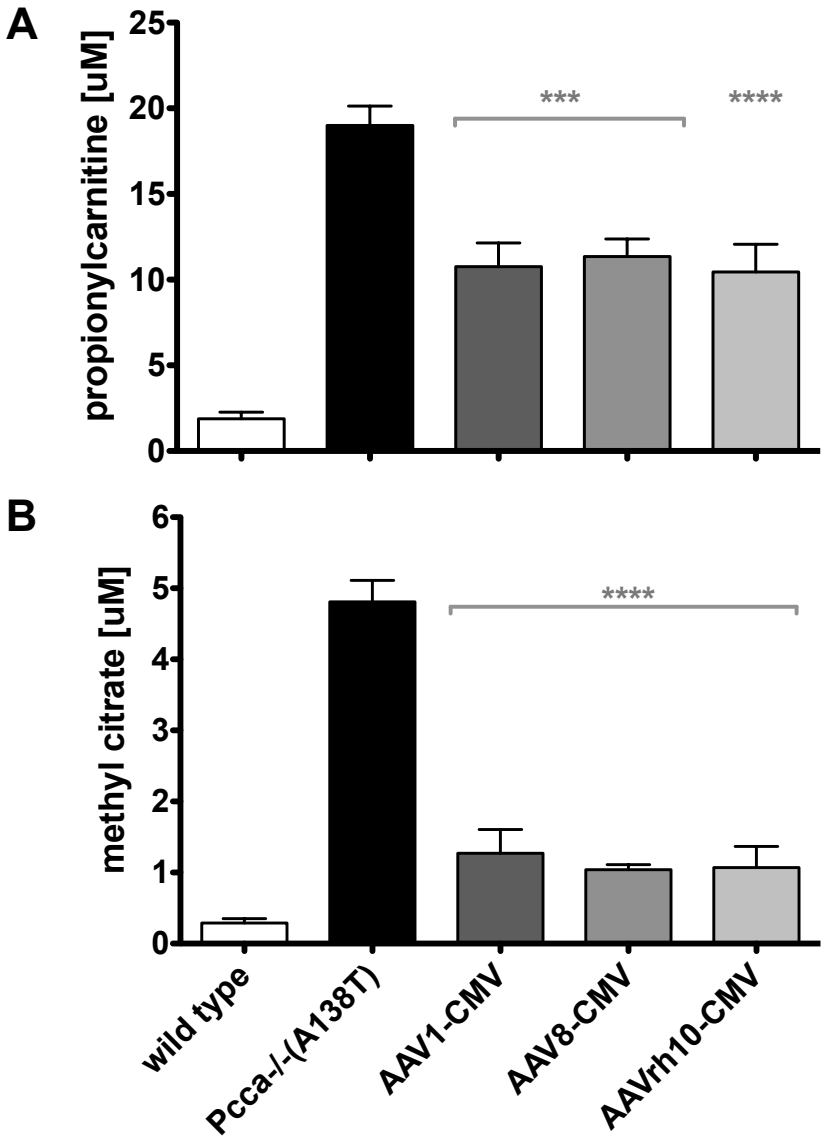

Figure 4.3. Biomarker Concentrations in *Pcca-/-*(A138T) Mice in Response to Treatment.

C3 (A) and MeCit (B) were assayed by tandem mass spectrometry 1 week after intravenous administration of $5x10^{11}$ vg of AAV1 (n=7), AAV8 (n=10), and AAVrh10 (n=10) expressing PCCA cDNA. Age-matched wild type mice were also analyzed (n=10). Asterisks indicate statistical significance relative to untreated *Pcca-/-*(A138T) mice (n=11).****P<0.001, ****P<0.0001.* Error bars depict SEM.

Pcca-/-(A138T) mice were administered $5x10^{11}$ vg of each vector by tail vein, 25 days after injection liver, heart, brain, gastrocnemius, kidney, and pancreas tissues were assayed by Western blot (Figure. 2b). In contrast to the widespread expression mediated by CMV-PCCA vectors, AAV8-TTR-PCCA mediated expression restricted to the liver and AAV1-MCK-PCCA produced expression that was restricted to the skeletal and cardiac muscles. Muscle-specific expression driven by the MCK6 promoter was predictably much weaker than what we have observed from CMV promoters [23], but heart PCCA levels in AAV1-MCK treated mice were approximately equal to heart PCCA levels in wild type mice (Figure 2a, b heart lanes).

Therapeutic Effects of Tissue-restricted PCCA Expression.

$5x10^{11}$ vg of each AAV1-MCK-PCCA and AAV8-TTR-PCCA vector were administered via single tail vein injection to 5-week-old *Pcca-/-*(A138T) mice and disease-associated metabolites were measured in the blood at 1 week and 45 weeks after injection (Figure. 4). Both the liver-specific and muscle-specific AAV vectors mediated significant decreases in the levels of circulating MeCit compared to untreated mice. MeCit correction in the AAV1-MCK group was statistically less than that mediated by AAV8-TTR or the CMV vectors ($p < 0.001$ for AAV8-CMV and AAV8-TTR compared to $p < 0.05$). Propionylcarnitine levels were significantly lower in all treated animals than untreated *Pcca-/-*(A138T) mice at the one-week time point and remained fairly constant throughout the 45-week study. However, the C3 concentration in the blood of untreated *Pcca-/-*(A138T) mice decreased naturally over time making analysis at the late time point difficult. Additionally, mice were administered a combinatorial therapy including $5x10^{11}$ vg of both AAV1-MCK-PCCA and AAV8-TTR-PCCA in a single injection. The levels

of both metabolites in response to the combination treatment was similar to that observed in the AAV8-TTR and AAV8-CMV treated mice.

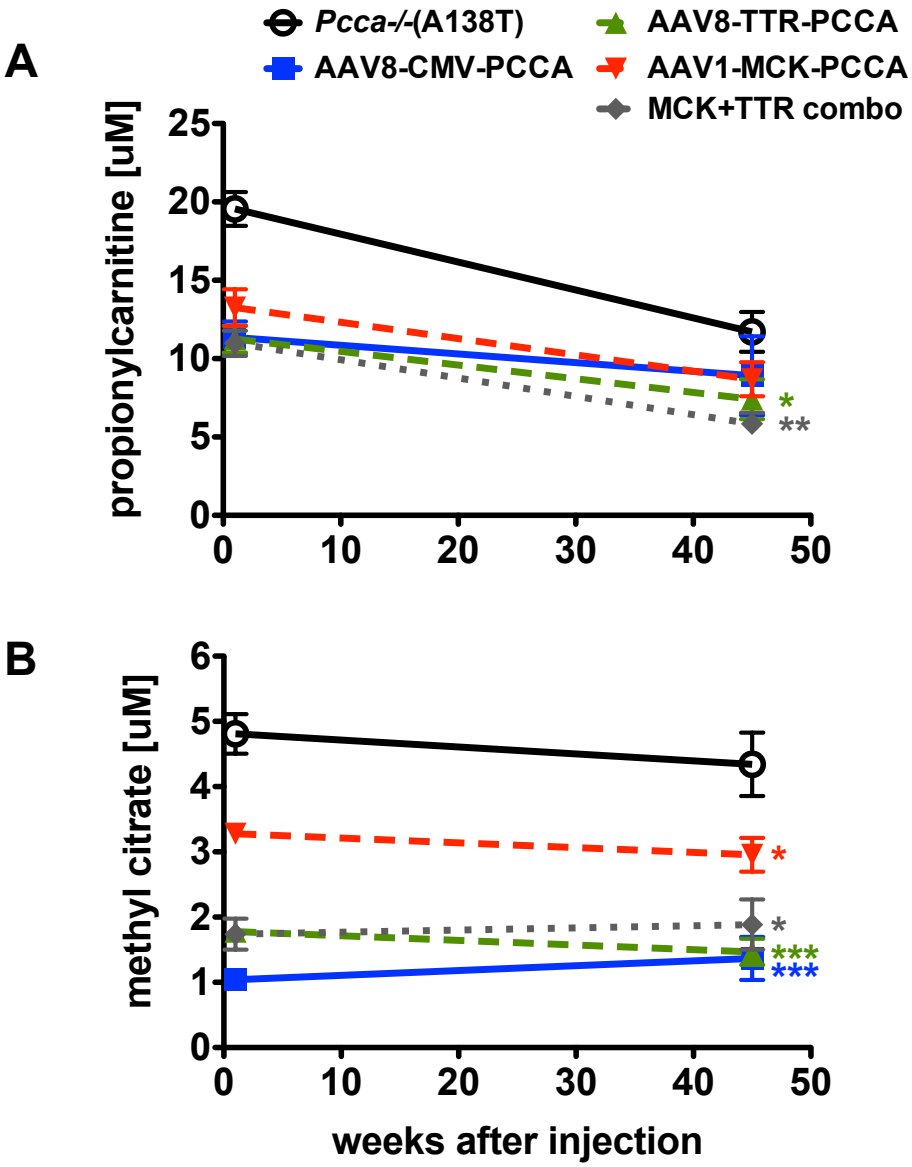

Figure 4.4. Circulating Biomarker Response to Tissue-Specific Therapy.

C3 (A) and MeCit (B) were assayed by tandem mass spectrometry 1 and 45 weeks after injection of $5x10^{11}$ vg of AAV1-MCK-PCCA, AAV8-TTR-PCCA, AAV8-CMV-PCCA, or AAV1-MCK-PCCA/AAV8-TTR-PCCA combination treatment (n=10 each). Asterisks indicate statistical significance relative to untreated *Pcca-/-*(A138T) mice at the 45-week time point. *$P<0.05$ and ***$P<0.001$. Error bars depict SEM.

Discussion

Propionic acidemia (PA) is a genetic disease that affects PCC activity in many cell and tissue types in the body. Current approaches to PA treatment involve stringent dietary restriction and more recently, elective liver transplantation. Organ transplantation is an invasive procedure that corrects only the phenotype in the liver and does not address defects elsewhere in the body. For PA, liver transplantation is able to reduce the frequency of neurologic side effects, but is unable to reduce the levels of PA metabolites. This lack of metabolite correction may explain why other phenotypes are not always corrected by liver transplantation. These observations in liver transplant recipients suggest that correction of PA in only the liver is inadequate to fully treat disease. This work explores the role of treatment of non-hepatic tissues for PA as well as tissue-specific and non-specific gene delivery by select AAV serotypes.

Our previous work and the work of others showed that treatment of PA mice with liver-biased AAV8 and Ad5 vectors is able to significantly reduce systemic PA metabolites [16, 24]. However, liver-biased vectors are not absolutely liver specific, so they may also deliver PCCA protein to additional non-hepatic cells (Figure. 1). In this study we explored the ability to modulate PA phenotypes by directing genetic correction to different organ and tissue systems – primarily the muscle.

To explore this question, we compared the ability of muscle-biased AAV1 with liver-biased AAV8 and the more broadly tropic AAVrh10 vector to reduce systemic PA metabolite levels. We found that regardless of their natural tissue bias, all three vectors mediated significant decreases in circulating levels of both C3 and MeCit at a dose of

$5x10^{11}$ vg per mouse. Restricting PCCA transgene expression to the liver and muscle by use of TTR and MCK promoters showed that both were still able to reduce systemic MeCit and C3 levels. AAV8-TTR-PCCA reduced PA metabolites to concentrations nearly as low as in mice treated with the promiscuous AAV-CMV-PCCA vectors. Muscle-specific AAV1-MCK-PCCA also resulted in significant decreases in metabolites one week after injection, albeit not to the reduced levels that were observed after administration of AAV8-TTR-PCCA or the broadly active AAV-CMV vectors. At least some of these results are similar to work restricting genetic correction to muscle for evaluating therapeutics for the treatment phenylketonuria [25]. Importantly, reductions in MeCit observed in mice treated with both tissue-specific vectors were maintained for the duration of the 45-week study. MeCit and C3 have been implicated in energetic inhibition leading to organ-specific defects as well as hyperammonemia and lactic acidemia in PA patients [26, 27]. We believe that MeCit more directly reflects propionyl-CoA levels and since these two analytes have together been implicated in bioenergetic inhibition in PA disease, MeCit is likely more significant than propionylcarnitine for evaluating efficacy of PA treatment options.

The decrease in C3 and MeCit in response to treatment with AAV8-TTR-PCCA was expected and reinforces the notion that liver gene therapy provides a therapeutic benefit. The results with AAV1-MCK-PCCA showed that treatment of muscle alone is able to reduce circulating metabolite levels as well. We expected that the combination of AAV8-TTR-PCCA and AAV1-MCK-PCCA might mimic the results of the CMV vectors and would mediate better metabolite control than either vector alone. This was surprisingly not the case, as the combination, at least at this dose, was not statistically

better than AAV8-TTR-PCCA alone. We hypothesize that treatment of the liver is still the best choice for a single-organ treatment and that liver correction may be helping to clear out some of the additional circulating C3 and MeCit produced in other tissues, but there is still locally-produced C3 and MeCit that likely remains in the tissue and can cause pathology. However, it was interesting that the MCK/TTR combination treatment did provide the greatest reduction in propionylcarnitine over time.

An ideal treatment for PA would correct the genetic defect in every cell that normally expresses the protein. Since such a treatment does not exist, one must consider all of the causes of pathology relating to circulation of toxic metabolites and local accumulation of metabolites in the tissues that needs to be reflected in the vector design.

If we are forced to treat one tissue, the liver is still the best choice for a single-organ treatment where it may serve to metabolize at least some C3 and MeCit produced by other tissues. The use of muscle-targeted therapy alone is not a preferred treatment option, but has been used in this case to demonstrate the need for expression in non-hepatic tissues. In that context, this study provides sound evidence that genetic correction of non-hepatic tissue mitigates systemic PA metabolites.

Local treatment of the cell autonomous aspects of PA may well provide benefits even if it does not affect systemic metabolite levels. It is likely that some aspects of PA result from local accumulation of disease metabolites. Preventing the buildup of propionyl-CoA and MeCit in the heart, skeletal muscle, or brain may reduce the amount of these metabolites in mitochondria and temper energetic inhibition that has been observed in these tissues. While systemic therapy likely mitigates neurologic damage due to

hyperammonemia, direct neurologic gene therapy may be the only effective method to protect the brain from locally produced toxic metabolites that may cause developmental delay, stroke, and other neurologic phenotypes.

A valid treatment option for PA must consider all of the causes of pathology relating to circulation of toxic metabolites and local accumulation of metabolites in the tissues that needs to be reflected in the vector design. We believe that treating a wide array of tissues represents the best option for PA disease correction and therefore favor an approach with PCCA expression being driven by a constitutively active promoter delivered in a broadly tropic vector serotype to transduce as many tissues as possible. Given this, broadly tropic vector serotypes like AAV9 and rh10 may have value when used in combination for systemic as well as direct neurologic gene therapy. An ideal therapy could be administered by intravenous injection as in this study, however, intracranial treatment may also be necessary to effectively transduce brain tissue.

Materials and Methods

AAV vector production. AAV serotype 1, 8, and rh10 vectors carrying *Cre* transgene were obtained from the University of Pennsylvania Vector Core (Philadelphia, PA). All PCCA expression vectors were made according to previously published protocol using triple transfection of HEK293 cells and subsequent concentration of media by tangential flow filtration (Guenzel et al 2013). All vectors were quantitated using Taqman primer/probe sets recognizing sequence in the codon-optimized PCCA gene (Life Technologies, Grand Island, NY).

Cloning. The transthyretin (TTR) promoter in combination with an intron from the small minute virus of mice (MVM) [21] were kind gifts from Dr. Paul E. Monahan (University of North Carolina at Chapel Hill). The muscle creatine kinase (MCK6) promoter was a kind gift from Dr. Jeffrey Chamberlain (University of Washington). pAAV-*TTR –PCCA* co and pAAV-*MCK -PCCA*_co were generated by addition of a MluI sites upstream of the *TTR* and *MCK* promoter regions and an EcoRI site downstream of the intron via PCR using Platinum® Taq DNA Polymerase (Life Technologies, Grand Island, NY). The PCR product was then cut with MluI and EcoRI and the fragment was ligated into pAAV-PCCA_co in place of the CMV promoter and β-globin intron.

Intravenous vector administration. Animal experiments were conducted with Institutional Animal Care and Use Committee approval in accordance with National Institutes of Health guidelines. All animals were housed and bred in Department of Comparative Medicine animal facilities (Mayo Clinic, Rochester, MN). Intravenous administration of AAV vectors was performed by single tail vein injection to 5-week-old

Pcca-/-(A138T) mice of $1x10^{12}$ vg of all AAV-cre vectors and $5x10^{11}$ vg of all AAV-PCCA vectors. AAV was administered in a volume of 100 µl for all injections. Groups of mice injected with AAV-PCCA vectors were composed of approximately 50% male and 50% female animals.

Confocal microscopy. Upon euthanasia organs were quickly removed and fixed in 4% paraformaldehyde-PBS for 4 hours at room temperature. Tissue segments were then immersed overnight in 30% sucrose-PBS solution at 4°C and transferred to 10% sucrose-PBS for an additional overnight incubation at 4°C. Tissue sections were then embedded in optimal cutting temperature (O.C.T.) medium (Sakura Finetek USA, Torrance, CA) and flash frozen in liquid nitrogen. 8µm sections were prepared using a Leica CM-850 cryostat, mounted on slides and stained using VECTASHIELD with DAPI (Vector Laboratories, Burlingame, CA). Confocal imaging was then performed using a Zeiss Axiovert LSM510 laser confocal microscope (Carl Zeiss Jena, Jena, Germany) in the Optical Morphology Core facility at Mayo Clinic (Rochester, MN).

Metabolic assays. Quantitation of propionyl carnitine and methyl citrate was performed by spotting blood on to filter paper. Blood was collected by submandibular puncture of mice with a Goldenrod Lancet (MEDIpoint Inc., Mineola, NY) and spotted on a Whatman 903 Protein Saver card (GE Healthcare, Westborough, MA). Punches of blood spots were then taken and acylcarnitine and methyl citrate levels were analyzed by tandem mass spectrometry as published previously (Turgeon 2008, 2010).

PCCA protein assays. Tissues for PCCA protein analysis were removed from euthanized mice 25 days after vector administration and homogenized in T-Per Reagent and

quantitated using Pierce BCA Protein Assay Reagent (Thermo Fischer Scientific, Rockford, IL). 50 μg of total protein in equal volumes were loaded onto 7.5% Mini-PROTEAN® TGX™ gels (Bio-Rad, Hercules, CA). Blots were then probed with anti-PCCA antibody (ProteinTech, Chicago, IL).

Data analysis. Statistical tests and graphing were performed with GraphPad Prism software.

Acknowledgements. AAV serotype 1, 8, and rh10 vectors carrying *Cre* transgene were obtained from the University of Pennsylvania Vector Core (Philadelphia, PA). The transthyretin (TTR) promoter in combination with an intron from the small minute virus of mice (MVM) was provided by Dr. Paul E. Monahan (University of North Carolina at Chapel Hill). The muscle creatine kinase (MCK) promoter was a kind gift from Dr. Jeffrey Chamberlain (University of Washington). We thank the *Clinical Core* of the Mayo Clinic Center for Cell Signaling in Gastroenterology (P30DK084567), Optical Microscopy Core, the Gene Expression Core, and the Advanced Genomics Technology Core at Mayo Clinic for assistance with the work. This work was supported by funding to M.A.B. from the Propionic Acidemia Foundation (PAF) and the Organic Acidemia Association (OAA). This work was also supported by the Mayo Clinic Department of Laboratory Medicine and Pathology (DLMP), the Liver Regeneration Program in the Center for Regenerative Medicine, and the Department of Molecular Medicine (DMM). M.L.H. received supported from the Kidney Disease Research Training Program T32-DK007013.

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CHAPTER V: SUMMARY AND FUTURE WORK

Summary

There are still barriers to overcome before gene therapy becomes a commonly approved and recognized option for the treatment of disease. This thesis was initiated with the intent of furthering gene therapy for the treatment of propionic acidemia and to hopefully provide impetus for formal toxicology studies and subsequent clinical trials. The **overall hypothesis** was that using viral gene therapy vectors to treat mice affected by propionic acidemia would result in correction of measurable disease markers. Further hypotheses were generated focusing on the optimal method of treatment relating to both the vector and the molecular basis of the disease. One such hypothesis was that treatment with AAV vectors would result in meaningful long-lasting disease correction in the liver and muscle of animals with PA, this was addressed in **chapter 3**. Another hypothesis was that full correction of the PA phenotype requires treatment of multiple tissue types, this was addressed in **chapter 4**.

Initial studies focused on generation and characterization of a novel propionic acidemia animal model by creating a hypomorphic mouse using an A138T mutation in the PCCA gene that was identified previously in human patients. This mouse model (designated *Pcca-/-*(A138T)) shared many characteristics with humans affected by PA such as decreased size and growth rate, elevations in propionylcarnitine, methyl citrate, glycine, alanine, ammonia, and BNP levels indicating cardiovascular involvement (**chapter 2**). These findings indicated that the *Pcca-/-*(A138T) model was an authentic representation of PA biochemistry in a mouse. Gene therapy vectors were then created with a codon-optimized human PCCA transgene packaged in the context of an AAV8 vector and a

first-generation adenoviral (serotype 5) vector. Studies with both of these vectors were encouraging as they were able to mediate rapid correction of metabolite levels (propionylcarnitine and methyl citrate) and cause significant weight gain as early as 1 week after intravenous administration (**chapter 2**). Correction of metabolite levels was maintained throughout the entire 13-week duration of the study in mice treated with AAV8 vector, but benefit was lost in mice treated with adenoviral vector between 8 and 13 weeks after injection. These data were presented as proof that AAV8 and Ad5 vectors expressing PCCA were able to mediate the effects of Pcca deficiency in the PA mice and the long-lasting benefit of AAV treatment led to additional studies based on that promising platform.

Although results in the initial study were very promising, 13 weeks of disease correction is insufficient for an inherited metabolic disorder with a high mortality rate. To explore the long-term efficacy of AAV-based therapy, treated mice were allowed to age for 1.5 years after injection (**chapter 3**), since the animals were treated at 5 weeks-of-age this study examined therapeutic efficacy in mice that were approximately 575 days old. This time point represents a significant portion of the lifespan of a mouse because 50% of wild type female FVB mice die by 760 days old and 50% of wild type male FVB mice die by 591 days [1]. Even at this late time point circulating methyl citrate levels remained decreased in males when compared to age-matched untreated PA mice. Moreover, analysis of tissue samples by Western blot revealed significant levels of PCCA protein in the liver, heart, and skeletal muscle of treated males and the heart of treated females. PCC activity in the liver of these aged males was elevated from 2% of wild type to 13% of wild type at this late stage as well. The implications for this long-term correction of

metabolite levels and increase of PCC activity were encouraging, and the persistent PCCA expression in muscle tissues, particularly the heart, led to another study aimed at examining the effect of gene therapy directed specifically to the liver or muscle of PA mice.

The third major study presented in this thesis attempted to determine how treatment of individual tissues impacts disease correction, the variable degree to which different AAV serotypes transduce different tissue types was initially used to probe this hypothesis. As summarized in **chapter 1** and reinforced in **chapter 4** AAV8 transduces liver to a high degree and muscle to a lesser degree while AAV1 transduces muscle to a high degree and liver to a lesser degree, AAVrh10 transduces both tissue types well but not as thoroughly as the ideal vector type for each tissue. It was hypothesized that using these different vector serotypes to deliver identical CMV-PCCA transgene cassettes to mice would result in variable amounts of disease metabolite correction based on the primary tissue type being treated. However, this was not the case as all three vectors worked equally well and resulted in significant metabolite correction (**chapter 4**). Examination of tissues by Western blot revealed that although there appeared to be subtle differences in the amount of PCCA protein expressed from these vectors in the liver, heart, and skeletal muscle, high levels of PCCA were present in all three of these tissues taken from mice treated with all three vectors. To better address the hypothesis transgene expression was further restricted using liver-specific TTR and muscle-specific MCK6 promoters in the context of AAV8 and AAV1 respectively. After administration of these vectors to PA mice metabolite levels were significantly lower in both the liver- and muscle-specific treated animals indicating that there was likely a significant amount of these metabolites

being produced within the muscle (**chapter 4**). These data provide evidence that the optimal treatment for individuals with PA will include correction of PCC activity in liver and muscle at a minimum to decrease the amount of PA metabolites such as methyl citrate being produced in these tissues. These studies also provide previously unknown insight into the molecular basis of the disease.

Hurdles to Propionic Acidemia Gene Therapy

Simply considering the molecular biology of the PCC enzyme complex reveals potential challenges to treatment with gene therapy vectors. The PCC enzyme is composed of 6 α subunits and 6 β subunits that must come together along with biotin to form the dodecameric holoenzyme. Mutations in the gene for either subunit will cause PA disease so vector design strategies must either include two different therapeutic vectors – one for deficiency in either subunit, or one therapeutic vector expressing both PCCA and PCCB to treat all individuals with PA.

Most individuals with PA are compound heterozygotes for one of the two subunits, and some mutations result in decreased protein stability while some do not. Consider a person with PA that has two different mutations in the PCCA gene, they would have normal β subunits present at high concentration in most cell types and two different hypomorphic α subunits present at variable concentrations depending on the protein stability. The location of the mutations may alter the ability of the α subunits to interact with β subunits, biotin, or the propionyl-CoA substrate. When a gene therapy vector introduces corrected PCCA cDNA, wild type α subunit is then present in transduced cells along with hypomorphic α subunits. The stability and interactions of the patient's

original mutant α subunits will likely determine the degree of correction realized by introduction of the corrected PCCA. At this point it is unknown to what degree enzyme activity may be reduced if some hypomorphic and some wild type subunits are present in the same PCC holoenzyme, so without actually removing the patients genomic PCCA, full correction of PCC activity may not be possible. However, studies performed in mice suggest that even an increase of PCC activity from 2% to 13% of wild type will result in significant systemic disease correction (**chapter 3**).

When the focus is expanded from a molecular biology perspective to a physiological perspective it is necessary to recognize PA as not just a "metabolic liver disease" but as a cell autonomous disease affecting multiple body systems. This concept has been suggested since publication of the case reports on cardiovascular pathophysiology highlighted in **chapter 1** and findings in this thesis (primarily **chapter 4**). While liver transplantation has a positive influence on the PA disease phenotype, it does not correct cells in other tissues that still have PCC deficiency. Liver-directed gene therapy has the potential to decrease the morbidity and mortality associated with transplant operations and subsequent immunosuppression required for graft survival, but other tissues would suffer the same fate as in liver transplant patients. Additionally, a typical hepatocyte life span in a human is only 5 months, meaning that any transgene present in the liver will be diluted over time and eventually lost as hepatocytes turn over. In contrast, cardiomyocytes have extremely long life spans elucidated in a study by Bergmann *et al*, which found that less than 50% of a human's cardiomyocytes are replaced during a typical person's life [2]. Targeting more stable tissues such as muscles will provide

long-lasting benefit to that tissue and potentially reduce overall circulating levels of harmful metabolites as indicated by the findings presented in **chapter 3**.

Future Work

Characteristics of an Ideal Vector for Propionic Acidemia Gene Therapy

In order to build a successful gene therapy vector for the treatment of PA there are some hurdles discussed in the previous section that must be considered. Based on the hurdles described above the ideal PA gene therapy vector would:

1. *Facilitate excision of defective PCCA or PCCB DNA to remove hypomorphic protein subunits.*
2. *Provide both PCCA and PCCB genes to enable treatment of individuals with mutations in either subunit.*
3. *Treat every cell in the body to enable correction of the cell-autonomous aspects of the disease.*
4. *Provide long-term (multiple years) transgene expression in transduced cells.*
    a. *This could be accomplished by integrating into cellular DNA at a safe site to avoid insertional mutagenesis and provide sustained expression that is passed on to subsequent daughter cells.*
    b. *In lieu of integration a vector that can be administered multiple times could also satisfy the desire for long-lasting expression.*

Some of these hurdles are more easily overcome than others and some are more important. What follows is a discussion of each of these points in the context of the common viral vectors (retroviruses, adenoviruses, and adeno-associated viruses) highlighted in **chapter 1** with focus on realistic technical design and regulatory agency approval.

Facilitate excision of defective PCCA or PCCB DNA to remove hypomorphic protein subunits. Recent Developments in several genome-editing platforms has made it possible to modify an organism's genome with relative ease. Zinc-finger nucleases, transcriptional activator–like effector nucleases (TALENS), and CRISPR-Cas technologies all use DNA or RNA probes coupled to nucleases to create double-strand breaks in specific DNA sequences and facilitate the removal or substitution of an area of DNA in the genome. At face value gene-editing technologies appear to be ideal for the removal of DNA encoding hypomorphic proteins as in PA, however, these technologies are still relatively untested in clinical settings and would exceed the size limitation present with AAV and possibly retroviral vectors when packaged along with a therapeutic transgene. While removal of endogenous mutant *PCCA* or *PCCB* genes may provide some benefit in terms of increasing PCC enzyme activity, an 11% increase of PCC activity in liver of the *Pcca-/-*(A138T) murine model significantly improves metabolic markers (as described in **chapter 3**), therefore it is unlikely that removal of hypomorphic genes will truly be required. Essentially genome editing to remove defective genes in PA patients should be considered as studies and possible clinical trials move forward, but it is probably unnecessary and would represent energy that could be better spent addressing other aspects of therapy at this point.

Provide both PCCA and PCCB genes to enable treatment of individuals with mutations in either subunit. As mentioned previously either of the PCC subunits can be implicated in PA. Ideally a vector would provide both subunits. This could not be accomplished with AAV vectors due to size restrictions, but the 2.1 kb PCCA and 1.7 kb PCCB genes along with regulatory elements could fit into both retroviruses and adenoviruses. Realistically a vector that provides both genes would likely result in more problems from a regulatory and safety standpoint than solutions from a scientific standpoint. Expressing high levels of a deficient protein in a person has a strong foundation for providing benefit, but it is possible that overexpressing high levels of a normal protein could have deleterious effects. At the very least regulatory agencies may question the merit of approving a dual gene therapy vector considering that one gene will likely have no benefit. Therefore, retroviruses, Ad, and AAV are all suitable to carry either the *PCCA* and *PCCB* genes individually in separate vectors. Unfortunately a single vector carrying only one of the PCC subunits and addressing only about half of the individuals with PA will probably be developed initially for clinical trials. With positive results an identical vector expressing the other subunit should follow the same path to clinical trials, but must not be neglected.

Treat every cell in the body to enable correction of the cell-autonomous aspects of the disease. Due to the fact that liver transplantation results in overall disease improvement treatment of every cell in the body for PA gene therapy is likely not necessary to obtain a significant benefit. However, treatment of non-hepatic tissue will likely relieve tissue-specific symptoms of PA present in the brain, heart, pancreas, and eyes of some patients. Although it will be difficult to devise one vector that can adequately treat all these tissues

at once it is possible to treat them individually when necessary. AAV vectors are very efficient at treating the liver and muscles when administered intravenously (**chapters 2, 3, 4**) and has been utilized to treat the eyes [3-6] and brain [7-9] when injected directly into those sites. Adenoviruses are often more restricted in their tissue tropism. Ad5 readily transduces liver tissue when administered intravenously, but lacks strong muscle transduction, however, addition of Ad3 knob protein significantly enhances skeletal muscle transduction [10]. Additional Ad serotypes have tropisms for the eyes, lungs, and kidneys [11] and it is likely that tropisms could be uncovered that would allow treatment of other tissues as well. Like the fiber or knob proteins of adenoviruses, the envelope glycoproteins of retroviruses can be exchanged to expand tropism to the liver, retina, CNS, and muscles among other tissues [12]. From a practical standpoint it appears that AAV vectors are best suited to treat multiple tissue types. Retro and adenoviruses have the capability, but the natural tropisms of AAV vectors are well documented and will provide a more straightforward path to successful therapy with minimal additional testing.

Provide long-term (multiple years) transgene expression in transduced cells. Achieving long-term transgene expression is likely the most important hurdle that must be addressed for an effective PA gene therapy. One way to achieve expression for multiple years is to integrate a transgene into the host genome. Retroviruses do this naturally but safety is an issue as previous trials have shown that transgenes may integrate in proto-oncogenic sites and cause mutagenesis [13, 14]. AAV vectors exist primarily as episomes but have the potential to persist for long periods of time as demonstrated clinically in hemophilia studies [15, 16] and in PA mice (**chapter 3**). First-generation adenoviruses do not

provide long-term expression in mice (**chapter 2**) or humans [17], but expression from helper-dependent or "gutless" adenoviral vectors has the ability to persist long-term as seen with AAV vectors [18]. Transgene expression can also be extended through repeat administration of vectors. Readministration of an identical vector will likely be ineffective as neutralizing antibodies resulting from the initial administration will render any subsequent vectors inactive. The multiple serotypes of Ad and AAV allow "capsid switching" where identical transgenes are packaged in different capsids to allow repeat administration. The downfall of this tactic is that each capsid would likely have to undergo full clinical trials to gain approval, making this a costly and time-consuming tactic. There are additional methods under development to extend transgene expression that will be discussed in the following section.

The three vectors discussed here are able to address the hurdles to PA gene therapy in different ways. Retroviruses, gutless Ads, and AAV are able to transduce multiple tissue types and result in long-term expression of transgene rendering any of the three a reasonable candidate for PA gene therapy. But which one represents the current best option moving towards clinical trials? Retroviruses provide the best long-term expression as they naturally integrate, but even ignoring some safety concerns it is unlikely that a replication defective retrovirus will transduce enough cells to elicit a strong therapeutic benefit. Moreover, pseudotyping with alternative glycoproteins will significantly increase the amount of work required from a regulatory approval standpoint. First-generation adenoviruses provide very high levels of expression, but rapid attenuation of this expression render them ineffective. Gutless Ads can offer both strong levels of expression and persistent expression, but multiple serotypes will likely be

required to treat at least the liver and muscles. AAV vectors solve all of these problems as single serotypes can be used to treat the liver and muscles intravenously while providing long-term expression. That is the reason why AAV vectors were chosen for a bulk of the studies presented in this thesis. Work presented here has provided sound data to support formal toxicology and preclinical trials. The following section will explore additional studies aimed at enhancing the efficacy of AAV gene therapy for PA as well as some potential studies that could be performed to probe the utility of retroviruses and gutless Ads for therapeutic use.

Additional Studies

The studies presented in this thesis have provided a framework for further gene therapy studies in PA. However, some additional studies may expand the understanding of gene therapy for PA and could help to guide future gene therapy trials.

Hypomorphic subunit interference. As mentioned in the discussion of hurdles to PA gene therapy, the effect of hypomorphic proteins on enzyme activity in corrected cells is unknown. Gene editing technology to remove hypomorphic transgenes is likely not necessary, but basic studies could be performed to determine if removal of hypomorphic genes could provide a benefit. This study would be relatively straightforward and could be as simple as transfecting hypomorphic and wild type PCCA genes into a PCCA knockout cell line. When compared to wild type PCCA transfected alone it should be clear if some mutants actually decrease optimal PCC enzyme activity. Several mutants could be tested in this way

Neurological gene therapy treatment. One of the hallmarks of PA disease is neurological involvement in the form of delayed development, seizures, cognitive disability, and movement disorders (**chapter 1**). At this juncture it is unclear whether direct treatment of the brain will be necessary to alleviate these symptoms or if the systemic treatment explored previously will correct the neurological phenotype. Since there is limited data available in humans it is critical that neurological studies using model animal systems explore the necessity of direct brain treatment. To date, neurological studies using the *Pcca-/-*(A138T) mouse model have not revealed any physiological abnormalities through behavioral, protein energetic, and histology studies (data not presented). Therefore studies designed at stressing the animals through the use of high protein diets and infection have commenced. If these studies prove ineffective it may be necessary to generate additional PA animal models using other hypomorphic genes or perhaps tissue-specific promoters to completely ablate Pcca or Pccb expression in the brain. A TTR-PCCA(A138T) construct could be made and used to create transgenic mice only expressing PCCA in the liver to enhance the neurological phenotype. Subsequent gene therapy studies can then focus on the utility of systemic treatment versus intracranial treatment with neurotropic vectors.

Prolonged liver expression. Although this thesis has focused a great deal on the utility of non-hepatic treatment, the liver is still the primary organ that must be transduced for successful PA gene therapy. The liver is also one of the hardest organs in which to maintain expression because of the high turnover rate of hepatocytes. Integration of vector genome would result in transgene being passed to daughter cells after cell division and extend expression. A recent study by Lisowski *et al* using AAV vectors

demonstrated that addition of genomic DNA homology regions to the ends of the transgene results in integration at the site of homology. Therefore "safe harbors" can be chosen that will not result in insertional mutagenesis, such as regions of ribosomal DNA [19]. This technology could be used with both AAV vectors and gutless Ad vectors to encourage integration and enhance long-term expression. These studies would be relatively straightforward as redesign of current vectors to include homology regions will allow this theory to be tested for PA. Additionally, as noted previously retroviral vectors have been avoided because of comparatively low transduction efficiency, but they are ideal vector choices in treatment of hematological disorders because *ex vivo* stem cell treatment has shown great clinical utility. Recent work identifying stem cell niches may allow selective transduction of hepatic stem cells ex vivo, with reintroduction leading to possible life-long renewing expression of transgene [20]. Current technology may make this study unfeasible at this point, but as future stem cell therapies progress it may be possible to introduce transgenes in this manner and combine cellular and gene therapy.

Gutless adenovirus studies. The studies presented in this thesis utilized both FG-Ad and AAV. While AAV proved to be superior, the FG-Ad therapy actually mediated very strong expression of PCCA transgene that lasted up to 2 months (**chapter 2**). These FG-Ad results are not insignificant and similar studies with gutless Ad could provide long-lasting expression akin to that seen with AAV. The AAV studies presented in **chapter 2 and 3** provide a framework for comparative gutless Ad trials.

Toxicology. Considering the studies presented here using AAV vectors formal toxicology studies represent a logical next step in the path towards clinical trials. Ideally toxicology studies could directly compare vectors operating off of a ubiquitous promoter

such as CMV, RSV, or CBH with a liver-specific promoter such as TTR. No negative results have been observed to date with non-hepatic expression, but a large-scale comparison would help to identify the optimal treatment method.

Concluding Remarks

Little attention is given to disorders such as propionic acidemia due to its low prevalence in most populations, but if an incidence of 1 in 100,000 births is assumed that still represents thousands of affected children in the United States alone and many more worldwide. Moreover PA is just one of many inborn errors of metabolism that could likely be treated with gene therapy and approval of one therapeutic agent will lay a framework for approval and encourage the study of therapies for other metabolic diseases. The studies and data presented here show that PA can be successfully treated using gene therapy. Additional studies may fully optimize the gene therapy vector and expression cassette, but proof-of-principle has demonstrated that we have the ability to treat this disease. With some additional testing we are hopeful that gene therapy for propionic acidemia soon becomes a reality.

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