CORRECTION OF LIVER DISEASE AND RENAL TUBULAR DYSFUNCTION BY GENETIC SELECTION

by

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LIST OF ABBREVIATIONS

AAV	adeno-associated virus
ADA	adenosine deaminase deficiency
AFP	α-feto protein
AKU	alkaptonuria
ALA	δ-aminolevulinic acid
ATN	acute tubular necrosis
AV	adenovirus
BMT	bone marrow transplantation
CNS	central nervous system
DNA	deoxyribonucleic acid
ENU	N-ethyl-N-nitrosourea mutagenesis
ES	embryonic stem cell
FAA	fumarylacetoacetate
FAH	fumarylacetoacetate hydrolase
FISH	fluorescent in situ hybridization
GABEB	generalized atrophic benign epidermolysis bullosa
GSH	glutathione
HCC	hepatocellular carcinoma
HGA	homogentisic acid
HGD	homogentisic acid dioxygenase
HPD	4-hydroxyphenylpyruvate dioxygenase

4-HPP	4-OH-phenylpyruvic acid
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HSC	hematopoietic stem cell
HTI	hereditary tyrosinemia type I
IR	inverted repeats
KLS	c-Kit+, Lin-, Sca-1+--markers for HSC
LOH	loss of heterozygosity
MAA	maleylacetoacetate
MAI	maleylacetoacetate isomerase
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cylohexanedione
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase-polymerase chain reaction
SA	succinylacetone
SAA	succinylacetoacetate
SB	Sleeping Beauty transposase/transposon
SCF	stem cell factor
SGPT	alanine aminotransferase
TAT	tyrosine amino transferase
WT	wild-type
X-SCID	X-linked severe combine immune deficiency

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ABSTRACT

The only known cure for inherited diseases of the liver and kidney is whole organ transplantation. However, organ transplantation is expensive and carries significant mortality. In addition, the number of available organ donors is limited. Thus, there is a need for researchers to develop other treatments for liver and kidney diseases. This dissertation investigates a novel gene therapy system for liver disease and offers promising evidence for cell therapy of renal disease using a mouse model ($Fah^{\Delta exon5}$) of the human disease, hereditary tyrosinemia type I (HTI).

Phage ϕC31 integrase is a site-specific recombinase that has been adapted for non-viral gene therapy applications. Specifically, ϕC31 integrase can recognize partially identical pseudo *attP* sites present in the host genome and facilitate a recombination event between the pseudo *att*P site and a vector carrying the transgene and the complementary *att*B site. Previous *in vivo* studies using ϕC31 integrase have shown that gene delivery is site-specific and stable. I further characterized the ϕC31 integrase system for liver-directed gene therapy in the $Fah^{\Delta exon5}$ mouse. Specifically, I demonstrated that a total of seven different integration sites (pseudo *attP* sites) were present within the mouse genome, which accounted for greater than 90% of all the integration events. In addition, integration of h*FAH* by ϕC31 integrase was stable and corrected hepatocytes were selectively expanded, leading to functional restoration of the liver. Although, I identified an initial abnormal hepatocyte morphology associated with integrase expression, the abnormal phenotype was transient and did not effect functional

correction of the liver disease. Thus, the integrase system may have significant utility in gene therapy settings because of the stability of transgene expression, relatively high integration frequency, and significant site-specificity.

There are few effective gene therapy systems for treatment of renal diseases; however recent studies have shown that cell therapy may be a viable approach. Bone marrow cells have been shown to localize to kidney in the presence of acute injury and aid in the regeneration process of damaged renal tubule epithelium. Given this result, I hypothesized that bone marrow cells could give rise to renal tubule epithelium and correct the renal disease associated with HTI. I found that repopulation of renal proximal tubules does occur by bone marrow-derived cells, but only in the genetically altered HTI mouse model, where renal injury is exaggerated. These bone marrow-derived renal tubule cells were obtained by cell fusion process of the hematopoietic cell with the renal tubule cell. In addition, a second mechanism for obtaining renal tubule repopulation was identified. A portion of the endogenous renal tubule cells underwent spontaneous genetic alterations, yielding a selective repopulation advantage over unaltered tubule cells. These experiments represent proof-of-principle that *in vivo* selection for genetically corrected renal tubule cells is feasible in the altered HTI mouse model.

In summary, this dissertation demonstrates correction of both liver disease and renal tubule dysfunction by genetic selection. These findings represent an important step for developing novel therapies for genetic diseases of the liver and kidney, thereby challenging the need for organ transplantation.

Chapter 1

Introduction

I. Introduction

In the United States, liver and kidney transplants for organ malfunction due to acquired or genetic disease is in large demand. Approximately, 17,000 people are waiting to receive a liver transplant and approximately 55,000 people are waiting for a kidney transplant (Donation 2005). Although the number of donors is relatively constant, increased demand has led to an overall shortage of organs. In addition, organ transplantation is expensive and carries significant mortality (Grompe et al. 1999; Donation 2005). These problems have led investigators to explore alternative treatments. For genetic diseases associated with liver failure, the option of gene or cell therapy has become increasingly promising; however, treatment of genetic renal diseases by similar therapies has been relatively unsuccessful. Thus further analysis of liver disease therapies and new approaches for treatment of renal diseases are imperative.

The long-term goal of my research is to develop innovative treatments for liver and kidney disease. Specifically, the purpose of this dissertation is two-fold. First, I characterized a novel non-viral gene therapy vector for correction of liver disease. Second, I demonstrated the feasibility of cell therapy for the treatment of renal tubule disease. All of these experiments were performed in a mouse model of hereditary tyrosinemia type I (HTI), a human disease that causes liver failure, leading to development of hepatocellular carcinoma, and renal dysfunction. Patients with HTI can be treated with a drug that minimizes the risk for liver and kidney disease, but currently, the only known cure is liver transplantation (Mitchell et al. 1999).

II. The Tyrosine Catabolic Pathway and its Diseases

Hereditary tyrosinemia type I (HTI) is a disease associated with a defect in the tyrosine catabolic pathway. L-tyrosine is a semi-essential amino acid that can be derived from either the hydroxylation of phenylalanine or the hydrolysis of dietary or tissue protein. Tyrosine has two major fates: either tyrosine is incorporated into proteins or tyrosine is degraded into two products, fumarate (a Krebs cycle intermediate) and acetoacetate (a ketone body). Tyrosine is degraded in a five-step enzymatic process, and deficiencies in four of the five enzymes in the pathway have been shown to cause autosomal recessive disease in man (La Du 1995; Mitchell et al. 1999) (Figure 1.1). The most severe disease, hereditary tyrosinemia type I (HTI), involves the last enzyme of the catabolic pathway, fumarylacetoactetate hydrolase (FAH), and is the focus of this thesis.

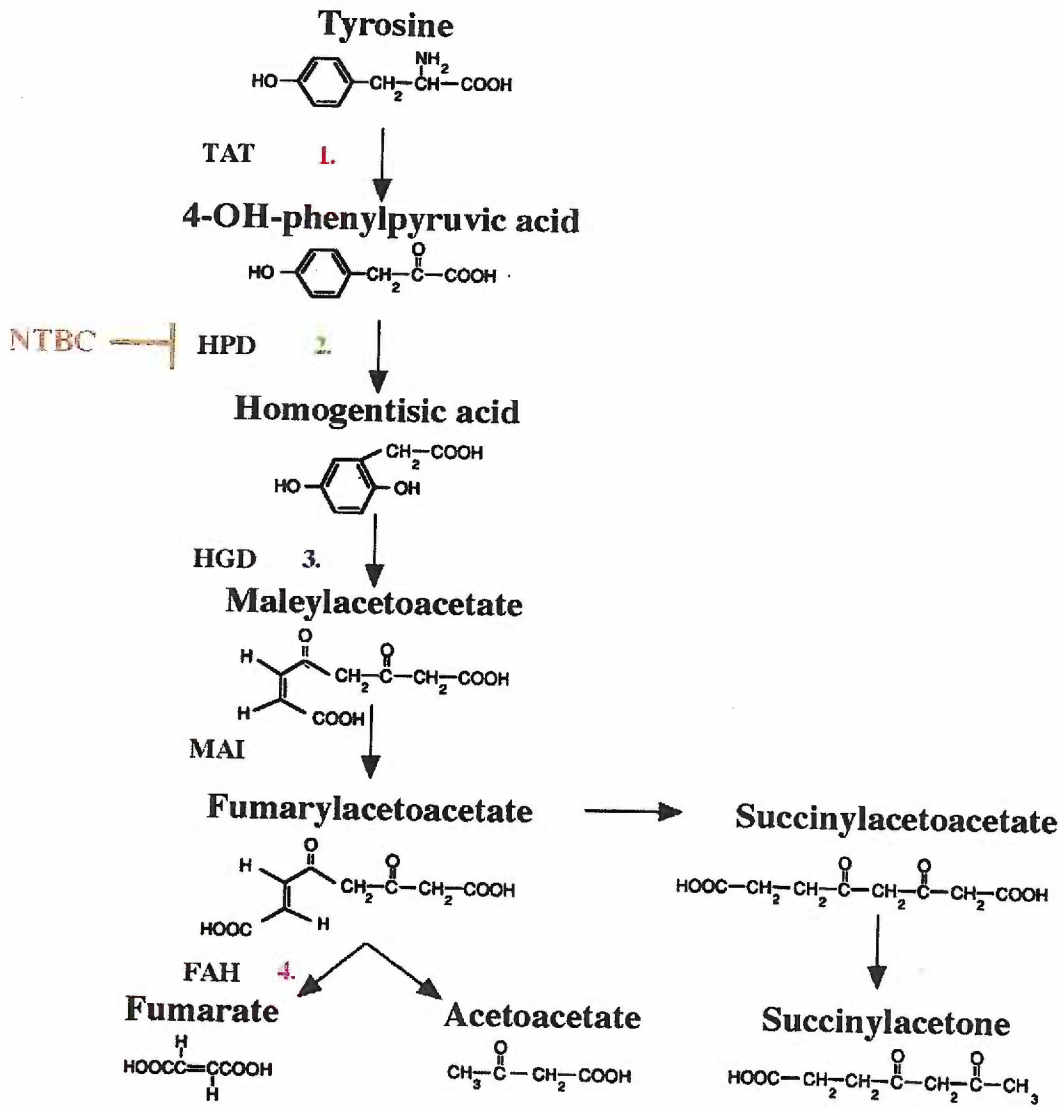


Figure 1.1. Tyrosine catabolic pathway and associated diseases.

Structures of substrates/products are represented. Defects in four enzymes of the pathway are associated with disease. 1. Defect in TAT causes tyrosinemia type II. 2. Defect in HPD causes tyrosinemia type III. 3. Defect in HGD causes alkaptonuria (AKU). 4. Defect in FAH causes tyrosinemia type I (HTI). NTBC inhibits the enzyme function of HPD and is used as a treatment for patients with HTI.

Understanding defects in other enzymes of the tyrosine catabolic pathway aids in our understanding of HTI. Tyrosinemia type II (also known as oculocutaneous tyrosinemia) is associated with the loss of tyrosine amino transferase (TAT), which is the first enzyme in the catabolic pathway. The most common clinical feature of this disease is the buildup of tyrosine, which affects the eyes, skin, and central nervous system (CNS) (Fois et al. 1986; Mitchell et al. 1999). Patients with HTI often have a decreased expression level of TAT and therefore are susceptible to the same symptoms of elevated tyrosine as tyrosinemia type II patients (Mitchell et al. 1999). Tyrosinemia type III is caused by a lack of 4-hydroxyphenylpyruvate dioxygenase (HPD), the second enzyme in the catabolic pathway. Tyrosinemia type III is relatively rare and only five cases have been documented. Patients with tyrosinemia type III accumulate less tyrosine than tyrosinemia type II patients, thus the clinical features are less severe, involving the CNS and mental retardation, but a clear phenotype has not emerged (Cerone et al. 1997; Mitchell et al. 1999). However, there is growing interest in this disorder and the consequences of the enzyme block at HPD. HTI patients are treated with the drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), which inhibits HPD (Lindstedt et al. 1992). Thereby, understanding the disease tyrosinemia type III may help us understand the effects of NTBC treatment for patients with HTI.

Deficiencies of the third enzyme of the catabolic pathway, homogentisate 1,2-dioxygenase (HGD), causes the disease alkaptonuria (AKU). Alkaptonuria is characterized by darkly colored urine in affected patients. The disease, alkaptonuria, was

the first documented example of an autosomal recessive inheritance pattern in human disease (Garrod 1902). However, it was not until 50 years later that La Du and colleagues demonstrated the reason for this dark urine phenotype—the absence of HGD activity in patients with AKU (La Du et al. 1958). Homogentisic acid (HGA), the substrate of HGD, is excreted in the urine and is oxidized, causing the dark color (Zannoni et al. 1969). The dark pigment is also deposited in tissues, causing arthritis in adults (La Du 1995). The gene mutated in AKU was first cloned in fungus (Fernandez-Canon et al. 1995) and then subsequently the human homolog was found, allowing for mutation detection (Fernandez-Canon et al. 1996).

A mouse model of AKU was created through random N-ethyl-N-nitrosourea (ENU) mutagenesis (Montagutelli et al. 1994). Similar to the human disease, mice with AKU excrete large amounts of HGA in their urine giving it a black color. However, the mutant mice do not show any evidence of joint disease. The identification of the specific mutation in the *Hgd* gene of AKU mice was recently reported (Manning et al. 1999). Mice that are both heterozygous for *Hgd* and mutant for *Fah* (the gene that causes HTI) were used for studies described in chapter three.

The fourth enzyme in the catabolic pathway is maleylacetoacetate isomerase (MAI), and to date a human disease has not been associated with a defect in this enzyme (Mitchell et al. 1999). Interestingly, mice deficient for MAI are healthy, even though the substrate of MAI, maleylacetoacetate (MAA) is toxic. It was shown that MAA can be

metabolized independent of MAI, thereby alleviating the toxic effects of MAA and yielding healthy MAI deficient mice (Fernandez-Canon et al. 2002).

Hereditary tyrosinemia type I

The most severe disease of the tyrosine catabolic pathway is hereditary tyrosinemia type I (HTI), which is also known as hepatorenal tyrosinemia (Lindblad et al. 1977). Hereditary tyrosinemia type I is characterized by severe liver damage, liver cancer, and renal tubule dysfunction. HTI is caused by defects in the last enzyme of the catabolic pathway, fumarylacetoacetate hydrolase (FAH). FAH is mainly expressed in hepatocytes and renal proximal tubular epithelium, but can also be detected in other tissues, such as lymphocytes, fibroblasts, amniotic fluid cells, and chorionic villus material. However, all five enzymes of the catabolic pathway are present together only in the liver and proximal tubules of the kidney and therefore affected in HTI patients develop both liver disease and renal failure (Mitchell et al. 1999).

Both acute and chronic forms of HTI exist. The acute form generally presents in the first months of life with liver failure and if left untreated will result in death. Patients with the chronic form have a longer progression of liver and renal proximal tubular disease. Patients with the chronic form are generally diagnosed in early childhood with symptoms of hepatomegaly, liver cirrhosis, hypophosphatemic rickets or some combination of these symptoms (Russo et al. 1990). The risk of developing hepatocellular carcinoma (HCC) can be as high as 37% early in life (Weinberg et al. 1976). HTI patients can also have episodes of porphyria-like neurologic crisis (Mitchell

et al. 1990). Biochemically, patients show increased α-fetoprotein (AFP) levels and plasma amino acids, including tyrosine and methionine (Mitchell et al. 1999). In addition, patients accumulate succinylacetoacetate (SAA) and succinylacetone (SA); the latter can be detected in the urine and is diagnostic for the disease (Lindblad et al. 1977; Mitchell et al. 1999).

HTI occurs worldwide, but the frequency is variable. In general the incidence rate is estimated to be approximately 1/100,000. However, in some parts of Quebec, the frequency is as high as 1/700 and 1/14 individuals is a carrier of the tyrosinemia trait. The *FAH* gene has been cloned (Phaneuf et al. 1991), and many mutations have been identified, including the predominant mutation in the French Canadian population (Grompe et al. 1994; St-Louis et al. 1997).

Current treatment for hereditary tyrosinemia type I

For a long time, dietary restriction of phenylalanine and tyrosine was the only treatment available for patients with HTI (Bodegard et al. 1969). Disease progression was slowed by dietary restriction, but patients still developed HCC (Mitchell et al. 1999). In the early 1980's, liver transplantation became the first cure for HTI and has been used successfully in many patients (Mohan et al. 1999). However, liver transplantation is expensive, carries significant morbidity, and requires long-term immunosuppressive therapy (Grompe et al. 1999).

In 1991, clinical trials for HTI began using the drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1.3-cyclohexanedione (NTBC) (Lindstedt et al. 1992). NTBC is a bleaching herbicide that inhibits the activity of HPD. Inhibiting HPD blocks the catabolic pathway, preventing formation of fumarylacetoacetate (FAA) and its derivative, succinylacetone (SA), which are thought to be the cause of the liver disease in HTI (Mitchell et al. 1999). Currently, over 300 patients world wide have been treated with NTBC and most showed signs of improved liver function with a decreased risk of HCC (Holme et al. 1998). However, in many NTBC-treated HTI patients, tyrosine blood levels are elevated, causing symptoms such as corneal ulcers (Giardini et al. 1983). Therefore, dietary restriction of phenylalanine and tyrosine are recommended, in addition to NTBC treatment (Holme et al. 1998).

Mouse models of hereditary tyrosinemia type I

There are four different mouse models of HTI. The first model described was a lethal albino mouse, C^{14CoS}, which was created by X-ray induced deletion (Russell et al. 1979). The gene encoding *Fah* was mapped to the deletion interval in these mice and later suggested to be the primary cause of lethality in this mouse model (Klebig et al. 1992; Ruppert et al. 1992). This was later confirmed by rescue of the C^{14CoS} mouse by introduction of the *Fah* gene (Kelsey et al. 1993). Creation of a second HTI mouse was accomplished by targeted disruption of exon 5 of the *Fah* gene (Grompe et al. 1993). The $Fah^{\Delta exon5}$ mouse has an identical phenotype to the C^{14CoS} mouse, including death in the neonatal period from hypoglycemia and liver dysfunction (Grompe et al. 1993). NTBC was shown to rescue the lethal phenotype in the $Fah^{\Delta exon5}$ mouse. However, when

Fah$^{\Delta exon5}$ mice are taken off NTBC liver disease and hallmark features of HTI, such as increased AFP and SA in the blood and urine, are present within two weeks. *Fah*$^{\Delta exon5}$ mice also demonstrate renal tubular disease along with elevated urine amino acid levels (Grompe et al. 1995).

Two additional mouse models were created by ENU mutagenesis, *Fah*^{5961SB} and *Fah*^{6287SB}. The *Fah*^{5961SB} mice have a phenotype similar to that of patients with the acute form of HTI, while *Fah*^{6287SB} mice display a phenotype similar to the chronic form (Aponte et al. 2001).

III. Pathophysiology of Hereditary Tyrosinemia Type I

The complete tyrosine catabolic pathway functions only within hepatocytes and renal proximal tubular epithelium, and therefore FAH is mainly expressed within these tissues (Ruppert et al. 1990) (Figure 1.2). However, the exact mechanism by which hepatic and renal symptoms arise when FAH is absent is unknown. One hypothesis is that FAA and FAA's derivative, succinylacetone (SA), are the primary metabolites that cause HTI because hepatic and renal symptoms do not occur in diseases associated with deficiencies of upstream enzymes in the pathway (Mitchell et al. 1999). SA is measured at high concentrations in the urine and plasma of HTI patients, but FAA levels are not elevated (Mitchell et al. 1999). This data suggests that FAA may act in a compartmentalized manner, while SA acts globally. Many studies have provided evidence that FAA and SA are main contributors to the hepatic and renal disease. These studies on HTI physiology are outlined below.

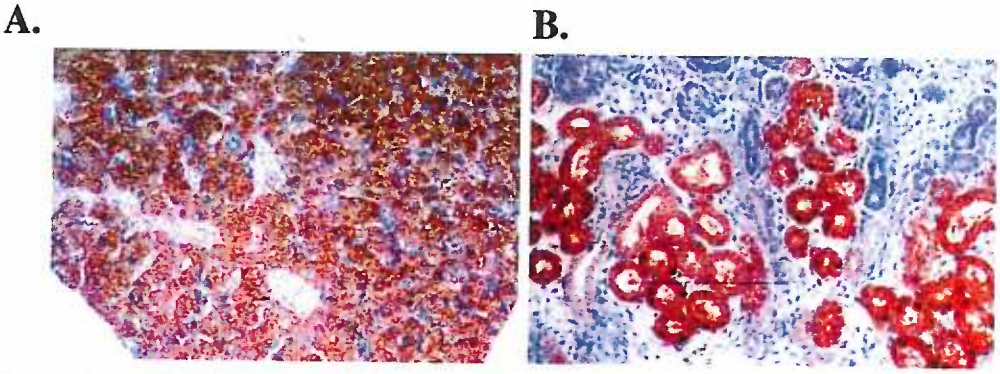


Figure 1.2. Fah immunohistochemistry in the liver and kidney of wild-type mice. Red/brown color represents Fah positive cells in A. Hepatocytes of wild type mouse liver. B. Renal proximal tubules in wild type mouse kidney. These are the only two cell types that express significant levels of Fah in the mouse and the human.

Fumarylacetoacetate and its role in oxidative stress

The structure of FAA predicts that it can be alkylated and thereby cause liver and kidney damage (Lindblad et al. 1977; Nebert et al. 2000). The alkylation of compounds, such as FAA, by sulfhydryl proteins affects the redox state of cells by the creation of new metabolites (Nebert et al. 2000). For example, previous studies have shown that FAA can form adducts with glutathione (GSH). Therefore, GSH may act as the nucleophiles that attacks FAA making this metabolite a disease-causing agent, in addition to reducing the levels of free GSH available (Edwards et al. 1956; Nebert et al. 2000). The main function of GSH in the cell is to alleviate oxidative stress by maintaining thiol groups and through detoxifying electrophiles and oxygen radicals (Lu et al. 1999). Thus, reduced GSH due to the increase in FAA production may contribute to the liver damage seen in HTI patients. Interestingly, free GSH levels are significantly reduced in the liver and blood of patients with HTI (Stoner et al. 1984).

Alteration of gene expression levels in HTI patients and mice

Perturbations of the mRNA level of several genes are found in the hepatocytes and renal proximal tubule cells of the lethal albino mouse, C^{14CoS}, (Ruppert et al. 1990) and in the $Fah^{\Delta exon5}$mouse (Ruppert et al. 1992; Grompe et al. 1995). For example, some genes are upregulated in the liver, such as NADPH: menadione oxidoreductase (NMO-1), which affects oxidative detoxification, and gadd153, which is typically induced by alkylating agents (Fornace et al. 1989). Other genes that regulated gluconeogenesis, ammonia detoxification, and synthesis of secreted proteins were down regulated, such as

TAT, glucose-6-phosphatase, and phophoenolpyruvate carboxykinase (Ruppert et al. 1992). These changes in gene expression in HTI mice, whether up or down regulation, are attributed to a protective response against severe oxidative stress.

Many HTI patients have elevated plasma tyrosine levels. Elevated tyrosine levels may be caused by the secondary inhibition of the upstream enzymes, such as HPD and TAT. Indeed, HPD enzyme levels are reduced in livers of HTI patients (Lindblad et al. 1977), and TAT mRNA levels are also decreased in the HTI mouse models C^{14CoS} and $Fah^{\Delta exon5}$ (Ruppert et al. 1992; Grompe et al. 1995). Therefore, reduction of either enzyme may cause the accumulation of tyrosine in HTI patients and disease symptoms similar to patients with tyrosinemia type II and III.

FAA role in inducing apoptosis

Acute accumulation of the FAH substrate, FAA, triggers apoptosis in hepatocytes and renal tubular cells. This observation was demonstrated in a mouse model, which was created to study the effects of excessive FAA production. In this model, mice mutant for both *Hpd* (tyrosinemia III) and *Fah* (C^{14CoS}) do not exhibit liver or renal failure, similar to the effect of *Fah* mutant mice treated with NTBC. However, when $Fah^{-/-}Hpd^{-/-}$ mice are challenged with HGA, the tyrosine pathway is reopened and massive apoptosis occurs in the liver because of FAA production (Endo et al. 1997). Later, FAA production was shown to trigger the release of cytochrome C from the mitochondria, which leads to apoptosis. However, administration of caspase inhibitors prior to HGA injection prevents

apoptosis (Kubo et al. 1998). This result was also demonstrated using a cell culture assay (Jorquera et al. 1999).

Two recent studies were published regarding cell death resistance in the liver and kidney of mice with HTI. Exposure of $Fah^{\Delta exon5}$ mice on NTBC to HGA induces rapid cellular death by apoptosis. Surprisingly, $Fah^{\Delta exon5}$ mice that have preexisting liver and kidney damage induced by NTBC withdrawal are resistant to cellular apoptosis after administration of HGA. Therefore, chronic liver and kidney disease, such as HTI, can paradoxically establish cell death resistance *in vivo* (Luijerink et al. 2004; Vogel et al. 2004). Failure of cell death in $Fah^{\Delta exon5}$ mice off NTBC may lead to an accumulation of damaged cells and therefore an enhanced risk for HCC (Vogel et al. 2004).

Fumarylacetoacetate acts a mutagen

HCC is a frequent outcome of HTI (Russo et al. 1990) and the development of this cancer is thought to be due to DNA mutations caused by FAA (Weinberg 1996). The first demonstration of the mutagenic effects of FAA was reported by exposing tissue culture cells to FAA and determining whether FAA induced mutations in the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene. Mutations in HPRT was detected by cellular resistance to 6-thioguanine (Tanguay et al. 1996). Using the same system, FAA was shown to be a powerful glutathione (GSH) depletor; the mutagenicity of FAA is, furthermore, potentiated by the depletion of GSH (Jorquera et al. 1997).

A second study found that a sub-apoptogenic, acute dose of FAA induces mitotic abnormalities and causes chromosomal instability in rodent and human cells. FAA activates the Ras/ERK pathway through mechanisms involving GSH-dependent events, and this activation leads to chromosomal instability and mitotic aberrations. However, replenishing intracellular GSH reduces the chromosomal instability induced by FAA by 80% (Jorquera et al. 2001). Similar mitotic aberrations, chromosomal instability, and sustained ERK activation were also observed in skin fibroblasts derived from HTI patients and these abnormalities may play a role in the carcinogenic process (Gilbert-Barness et al. 1990; Zerbini et al. 1992).

Succinylacetone

Studies have shown that SA, a derivative of FAA, plays two different roles, causing both the neurologic and renal symptoms associated with HTI. First, SA is believed to be responsible for the neurologic abnormalities in HTI patients. SA is a potent inhibitor of the porphyrin synthetic enzyme δ-aminolevulinic acid dehydratase. Inhibition of this enzyme causes increased levels of δ-aminolevulinic acid (ALA), which is a known neurotoxin and is implicated in certain peripheral neuropathies. Therefore, inhibition of ALA dehydratase by SA allows for ALA to accumulate at high levels. Interestingly, ALA was found to be elevated in the urine of children with HTI (Sassa et al. 1982).

Second, SA is considered to cause renal disease in HTI patients because studies in rats injected with SA demonstrated inhibition of sodium dependent phosphate uptake and

impaired sugar and amino acid uptake in renal tubule cells (Burke et al. 1989; Roth et al. 1991). This finding was further substantiated by experiments performed in mice mutant for both *Hpd* and *Fah*. When these mice were given HGA, the proximal tubules displayed evidence of apoptosis and decreased absorption of glucose and phosphate by the tubules. However, when caspase inhibitors are given, prior to HGA administration, reduced apoptosis was observed, but the ability of the renal tubules to absorb glucose and phosphate was not improved. Because apoptosis has been directly linked to FAA exposure, SA must affect the ability of the tubules to absorb glucose and phosphate by inhibiting cellular metabolism or transcription factors (Sun et al. 2000).

Understanding the disease pathology of HTI, aids in our understanding of how to treat this disease. FAA, the compound that accumulates in FAH deficiency, is the main cause of damage to the liver the renal proximal tubules of the kidney. FAA accumulation induces oxidative damage, apoptotic cell death, and alters gene expression. Importantly, FAA acts cell autonomously, only damaging the cells in which it is produced. Therefore, every *FAH* deficient hepatocyte or renal proximal tubule cell must be corrected in order to prevent accumulation of FAA. Researchers are developing innovative ways to correct *FAH* deficiency, or prevent the accumulation of FAA, in liver and kidney of HTI patients. However, currently, the only cure for HTI is organ transplantation.

IV. Somatic Reversion in Genetic Disease

A reversion event causes a genetic mutant to regain its wild-type phenotype. The reversion can occur by two different mechanisms: by correction of the original genetic mutation or by a secondary DNA sequence change resulting in a functional gene product. If the reversion event occurs in a somatic cell of a given tissue, only a portion of that tissue will show the reverted phenotype, thereby causing a mosaic expression pattern of the functional gene product (Jonkman 1999). This phenomenon occurs in HTI, as well as in other genetic disorders. Somatic reversion followed by selective expansion of the revertant cell, suggested that cell or gene therapy applications are a feasible option for treatment of HTI and other genetic diseases.

Somatic reversion in HTI

Somatic reversion occurs in the livers of HTI patients as originally determined by gross examination of the livers from two HTI patients, who received liver transplants. Several areas of the HTI affected livers stained positively for FAH and also showed a high amount of FAH enzyme activity. The authors speculated that some type of somatic reversion event had occurred in a fraction of hepatocytes that led to proliferation and the formation of a mosaic FAH expression pattern (Kvittingen et al. 1993). The livers of an additional 23 HTI patients were examined and similar regions of FAH positive hepatocytes were identified in 21 of these patients. Subsequently, in four patients showing this mosaic FAH staining pattern, mutation analysis was performed in the liver tissue. Two of the four patients were homozygous for the same HTI-causing mutation

and the other two patients were compound heterozygous. However, in all four of the patients, hepatocytes in the FAH positively stained section had reverted to the wild-type sequence in one allele, but hepatocytes in the FAH negatively stained section had retained the mutant sequence (Kvittingen et al. 1994). Precisely how this reversion to the wild-type sequence occurred is unknown. However, it is hypothesized that FAA, the mutagenic substrate of FAH, induced a point mutation at the specific base that was originally mutated, which resulted in genetic correction. This type of genetic correction would be an extremely rare event, but because of the high selection pressure for corrected hepatocytes, such an event could be detected.

Somatic reversion was also demonstrated in an altered HTI mouse model. $Fah^{\Delta exon5}$ mice that are heterozygous for *Hgd* ($Fah^{-/-}$,Hgd^{aku}/Hgd^{wt}) spontaneously develop clonal nodules of functionally normal hepatocytes (Figure 1.3). In these nodules, it was discovered that a mutation had occurred in the wild-type allele of *Hgd*, which resulted in a new cellular genotype, $Fah^{-/-}$,Hgd^{aku}/Hgd^{aku}. This loss of heterozygosity (LOH) in the wild-type allele was caused by FAA-induced deletions, insertions, and missense mutations (Manning et al. 1999). The $Fah^{-/-}$,Hgd^{aku}/Hgd^{aku} hepatocytes had a strong proliferative advantage and were able to rescue the liver of the HTI mice. Following this repopulation, the tyrosine catabolic pathway became blocked at Hgd in the liver. While accumulation of HGA, the substrate of Hgd, does not harm the liver, HGA does induce chronic injury in the $Fah^{-/-}$,Hgd^{aku}/Hgd^{wt} renal tubular cells (Manning et al. 1999). Therefore, this altered HTI mouse is an excellent model of chronic renal disease. Chapter three describes cell therapy experiments using this mouse model.

Figure 1.3. Liver from a *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mouse off NTBC.

Fah$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice undergo loss of heterozygosity of the wild-type *Hgd* allele in the absence of NTBC. Nodules of healthy *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{aku} hepatocytes are depicted by the white arrows. These hepatocyte nodules are capable of repopulating the entire organ, making the liver resistant to the effects of FAA substrate.

Somatic reversion as a basis for cell therapy

The principle of *in vivo* selection for revertant hepatocytes raised the question of whether transplanted cells could be selected in a similar fashion. Sandgren et al. developed a mouse model of somatic reversion. These mice, which express a liver-toxic urokinase transgene, develop multiple nodules of healthy hepatocytes, which allow the animals to survive. Thus, the hepatocytes in which the transgene was inactivated were healthy and able to repopulate the diseased liver, while the urokinase-expressing

hepatocytes in these mice were severely damaged and unable to survive (Sandgren et al. 1991). Using this same mouse model, transplantation of syngeneic wild-type hepatocytes was performed. These transplanted hepatocytes significantly repopulated the recipient mice after approximately four weeks, demonstrating that transplanted cells can be selected in a similar fashion as cells that had undergone somatic reversion (Rhim et al. 1994).

Because of the spontaneous emergence of revertant nodules in the liver of HTI patients (similar to the urokinase mice), the concept of therapeutic liver repopulation may be a viable treatment option. In previous studies, the HTI mouse model ($Fah^{\Delta exon5}$) was used to test the ability of transplanted hepatocytes to cure HTI liver disease. This is research is described in more detail below.

V. Current And Proposed Therapeutic Approaches for Hereditary Tyrosinemia Type I

Dietary and drug treatments for HTI

The current treatment for HTI patients is administration of NTBC along with dietary restriction of phenylalanine and tyrosine (Mitchell et al. 1999). However, because NTBC was a recent discovery (Lindstedt et al. 1992), the long-term effects of NTBC treatment and the ability of NTBC to prevent HCC have not been fully evaluated. There have been two recent cases where patients on NTBC developed severe HCC (Dionisi-Vici et al. 1997; van Spronsen et al. 2005), though it is unknown whether the HCC was present prior to the start of NTBC treatment, or whether NTBC failed to sufficiently inhibit HPD resulting in production of FAA. To answer these questions, researchers performed NTBC treatment studies in the $Fah^{\Delta exon5}$ mouse model. Initially, full correction of the liver and renal disease by NTBC was reported in these mice (Grompe et al. 1995). However, in a long-term, follow-up study, many of the $Fah^{\Delta exon5}$ mice treated with NTBC developed HCC after approximately 18 months (Grompe et al. 1998), even with the most stringent treatments (higher doses of NTBC and dietary restriction of tyrosine) (Al-Dhalimy et al. 2002). In addition, differences between gene expression profiles of NTBC-treated $Fah^{\Delta exon5}$ mice and wild-type mice demonstrate that NTBC does not totally normalize $Fah^{\Delta exon5}$ mice (Luijerink et al. 2003). Given these observations, there is an obvious need for a more effective therapy.

Cell therapy for HTI liver disease

As described previously, individual hepatocytes within the livers of HTI patients can undergo somatic reversion followed by repopulation in an attempt to overcome the liver disease (Kvittingen et al. 1993; Kvittingen et al. 1994). This phenomenon of *in vivo* selection for revertant hepatocytes suggests that therapeutic liver repopulation by transplanted wild-type hepatocytes may be possible for HTI patients. The principle of liver repopulation by transplanted hepatocytes, previously demonstrated in the albumin-urokinase mice (Rhim et al. 1994), was applied to the HTI mouse model. $Fah^{\Delta exon5}$ mice were transplanted with hepatocytes from a syngeneic wild-type donor. After transplantation, the mice were either taken off NTBC or kept on the drug. In the mice that were taken off NTBC, more than 95% of the hepatocytes were replaced by transplanted donor cells within 6 weeks, but in the NTBC-treated mice, transplanted hepatocytes were not able to repopulate the liver (Overturf et al. 1996). Figure 1.4 illustrates the degree of repopulation present at different time points off NTBC. Subsequent studies have shown that as few as 1000 donor cells were sufficient to repopulate the majority of mutant mouse liver (Overturf et al. 1996). Not only were the donor wild-type hepatocytes capable of repopulating the liver of $Fah^{\Delta exon5}$ mice, but they could also be serially transplanted into another $Fah^{\Delta exon5}$ mouse. Healthy hepatocytes were able to undergo at least 100 cell doublings without a loss of function (Overturf et al. 1997).

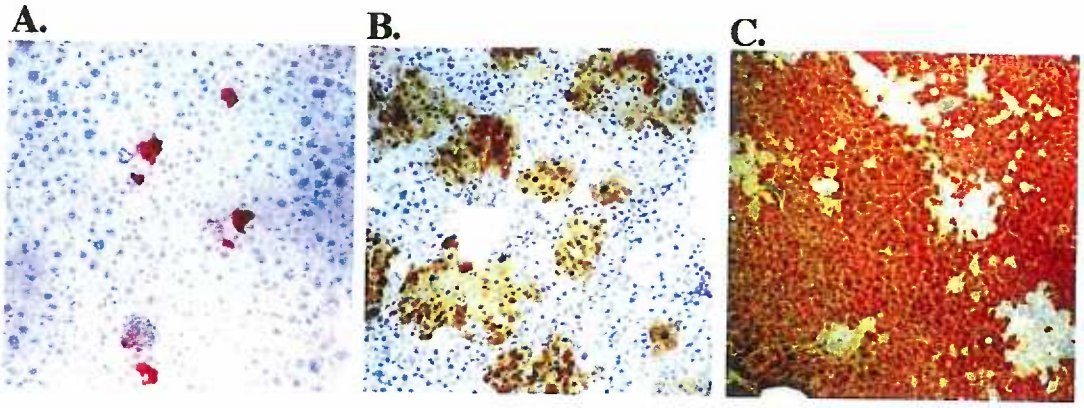


Figure 1.4. Repopulation of *Fah*$^{\neq exon5}$ mouse liver with wild-type hepatocytes. Immunohistochemistry for Fah positive hepatocytes in *Fah*$^{\neq exon5}$ mice transplanted wild type hepatocytes. In the absence of NTBC, Fah positive hepatocytes are capable of repopulating the liver of Fah deficient mice. The three panels represent the degree of repopulation after A. three days off NTBC. B. 21 days off NTBC. C. two months off NTBC. The red/brown colored cells are positive for Fah.

In the murine HTI model (*Fah*$^{\neq exon5}$), wild-type hepatocytes expressing Fah have a strong selective growth advantage and are capable of nearly complete liver repopulation. Therefore *Fah*$^{\neq exon5}$ mice are uniquely suited for the study of gene and stem cell therapy for the liver disease because corrected hepatocytes would have the same selective advantage as transplanted wild-type hepatocytes. A significant number of gene therapy studies have been performed in the *Fah*$^{\neq exon5}$ mouse and each treatments has advantages and disadvantages as outlined in the following section.

IV. Gene Therapy for HTI

Experimental gene therapy strategies for liver and renal disease have ranged from conventional virus-mediated gene transfer to novel non-viral gene transfer. However, for a gene therapy strategy to be successful, the system must have the following characteristics: (1) the ability to transduce quiescent cells, (2) the ability to be efficiently integrated into the host genome at a defined location, (3) easily produced in large quantities, (4) non-toxic, and (5) capable of being administered several times to the organism (Kren et al. 2002). Currently, no gene transfer system (viral or non-viral) meets all of the qualifications. However, some gene transfer systems meet more of these requirements than others (Table 1.1).

Types	*Viral or Non-viral*	*Transduce Quiescent Cells?*	*Integration?*	*Site-specific Integration?*	*Immune Response?*
Onco retrovirusus	Viral	No	Yes	Random Integration	No
Lentivirus	Viral	+	Yes	Random Integration	No
AV	Viral	+++	No (episomal)	None	+++
Helper-dependent AV	Viral	+++	No (episomal)	None	+
AAV	Viral	+++	Yes/No	Random Integration	+
SB Transposon	Non-viral	+	Yes	Random Integration	No
ϕC31 Integrase	Non-viral	+++	Yes	Site-specific	No

Table 1.1. Comparison of viral to non-viral vectors for liver-directed gene therapy. Five different vectors for gene therapy are compared for their ability to transduce quiescent cells, to integrate into host genome, to integrate in a site-specific manner, or to elicit an immune response. The five different vectors: retrovirus, adenovirus (AV), adeno-associated virus (AAV), Sleeping Beauty (SB) transposon, and ϕC31 integrase have varying characteristics, which make each vector system more or less effective as a gene therapy tool. However, ϕC31 integrase, is perhaps the best vector. Three pluses (+++) means the best system for tranducing quiescent cells, or the system that elicits a high immune response. One plus (+) means that the system can transduce quiescent cells, or that can elicits a small immune response, but other systems are more robust.

Liver-directed, viral gene therapy methods

Oncoretroviruses have commonly been used for gene therapy studies because they are capable of integrating into the host genome and because the immune response generated by the virus is minimal. Therefore, retroviral gene therapy was applied to $Fah^{\Delta exon5}$ mice (Overturf et al. 1996). The Moloney murine leukemia virus (Mo MLV) based retroviral vector carrying the human *FAH* gene was infused into the portal vein of Fah deficient mice. After the injection, selection for corrected hepatocytes was induced by removal of the mice from NTBC. Within 8 weeks, 90% of hepatocytes in the recipient liver were positive for FAH by immunohistochemistry and normal liver function was restored. However, the initial viral transduction efficiency of hepatocytes was less than 1% (Overturf et al. 1996). Efficiency of gene transfer into the liver is minimal using oncoretroviruses because they require cell division for integration and

hepatocytes do not divide frequently (Nguyen et al. 2004). Nonetheless, MoMLV based retroviral vectors were tested in neonatal dogs with mucopolysaccharidosis VII. Because of the rapid growth of the liver from birth to adulthood, the MoMLV carrying the β-glucuronidase gene was able to transduce hepatocytes and correct the liver disease in these dogs (Xu et al. 2002).

Another group of retroviruses, termed lentiviruses, have been engineered for gene transfer. Recombinant lentiviruses can transduce quiescent cells that are not undergoing cell divieion (Kafri et al. 1997). However, efficient gene transfer into intact liver by lentviral vectors cell cycling (Nguyen et al. 2002). For example, factor IX levels were four to six fold higher after lentiviral gene transfer in partially hepatectomized mice than in nonhepatectomized recipients (Park et al. 2000). Therefore, other viral vectors with greater ability to transduce non-dividing cells are currently under consideration.

Adenoviruses are an attractive gene transfer method because they can transduce non-dividing cells (Li et al. 1993). In sufficient doses, adenoviral vectors have been shown to transduce over 90% of all hepatocytes in the liver. However, the therapeutic benefit of transducing large numbers of hepatocytes is often lost because the virus remains as an episome and does not integrate. In addition, the immune system also eliminates transduced hepatocytes because of the overwhelming expression of virus carrying the transgene (Kren et al. 2002; Nguyen et al. 2004). Nonetheless, adenovirus gene therapy for liver correction in the *Fah*$^{\Delta exon5}$ mice was tested. In most instances, significant liver repopulation (>50%) and functional correction was shown. However,

HCC developed in some mice, possibly arising from non-corrected Fah-deficient hepatocytes or from immunological rejection of cells harboring the FAH-expressing adenovirus (Overturf et al. 1997).

In recent years, a variety of adenoviral vectors have been developed, which minimized the immune response generated. Currently, the most effective is a helper-dependent adenoviral vector in which all the viral open reading frames were deleted; leaving only the inverted terminal repeats necessary for replication and packaging (Parks et al. 1996). In animal models, such as canine and murine models of hemophilia A, the helper-dependent adenoviral vector mediated therapeutic factor VIII expression for several months (Reddy et al. 2002; Brown et al. 2004). In addition, helper-dependent adenoviral vectors have a reduced immune response, in comparison to first generation adenoviruses (Muruve et al. 2004).

The adeno-associated virus (AAV) is another promising for viral gene therapy system. AAV can infect dividing and non-dividing cells, and while AAV remains largely as an episome, it can integrate under certain conditions. However, AAV can also cause a humoral immune response, which may be a problem if the virus needs to be re-administered (Kren et al. 2002). Many studies have shown that AAV vectors are able to transduce hepatocytes in animal models for a prolonged period (Wang et al. 2000; Nathwani et al. 2002). AAV serotype 2 (AAV2) was used to deliver human *FAH* into hepatocytes of $Fah^{\Delta exon5}$ mice. After strong selective pressure, the integration sites were analyzed, and AAV2 was shown to preferentially integrate into active genes (Nakai et al.

2003; Nakai et al. 2005). AAV8, the newest member of the AAV family, is an attractive candidate for hepatic gene transfer applications because of the 10- to 100-fold improved transduction efficiency of murine and canine liver, in comparison to other serotypes (Gao et al. 2002; Wang et al. 2005). To date, integration sites have not been analyzed for AAV8. AAV has many different serotypes, and while one serotype may be preferential to another, random integration will always be a concern for viral gene therapy, regardless of which serotype utilized.

Viral gene therapy is an effective treatment for liver disease associated with HTI and other disorders. But, complications can be associated with the type of vector used for gene therapy. Recently, much work has been done to investigate the use of non-viral gene therapy for treatment of liver disease. Non-viral DNA is considered to be safe because it does not involve viral particles and therefore may not elicit an immune response. Non-viral gene therapy methods have nevertheless been hampered by the lack of integration and long-term gene expression (Liu et al. 2002). However, two different non-viral gene therapy methods do facilitate integration of the transgene: Sleeping Beauty (SB) transposase/transposon, and ϕC31 integrase.

Liver-directed, non-viral gene therapy methods

The *Sleeping Beauty* (SB) transposase/transposon is a plasmid-based, non-viral gene transfer system that facilitates stable integration and permanent transgene expression of plasmid DNA. SB transposon was reconstructed from a Tc1/mariner-like transposon derived from fish (Ivics et al. 1997). The SB system integrates transgenes by

a cut-and-paste mechanism that requires the binding of the enzyme, SB transposase, to short inverted repeat (IR) sequences flanking the transposon, which carries the transgene (Plasterk 1996). SB can mediate transposition of foreign DNA into host cells both *in vitro* (Ivics et al. 1997) and *in vivo* (Yant et al. 2000; Montini et al. 2002). SB has also been used in the HTI mouse model ($Fah^{\Delta exon5}$) for correction of the liver disease. In this study, integration was stable and expression of the transgene was maintained for several months. However, integration occurred at random sites in the genome and therefore could result in insertional mutagenesis, similar to the different viral vectors (Montini et al. 2002).

The newest non-viral gene therapy system, ϕC31 integrase, is a site-specific recombinase that mediates efficient integration of plasmid DNA into host cell genomes (Thyagarajan et al. 2001). In nature, ϕC31 integrase facilitates a unidirectional recombination event between the 39 bp *attP* site in the phage genome and the related 34 bp *attB* site in the *Streptomyces* chromosome (Groth et al. 2000). In mammalian cells, integrase can recognize partially identical *attP* sites (pseudo *attP* sites) in the host genome and facilitate a recombination event between the pseudo *attP* site and a vector carrying the transgene and the *attB* site (Olivares et al. 2002; Ortiz-Urda et al. 2002) (Figure 1.5). Pseudo *attP* sites are rare in both the human and murine genome and therefore the number of potential integration sites for the vector is limited. Two landmark papers have been published showing the *in vivo* applications of ϕC31 integrase. The first study showed the delivery and site-specific integration of the human factor IX transgene in mice, resulting in physiologically relevant levels of factor IX (Olivares et al.

2002). The second study reported the ability of ≥C31 integrase to transfect human keratinocytes and to correct inherited skin diseases in a xenotransplantation model (Ortiz-Urda et al. 2002; Ortiz-Urda et al. 2003). Together, these studies demonstrate ≥C31 integrase as a promising system for stable transgene integration. However, additional studies are needed to determine the actual number of potential integration sites, where they are located within the genome, and the frequency with which each site is used. Use of ≥C31 integrase for gene therapy in the liver is the subject of chapter 2.

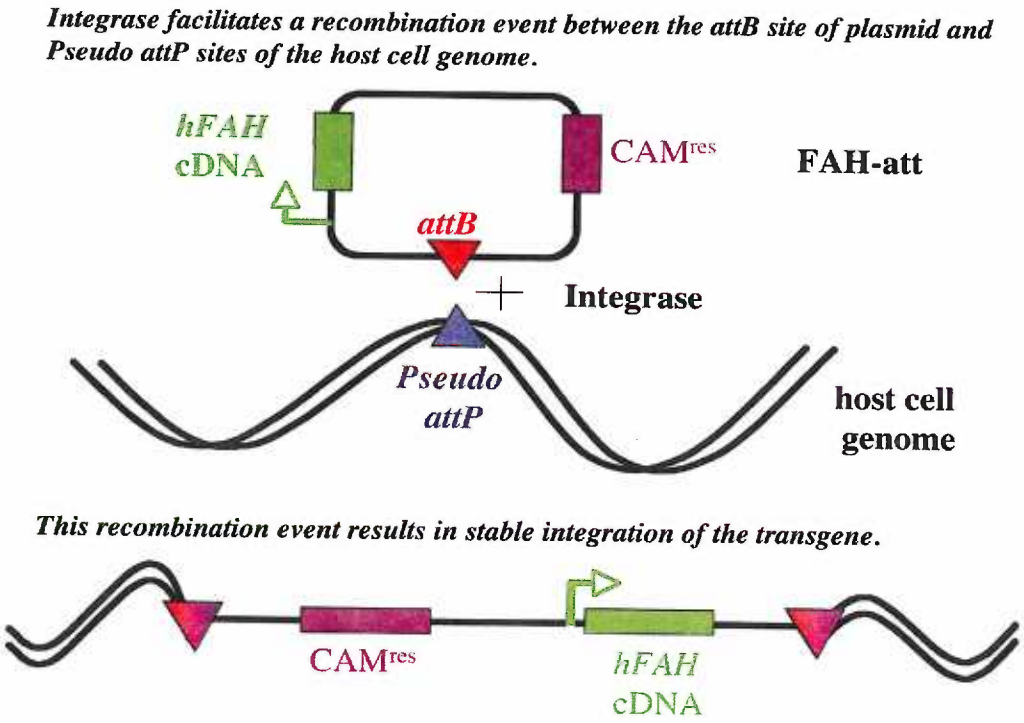


Figure 1.5. Depiction of ≥C31 integrase mediated integration of *hFAH* into the host cell genome.

It is important to state that even with highly efficient gene transfer methods, HTI mice will always be at a risk for developing HCC from uncorrected Fah-deficient hepatocytes. In addition, liver repopulation after any gene therapy method is not 100%, which also creates a risk for HCC development. Thus, while the HTI mouse can be used to develop and characterize new therapies for liver and potentially kidney diseases, translation of these therapies into the clinic for patient care will require additional studies on the pathophysiology of HTI and the molecular effects of FAA. Nevertheless, the HTI mouse will remain an excellent model for characterizing gene therapies for the liver because the toxicity of FAA levels in these mice create ideal conditions for liver regeneration.

Kidney-directed gene therapy

While liver-directed gene therapy for the cure of HTI liver disease has been extremely successful, no effective gene therapy treatment, either viral or non-viral, has been described for kidney diseases. Renal gene therapy is extremely difficult because of the unique structure and function of the kidney. The kidney is composed of four compartments, the glomerulus, the tubules, the vasculature, and the interstitium. Each compartment is separated by basement membranes. The glomerular basement membrane separates blood flow from urine flow and the tubular basement membrane separates urine flow from interstitial flow. These basement membranes make gene transfer in the kidney difficult because vectors are unable to pass through the barriers (Imai et al. 2004).

Four different gene delivery methods have been described for the kidney: injection of vectors into the renal artery or vein, intraparenchyma injection, or an injection performed retrograde up through the ureter (Imai et al. 2004). Each method has had varying success. Two reports described the introduction of an adenovirus into tubular epithelium of rats by injection into the renal artery or ureter. However, in both reports, transgene expression was transient and lasted for only three weeks (Moullier et al. 1994; Zhu et al. 1996). Longer expression (up to three months) in renal tubule cells was achieved by using AAV injected into the renal parenchyma of mice (Lipkowitz et al. 1999). An additional paper attempted renal gene therapy using a lipid-mediated gene transfer system injected into the renal pelvis or the renal artery of mice. Expression of the reporter gene lasted up to 6 weeks in this study (Lai et al. 1997). While these studies have helped to identify the advantages and disadvantages of different gene transfer methods, to date none of these methods has been used to successfully correct a renal disease.

VII. Stem Cell Therapy for HTI

Adult stem cells are defined by their ability to self-renew and to replenish multiple cell types within the tissue they reside (Camargo et al. 2004). However, the concept of stem cell plasticity suggests that adult stem cells can transdifferentiate into cells outside of their tissue of origin in response to environmental cues (Wagers et al. 2004). Recently, many studies have shown that adult hematopoietic stem cell (HSC) can give rise to other non-hematopoietic cell types (Wagers et al. 2004). Specifically, our laboratory demonstrated the ability of HSC to correct the liver disease associated with HTI (Lagasse et al. 2000). This section briefly outlines the development of HSC as a tool for treating diseases.

The normal function of the HSC, found in bone marrow, is to generate all of the lineages of mature blood cells (Kondo et al. 2003). However, a series of papers have shown that the HSC can also contribute to non-hematopoietic lineages. Specifically, the HSC can give rise to skeletal muscle (Ferrari et al. 1998), neurons (Mezey et al. 2000), cardiac muscle (Orlic et al. 2001), liver parenchyma (Petersen et al. 1999; Lagasse et al. 2000; Theise et al. 2000), and kidney epithelium (Kale et al. 2003). In the majority of these studies, transdifferentiation of the HSC into different lineages required significant injury to the tissue. For example, HSCs were capable of differentiating into cardiomyocytes, only after injury was applied to the heart (Orlic et al. 2001). However, functional replacement of the injured tissues by bone marrow-derived cells has not been reported in most cases, except in the liver.

Liver-directed stem cell therapy

Normally, the mature hepatocyte is responsible for proliferation in response to liver injury (Fausto 2000). However, in some instances of chronic injury where the differentiated hepatocyte is compromised and cannot divide, stem cells must respond to the injury and repopulate the organ (Fausto et al. 2003). In 1999, bone marrow cells was shown to give rise hepatic cells, specifically oval cells, in rats under certain pathological conditions (Petersen et al. 1999). This initial study was followed by additional reports that bone marrow cells can give rise to hepatocytes in mice (Theise et al. 2000) and in humans (Alison et al. 2000; Theise et al. 2000). In 2000, using the HTI mouse model, our laboratory demonstrated that the HSC found in the bone marrow was responsible for giving rise to hepatocytes. These hepatocytes derived from the HSC were capable of repopulating the liver of HTI mice and correcting the liver disease. This study is the only report of functional correction of injured tissue by bone marrow-derived cells (Lagasse et al. 2000).

Kidney-directed stem cell therapy

In 2002, a clinical observation suggested that extrarenal cells participate in the regenerative response following acute tubular necrosis (ATN) in patients. Specifically, in male recipients of kidneys from female donors, approximately 1% of the tubular cells contained a Y chromosome, suggesting that these cells were host-derived (Gupta et al. 2002). This observation prompted additional studies to determine if the extrarenal cells were possibly derived from the bone marrow, given the recent findings on the plasticity

of the HSC. In 2003, Kale *et al.* reported that bone marrow stem cells could contribute to the repair of ischemic epithelium following induction of ATN in mice (Kale et al. 2003). Furthermore, Poulsom *et al.* showed that bone marrow cells participated in the normal turnover of renal epithelium in addition to renal regeneration after damage (Poulsom et al. 2001). To date, however, it is unknown whether bone marrow cells can give rise to renal tubular epithelium that can correct an inherited renal disease. This prompted us to ask whether bone marrow-derived renal tubule cells could be generated in our HTI mouse model and whether these cells could repopulate the tubules and treat the renal disease. These experiments are described in chapter 3.

Stem cell plasticity or fusion

Currently, controversy exists over the mechanism of stem cell plasticity. When reports of stem cell plasticity were first published, it was assumed that the stem cell transdifferentiated into a cell type of a different lineage. Recently, however, there is increasing evidence for a different mechanism, cell fusion. For example, cell fusion between a bone marrow-derived cell and a non-hematopoietic cell forms a heterokaryon and thereby the gene expression pattern of the original bone marrow cell is converted into the gene expression pattern of the fusion partner. This phenomenon was first demonstrated *in vitro* when embryonic stem (ES) cells cultured with bone marrow cells fused and acquired phenotypic properties of each other (Terada et al. 2002). In 2003, two independent laboratories demonstrated cell fusion of hepatocytes with bone marrow-derived cells *in vivo* using the HTI mouse (Vassilopoulos et al. 2003; Wang et al. 2003). Specifically, the fusion event occurs between a myelomonocytic cell (derived from the

HSC) and a pre-existing hepatocyte (Willenbring et al. 2004). Cell fusion mechanisms have been implicated in the contributions of transplanted bone marrow cells to liver hepatocytes, cardiac myocytes, Purkinje neurons, and skeletal muscle (Wagers et al. 2004). Additionally, in Chapter 3, I present evidence that bone marrow-derived renal tubular epithelium is obtained through cell fusion. While cell fusion may be the main mechanism behind stem cell plasticity in the liver, heart, brain, muscle, and kidney, other mechanism may exist. This is the focus of continuing research in the field of stem cell plasticity.

VIII. Thesis Rationale

Patients with HTI develop both liver disease, leading to HCC, and renal tubular dysfunction (Mitchell et al. 1999). Previous research has been conducted using the HTI mouse model to develop treatment strategies for HTI liver disease. Among the most successful liver-directed therapies have been viral and non-viral gene therapy systems, even though each system also has major disadvantages. Therefore, there is a definite need to further identify and characterize alternative gene therapy systems.

In 2002, a new non-viral gene therapy system, ϕC31 integrase, was described. ϕC31 integrase is a site-specific recombinase that mediates effective integration of plasmid DNA into host cell genomes (Thyagarajan et al. 2001). ϕC31 integrase is advantageous in comparison to other non-viral gene therapy systems because it can integrate foreign DNA into the host genome only at specific locations, thus preventing random integration. In addition, following integration, transgene expression is stable (Olivares et al. 2002). I hypothesized that the ϕC31 integrase system would be a viable approach for treating HTI. I used the $Fah^{\Delta exon5}$ mouse because of its unique feature to selectively expand corrected hepatocytes. Therefore, I was also able to fully characterize all the integration sites and to assess the frequency with which each site is used. In addition, I was able to evaluate the histological appearance of cells corrected by integrase. This study provides a better understanding of both the advantages, such as integration site-specificity, and potential disadvantages, such as abnormal cellular morphology following exposure to integrase, of the ϕC31 integrase system.

While ϕC31 integrase is a promising treatment for liver disease, currently no effective gene therapy system has been described for renal diseases. Recently, two reports were published suggesting that hematopoietic cells are capable of repopulating the proximal tubules of the kidney after induction of ischemic injury. Therefore, I hypothesized that bone marrow cells could repopulate proximal tubular epithelium and correct renal dysfunction associated with an inherited disease, HTI. Interestingly, I found that repopulation of the damaged tubular epithelium by bone marrow cells did not occur in the standard HTI mouse model, but rather occurred after extensive chronic renal injury was applied to the kidney using our adapted HTI ($Fah^{-/-}$,Hgd^{aku}/Hgd^{wt}) mouse model. Also, cell fusion was the mechanism identified for obtaining bone marrow-derived renal tubule cells, which previously had not been known. In addition, I showed that genetic alteration of endogenous renal tubule cells also leads to repopulation and functional correction.

Together these two studies provide insight into potential therapies for the liver and kidney disease associated with HTI. In addition, this research may be applicable to other inherited diseases of the liver and kidney.

Chapter 2

In vivo Correction of Murine Hereditary Tyrosinemia Type I by ϕC31 Integrase Mediated Gene Delivery

Held, P.K., Olivares, E. C., Aguilar, C. P., Finegold, M., Calos, M. P. and Grompe, M. (2005) Molecular Therapy **11**(3): 399-408.

I. Abstract

Phage ϕC31 integrase is a site-specific recombinase that mediates efficient integration of circular extrachromosomal DNA into the host genome. Here, the integrase system was used to transfer the human fumarylacetoacetate hydrolase (*FAH*) gene into the liver of mice affected with hereditary tyrosinemia type 1 (HTI). Approximately 3.6% of transfected hepatocytes experienced an integration event. The absolute frequency of integration was 1/1,374. A higher proportion of integrase-transfected FAH positive hepatocytes displayed abnormal morphology (bizarre nuclei, enlarged cells) on day 25 after gene transfer, compared to cells not receiving integrase. The increased frequency of these abnormal cells correlated with the amount of integrase plasmid administered, suggesting some form of integrase toxicity in $Fah^{\Delta exon5}$ mouse livers. The abnormal hepatocyte appearance was transient and livers analyzed after longer selection (90 days) showed 70% repopulation with only normal healthy FAH positive hepatocytes. A total of seven different integration sites (accounting for > 90% of integration) were identified. Serial transplantation of integrase-corrected hepatocytes to $Fah^{\Delta exon5}$ recipients was successful, suggesting long-term viability of corrected cells and persistent gene expression through many rounds of cell division. The stability of transgene expression, relatively high integration frequency, and significant site-specificity that characterize ϕC31 integration system suggest that it may have utility in many gene therapy settings.

II. Introduction

Currently, most gene therapy applications use viral vectors to transfer therapeutic DNA into cellular targets. While these vectors can be effective *in vivo*, they also have several disadvantages. First, viral vectors can trigger immune responses at multiple level (Yang et al. 1995; Hernandez et al. 1999). Second, large-scale production of clinical grade vectors is difficult and costly. Finally, some viral vectors can cause insertional mutagenesis (Nakai et al. 1999; Hacein-Bey-Abina et al. 2002). In contrast, non-viral vectors are less likely to trigger immune responses and are cheaper to produce, but their efficacy in correcting genetic diseases has been hampered by lack of integration and long-term gene expression. Recently, plasmid based non-viral gene transfer systems capable of mediating stable integration of transgenes have been described. The *Sleeping Beauty* transposase/transposon system has been shown to mediate effective transposition of foreign DNA into host cells *in vitro* and *in vivo* (Ivics et al. 1997; Yant et al. 2000; Montini et al. 2002). However, *SB* transposase-mediated integration occurs at random sites in the genome and therefore could result in insertional mutagenesis similar to integrating viral vectors. Ideally the integration of foreign DNA into the genome must be site-specific and controlled to prevent activation of oncogenes and/or disruption of essential or tumor-suppressor genes.

Phage ϕC31 integrase is a site-specific recombinase that mediates efficient integration of plasmid DNA into host cell genomes including mammalian cells (Thyagarajan et al. 2001). In nature, it mediates a unidirectional recombination event

between the 39 bp *attP* site of the phage ϕC31 genome and related 34 bp *attB* site in the *Streptomyces* chromosome (Groth et al. 2000; Groth et al. 2004). In mammalian cells, ϕC31 integrase has been shown also to recognize partially identical *attP* sites in both the murine and human genomes (Olivares et al. 2002; Ortiz-Urda et al. 2002). These sites were termed "pseudo *attP*" sites. The efficiency of integration at genomic pseudo *attP* sites was estimated to be approximately 5-10% (Olivares et al. 2002), similar to that observed with *SB* transposase. Pseudo *attP* sites are rare in both the human and murine genomes and therefore the number of integration sites is limited. In one setting, only two integration sites were identified *in vivo* within the mouse genome (Olivares et al. 2002). Therefore, ϕC31 integrase mediated integration is non-random and efficient enough to make the system very attractive for non-viral gene therapy of genetic disorders. Several papers have been published showing *in vivo* applications of ϕC31 integrase. One study showed the delivery and site-specific integration of human factor IX transgene in mouse liver *in vivo* resulting in production of therapeutic Factor IX levels (Olivares et al. 2002). Further studies reported the ability of ϕC31 integrase to transfect human keratinocytes and to correct inherited skin diseases in a xenotransplantation model (Ortiz-Urda et al. 2002; Ortiz-Urda et al. 2003).

In order to further characterize the properties of ϕC31 integrase mediated gene transfer *in vivo* the murine model of hereditary tyrosinemia type 1 (HTI) was used (Grompe et al. 1993). This system has unique advantages for the characterization of stable integration events in hepatocytes *in vivo*. HTI is a genetic liver disease caused by deficiency of fumarylacetoacetate hydrolase (FAH) (Grompe et al. 1993), the enzyme

that catalyzes the last step in the tyrosine degradation pathway. In HTI, FAH-expressing hepatocytes display a strong proliferative advantage and eventually repopulate the entire diseased liver (Overturf et al. 1998; Manning et al. 1999). Because of this selective advantage, hepatocytes in which ϕC31 integrase has mediated integration of the FAH gene can be identified, counted, and histologically evaluated soon after transfection as well as after several rounds of growth and expansion of FAH+ hepatocyte clones.

Here we report the properties of ϕC31 integrase mediated gene transfer in the HTI mouse model.

III. Materials and Methods

Vector construction

Plasmid constructs of pCMVInt (CMV promoter driving integrase expression) and pCS (empty cassette) were previously described (Groth et al. 2000; Olivares et al. 2002). The full-length human FAH cDNA expression cassette contained in phFAFFA2 (1.4 kb) (Phaneuf et al. 1991) was cloned into a blunted *EcoR*I site of the expression plasmid pCD-SRα (Takebe et al. 1988). The resulting plasmid, pCD-SRαhFAH, expresses the FAH enzyme under the transcriptional control of the SRα promoter and contains the SV40 polyadenylation sequence (entire expression cassette=3.46 kb). This cassette was removed by digestion with *Sal*I and cloned into the *Xho*I sites of the construct pBC-hAAT-B, replacing the hAAT cassette (Olivares et al. 2002), resulting in a 6.77 kb plasmid, pBCB-FAH, which contains an *attB* site.

Animal husbandry, integrase injection, hepatocyte selection, and integration frequency

Fah$^{\Delta exon5}$ (Grompe et al. 1993) mice were handled according to NIH guidelines for animal care with the approval of the institutional animal care and utilization committee of the Oregon Health & Science University. All *Fah*$^{\Delta exon5}$ mice were treated with NTBC containing water at a concentration of 7.5 mg/L (provided by S. Lindstedt, Gothenborg, Sweden)(Grompe et al. 1995). *Fah*$^{\Delta exon5}$ mice (ranging in weight from 18 grams to 22 grams) underwent hydrodynamic tail vein injection over 5-8 seconds administering plasmid constructs diluted in 0.9% saline solution (approximately 2 mls) as previously described (Liu et al. 1999; Song et al. 2002). After an injection recovery period of three

days, the mice were removed from the protective drug NTBC in order to allow for expansion of FAH positive hepatocytes. NTBC therapy was reinstated for 1 week whenever animals lost more than 25% of their pretreatment weight. NTBC was removed again after weight gain equaled or exceeded the starting weight.

To measure the integration frequency, $Fah^{\Delta exon5}$ mice were hydrodynamically injected with different constructs and were removed from NTBC 3 days after injection for a selection period of 25 days (Liu et al. 1999; Song et al. 2002). The mice were then sacrificed and sections of at least 0.7 cm^2 were analyzed by FAH immunohistology. An observer, who was unaware of the sample being scored, counted the number of FAH positive hepatocytes using a Leica microscope DM RX (Leica Microsystems, Bannockburn, Il). The surface area of the liver section was determined by scanning the glass slides along with a size standard using a Microtec 2 scanner at a resolution of 254 dpi. Adobe PhotoShop 5.02 software was used to select and count the pixels corresponding to the liver sections. A typical mouse liver contains 186,000 hepatocytes/cm^2 (Wang et al. 2002).

The absolute frequency of integration was determined by dividing the number of FAH positive clusters and individual FAH positive cells by the total number of hepatocytes scored for a given section. In the $Fah^{\Delta exon5}$ mouse, hydrodynamic tail vein injection transfects only 2% of all hepatocytes (data not shown) (Montini et al. 2002). Therefore, the absolute frequency must be divided by 2% in order to calculate the actual transfection efficiency. The actual transfection efficiency reflects the number of

hepatocytes that integrated the integrase constructs assuming all hepatocytes were exposed to the construct.

Immunohistochemistry

Liver tissues fixed in 10% phosphate buffered formalin, pH 7.4, were dehydrated in 100% ethanol and embedded in paraffin wax at 58°C. Four-micrometer sections were rehydrated and stained with hematoxylin and eosin and with a polyclonal rabbit antibody against rat FAH (kindly provided by Robert Tanguay, University of Laval, Quebec, Canada). The antibody was diluted in PBS, pH 7.4, and applied at concentrations of 1:300,000 at 37°C for 30 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 and methanol. Avidin and biotin pretreatment was used to prevent endogenous staining. The secondary antibody was biotinylated goat anti-rabbit IgG used at 1:250 dilution (BA-1000); Vector Laboratories, Burlingame, CA). Color development was performed with the AEC detection kit (Cat. No. 250-020; Ventana Medical Systems, Tucson, AZ).

Dysplasia index

To quantitatively evaluate the dysplastic appearing hepatocytes, a dysplasia index scoring system was devised. FAH positive nodules in which more than half of the individual hepatocytes displayed an abnormal phenotype were scored as 3+. FAH positive nodules in which less than half of the individual hepatocytes displayed an abnormal phenotype were scored as 2+ and nodules in which all hepatocytes looked

normal were scored as 1+. The observer was blinded to the transferred plasmid DNA administered for each mouse.

Hepatocyte transplantation

Integrase injected *Fah*$^{\Delta exon5}$ mice that survived for more than 3 months off of NTBC were sacrificed and hepatocytes were isolated for serial transplantation according to a previously described protocol (Overturf et al. 1997). For each round of serial transplantation, 500,000 hepatocytes were intrasplenically injected into *Fah*$^{\Delta exon5}$ recipient mice. One week post injection, the recipient mice were removed from NTBC to allow for selection and expansion of FAH positive hepatocytes.

Liver function tests

Animals were sacrificed by decapitation and blood was collected in Microtainer plasma separator tubes with lithium heparin (Becton-Dickson Vacutainer Systems, Franklin Lakes, NJ). After brief centrifugation the plasma was frozen at –80^{0}C. Twenty microliters of plasma was mixed with 80 µl of a 7% (w/v) bovine serum albumin solution and assayed for aspartate serine aminotransferase, bilirubin, and creatinine levels using a Kodak Ektachem 700 chemistry analyzer (Eastman Kodak, Rochester, NY).

Southern blot analysis

Southern blot analysis was performed on DNA from freshly obtained or frozen (-80°C) liver tissue extracted from treated *Fah*$^{\Delta exon5}$ mice. Genomic liver DNA was isolated from ~350 mg of tissue using the Qiagen genomic DNA isolation kit. 10 µg of

genomic DNA was digested with the restriction enzymes *Hind*III or *Sca*I or *Pst*I (Roche, Indianapolis, IN). Capillary transfer and hybridizations were performed according to standard protocols (Sambrook et al. 2001). A 478 bp fragment from nucleotides 373-850 of the human *FAH* cDNA was isolated by PCR and radioactively labeled with dCTP to probe the digested DNA. Hybridization was detected by the radioactive signal (Al-Dhalimy et al.). The integration sites and copy number were evaluated.

Pseudo site rescue by nested inverse PCR

Mice that received FAH-att and integrase were sacrificed. Ten μg of liver genomic DNA was digested with a group of restriction enzymes (*Nhe*I, *Spe*I, and *Xba*I) that have incompatible cohesive ends and cut at least once in FAH-att. The digests were extracted with phenol/cholorform, precipitated with ethanol, and resuspended in 500 μl of 1X ligation buffer, to which was added 1,000 units of T4 DNA ligase (New England Biolabs, Beverly, MA). Low concentration ligations were incubated at 17^{0}C overnight, extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 20 μl TE. Primary amplification with primers attB-F2 and attL-PCR-1 were carried out as previously described (Olivares et al. 2002; Olivares EC 2002). The PCR products were purified using the QiaQuick PCR purification kit (Qiagen, Valencia, CA), and a portion of the eluate was reamplified with nested primers attB-F3 and attL-iPCR-2 using the same protocol cited above. All amplification products were excised from the gel, purified, and cloned into pCR2.1-TOPO using the TOPO Cloning Kit (Invitrogen, Carlsbad, CA). Insert-containing colonies were sequenced using standard primers previously described (Akerley et al. 1998). Using specific primers designed for each

integration site in combination with vector-specific primers attB-F3 and hAATB-R, the recombination junctions were then amplified from the genomic liver DNA.

To further validate putative integration sites, additional primers were designed corresponding to sequences on either side of the vector-genome junction and seven integration sites were confirmed by this method. The BlastN mouse genome database (www.ncbi.nlmm.nih.gov/genome/seq/MnBlast.html) was then used to identify the precise genomic location and scan for the presence of nearby genes.

IV. Results

Frequency of stable integration and histological evaluation

The efficiency of gene transfer into mouse liver is typically determined by the expression of histochemical markers such β-galactosidase or alkaline phosphatase (Gusella et al. 2002; Mujtaba et al. 2002). However, the simple presence or absence of such markers does not accurately measure integration frequency because gene expression can occur in both the presence and absence of chromosomal integration (Chen et al. 2001). In the $Fah^{\Delta exon5}$ mouse model, single FAH expressing hepatocytes can selectively expand and form clonal nodules only if stable integration occurred in the initially transfected cells (Overturf et al. 1998; Chen et al. 2000). This model is therefore well suited to measure the frequency of vector integration and stable transgene expression.

The integration frequency was calculated for experimental plasmids and controls. Group 1 (n=10) was injected with a FAH expressing construct containing the *att*B site (FAH-att) plus the integrase expression construct acting in trans. Group 2 (n=5) was treated with FAH-att plus an empty vector lacking integrase. A previously described 1:1 ratio (25 μg of each plasmid) of integrase (or empty vector) to the FAH expression construct (FAH-att) was used (Olivares et al. 2002). On day 3 after plasmid injection, NTBC was discontinued to permit the development of liver disease and the selection of FAH positive hepatocyte nodules. All animals were sacrificed 25 days post injection and the liver tissue was processed for histological evaluation. Several liver sections for each mouse were scored for FAH positive cells and nodules. The total number of sections

evaluated for each group was 30 for group 1 and 9 for group 2 (Figure 2.1)(Table 2.1). Approximately 2 to 3 sections were scored for each individual mouse within group 1 or 2.

In the liver sections from group 1 (mice treated with FAH-att and integrase), the average absolute frequency of FAH positive nodules was 7.3 +/- 3.8 x 10^{-4} (1/1,374 hepatocytes). The frequency ranged over 10-fold from a maximum of 1/465 cells in the best mouse to 1/4,880 in the worst. Group 2 (FAH-att and an empty vector) displayed minimal integration at only 2.8 +/- 4.4 x 10^{-5} (1/35,640 cells) and was significantly different from group 1 as evaluated by a student T-test (p value<.0001). In order to determine the efficiency of the ϕC31 integrase mediated integration on a per cell basis, the DNA transfection frequency must be considered. To measure this parameter, three *Fah*$^{\Delta exon5}$ mice were tail vein injected with a β-galactosidase expression construct and sacrificed three days later. Liver sections were stained for β-galactosidase and the hepatocyte transfection efficiency was calculated at ~ 2% (data not shown). This number confirms that *Fah*$^{\Delta exon5}$ mice have lower rates of transfection than typically seen after hydrodynamic injection (Montini et al. 2002). The average corrected integration frequency for group 1 mice was 3.6% (1/27), ranging from 1% to 10.7%. Group 2 mice had an average per cell integration frequency of 0.1% (1/714), ranging from 0 to 0.6% (Figure 2.1)(Table 2.1).

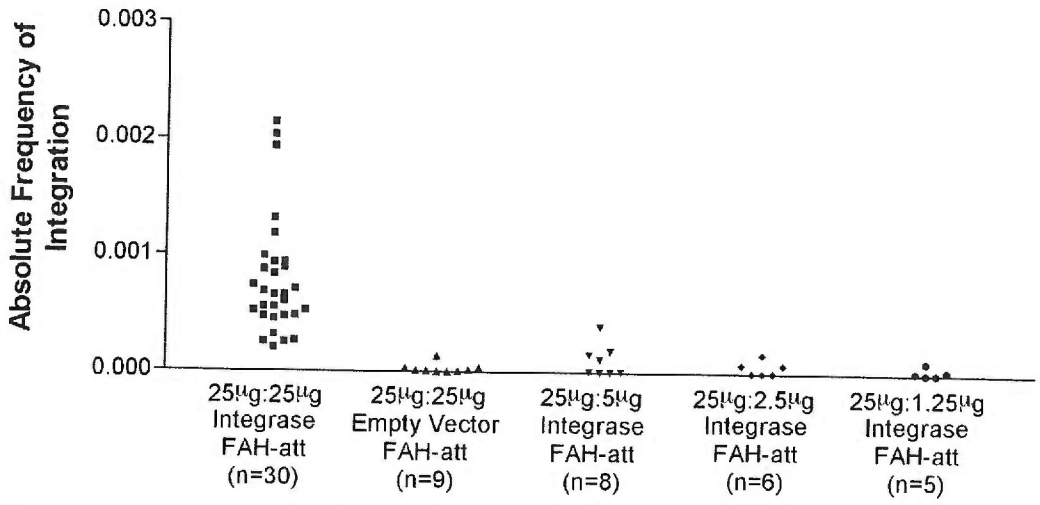


Figure 2.1. Frequency of ϕC31 integrase mediated stable FAH gene transfer

The frequency of integration in mice treated with different ratios of FAH-att + integrase plasmid or FAH-att + empty vector after 25 days of selection are shown. n = number of sections

Group	1	2
Plasmid Combinations	**25 μg FAH-att 25 μg Integrase (n=30)**	**25 μg FAH-att 25 μg Empty Vector (n=9)**
Absolute Integration Frequency	7.3 +/- 3.8 X 10^{-4}	2.8 +/- 4.4 X 10^{-5}
Minimum	1/4,880	0
Median	1/1,374	1/35,640
Maximum	1/465	1/7,165
Corrected Integration Frequency	3.6% 1/27	0.14% 1/714

Table 2.1. Absolute and actual integration frequencies of integrase-treated mice. FAH+ hepatocytes and nodules were counted as single integration events. Averages with standard deviations are given. In addition, the minimum (worst animal) and maximum (best animal) frequencies in each group are shown.

Integrase induced a higher frequency of hepatocyte dysplasia

FAH positive hepatocytes observed in histological sections from group 1 mice harvested after 25 days of selection displayed a unique, dysplastic phenotype at a higher frequency than controls. Specifically, individual FAH positive hepatocytes were often markedly enlarged in comparison to adjacent FAH negative hepatocytes (Figure 2.2 A). In addition, many of the nuclei in these abnormal cells were massively enlarged, giving the hepatocytes a "dysplastic" appearance. An index rating system was devised to quantitate the presence of these abnormal cells. Nodules in which more than half of the hepatocytes were clearly abnormal in phenotype were scored with a 3+ rating, while nodules in which less than half of the hepatocytes were abnormal were scored with a 2+ rating. Nodules that contained only morphologically normal hepatocytes were given a 1+ rating (Figure 2.2 D, E, and F). Seventy percent of the nodules in group 1 mice contained abnormal cells (2+ and 3+) (Figure 2.3). Group 2 mice, which did not receive integrase, did not have any 3+ nodules, but still had approximately 25% 2+ nodules, which contain a lower fraction of abnormal cells (Figure 2.2 C and Figure 2.3). As an additional control, $Fah^{\Delta exon5}$ mice were also injected with the *Sleeping Beauty* transposon and scored in parallel to the integrase transfected mice after sacrifice on day 25 (Montini et al. 2002). Specifically, 30 μg of the transposon containing the FAH gene (driven by the SRα promoter) and 1 μg of the transposase were delivered together by hydrodynamic injection into $Fah^{\Delta exon5}$ mice as previously described (Montini et al. 2002). Mice treated with the transposon showed hepatocyte dysplasia in a smaller percentage of the nodules, similar to the group 2 controls, which did not receive any integrase plasmid. Approximately 75% of the nodules were healthy (1+) and 25% of the nodules were scored as 2+ (Figure 2.3).

The 2+ nodules found in group 2 with no integrase and in the *Sleeping Beauty* transposon control suggested that a level of hepatocyte dysplasia is attributable to the Fah deficiency itself, FAH overexpression and/or the hydrodynamic injection method.

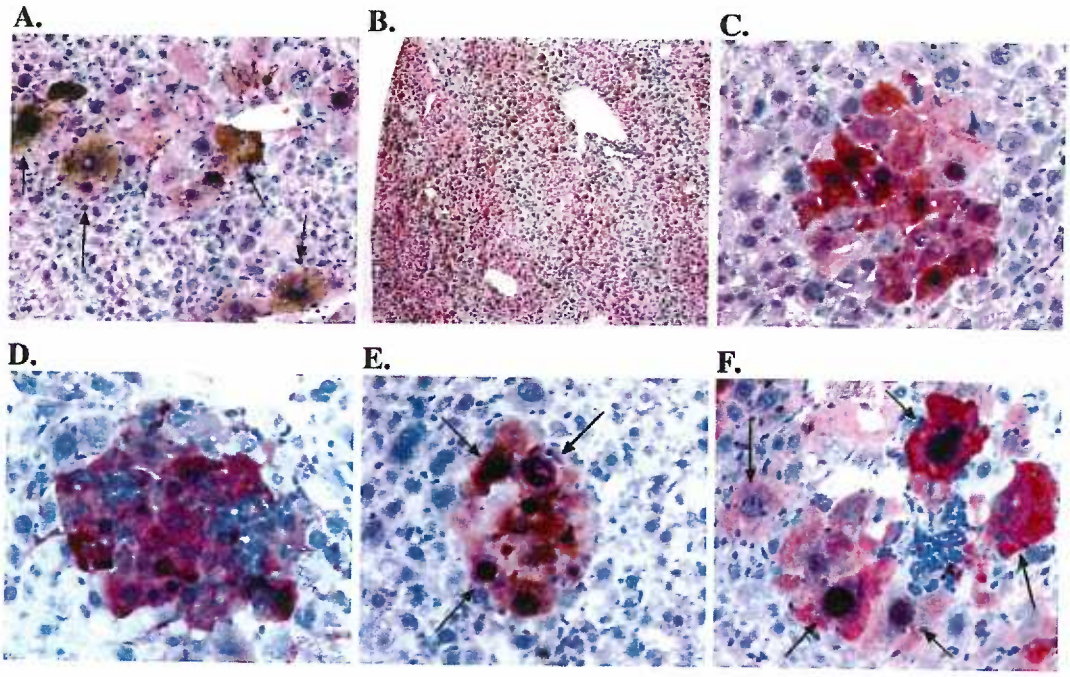


Figure 2.2. FAH immunohistochemistry in hepatocytes treated with ≥C31 integrase. (A.) Mice treated with FAH-att and integrase after 25 days of selection. Arrows denote hepatocytes with abnormal morphology. (B.) Mice treated with FAH-att and integrase after 90+ days of selection. (C.) Mice treated with FAH-att and empty vector after 25 days of selection. (D.) Nodule scored as a 1+ dysplasia index (no abnormal FAH+ cells). (E.) Nodule scored as a 2+ dysplasia index (less than half of the FAH positive cells are abnormal). (F.) Nodule scored as a 3+ dysplasia index (more than half of the FAH positive cells are abnormal).

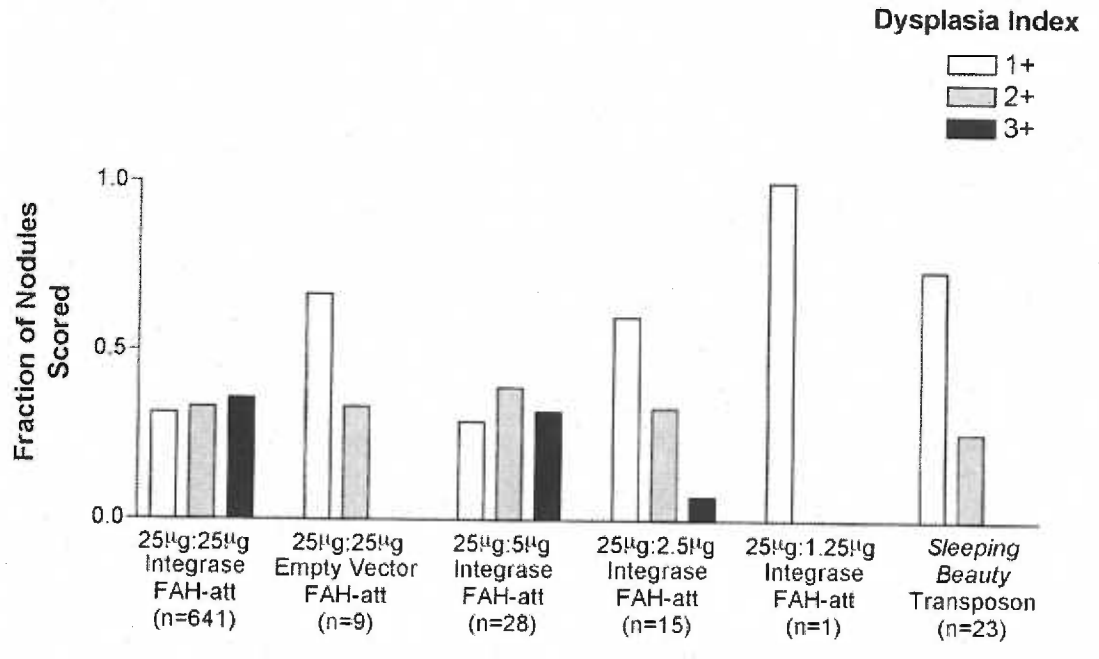


Figure 2.3. Nodule dysplasia index in hepatocytes treated with ϕC31 integrase. The nodule dysplasia index in mice treated with different ratios of FAH-att + integrase plasmid or FAH-att + empty vector after 25 days of selection are shown. n = number of nodules scored.

In fact, prevalent hepatocyte dysplasia as seen in the 3+ nodules is a common feature of *Fah*$^{\Delta exon5}$ mouse liver during NTBC withdrawal without correction (Al-Dhalimy et al. 2002; Luijerink et al. 2003). Here, NTBC was withdrawn three days after plasmid injection and it is therefore possible that this time was too short to initiate FAH expression. To determine whether removal of NTBC prior to full FAH expression was the cause of injury and dysplasia in the integrase transfected hepatocytes, three additional group 1 mice were given maximal NTBC therapy during and after plasmid injection.

They were treated with a higher dose of NTBC (30 mg/L drinking water = 4x the normal amount) for a longer period (one week instead of 3 days). However, this did not change the frequency of FAH integration or the dysplasia index. The average integration frequency for these three mice was 7.4 +/- 1.8 x 10^{-4} (1/1,332) and ~35% of the nodules were scored as highly abnormal (3+).

Importantly, the abnormal phenotype described above did not persist long-term (Figure 2.2 B). Integrase treated (n=4) mice sacrificed after more than 90 days of hepatocyte selection had ~70% liver repopulation with only normal, healthy hepatocytes as seen in Figure 2.2. This observation indicates that the abnormal hepatocyte phenotype found 25 days after gene therapy is transient. It is currently unknown whether individual abnormal FAH positive hepatocytes revert to normal cell morphology or whether normal FAH positive hepatocytes have a selective advantage and simply outgrow the dysplastic cells over time.

Effects of different levels of integrase

To determine whether the high levels integrase plasmid administered were the cause of the higher frequency of abnormal hepatocytes, additional experiments were performed to define the optimal amounts of integrase plasmid for effective gene therapy. Four different groups of *Fah*$^{\Delta exon5}$ mice were injected with the following dilutions of integrase: group A (n=10) was injected with 25 μg of both plasmids, integrase and FAH-att (1:1 ratio); group B (n=8) received 5 μg of integrase and 25 μg of FAH-att (1:5 ratio); group C (n=6) 2.5 μg of the integrase and 25 μg of FAH-att (1:10 ratio); and group D

(n=5) 1.25 μg of integrase and 25 μg of FAH-att (1:20). Liver sections from each mouse were evaluated for integration frequency and morphology 25 days after removal from NTBC. The highest percentage of abnormal 3+ nodules was seen in mice from group A mice receiving a 1:1 ratio of Fah-att to integrase (Figure 2.3). The proportion of 3+ nodules decreased and the frequency of 1+ normal nodules increased with a decreasing amount of the integrase plasmid. Group D mice, receiving only 1.25 μg of the integrase plasmid, had no abnormal nodules. Thus, the amount of the integrase plasmid administered directly correlated with the frequency of morphologically abnormal hepatocytes. The quantity of integrase plasmid was also strongly correlated with the level of gene transfer. The average absolute integration frequency ranged from 1/1,374 with a 1:1 ratio (Group A) to only 1/34,091 with a 1:20 ratio (Group D). These findings are summarized in Table 2.2 and Figure 2.1.

Group	A	B	C	D
Plasmid Combinations	**25 μg FAH-att 25 μg Integrase (n=30)**	**25 μg FAH-att 5 μg Integrase (n=8)**	**25 μg FAH-att 2.5 μg Integrase (n=6)**	**25 μg FAH-att 1.25 μg Integrase (n=5)**
Absolute Integration Frequency	7.3 +/- 3.8 X 10^{-4}	1.1 +/- 1.4 X 10^{-4}	5.2 +/- 6.6 X 10^{-5}	2.9 +/- 4.3 X 10^{-5}
Minimum	1/4,800	0	0	0
Median	1/1,374	1/9,375	1/19,201	1/34,091
Maximum	1/465	1/2,558	1/6,101	1/9,784
Corrected Integration Frequency	3.7% 1/27	0.55% 1/181	0.26% 1/385	0.15% 1/690

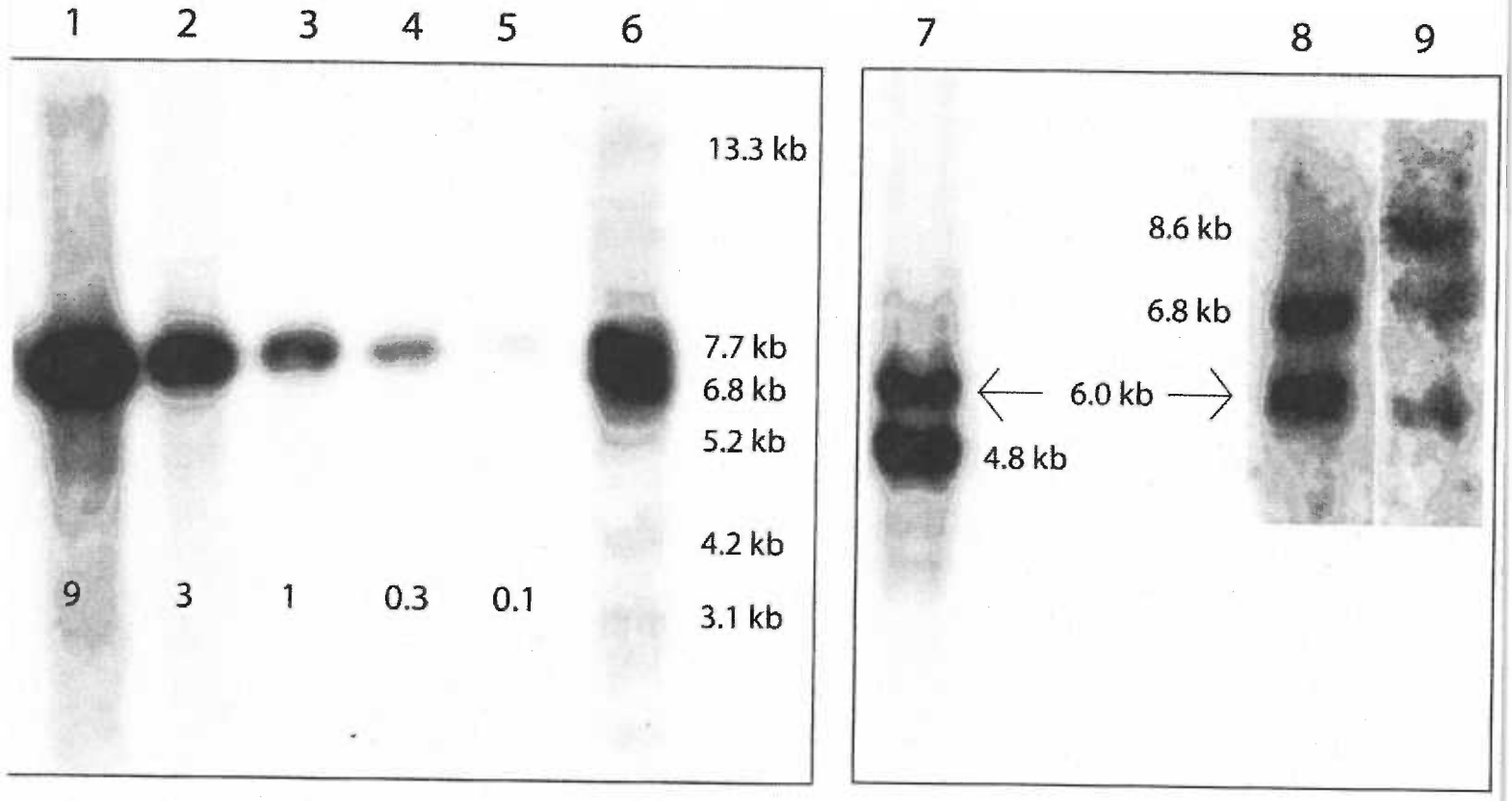
Table 2.2. Effect of integrase levels on integration frequency.

Fah$^{\Delta exon5}$ mice treated with varying ratios of integrase to FAH expression construct. FAH positive hepatocytes and nodules are counted as single integration events. In addition, the minimum (worst animal) and maximum (best animal) frequencies in each group are shown.

Integration site analysis

One major pseudo *attP* site (mpsL1) favored for integration into the mouse genome has been described previously (Olivares et al. 2002). Here we determined whether integration into other sites also occurred. Approximately 1/1,000 hepatocytes were stably transfected by hydrodynamic injection of the integrase construct and FAH-att, but under selective pressure, the FAH positive hepatocytes expanded to approximately 70% of the liver mass (~3 x 10^7 hepatocytes). Group 1 mice (n=3), treated with FAH-att and integrase, underwent a period of selection for ~ 90 days. These long-term survivors were then used as hepatocyte donors for serial transplantation into naïve $Fah^{\Delta exon5}$ secondary recipients. After the recipient mice survived 90+ days off NTBC, they were used as hepatocyte donors for a second round of serial transplantation. Tertiary $Fah^{\Delta exon5}$ recipients also underwent a selection period of 90+ days off NTBC. Liver tissue harvested and analyzed after the selection period showed high levels of repopulation in the tertiary recipients (>70% by immunohistochemistry) (Figure 2.2 B). All FAH positive hepatocytes were healthy and showed no signs of dysplasia. Additionally, liver function tests were performed on eight tertiary recipient mice. The average alanine aminotransferase level was 232 units/L (ranging from 90-755 units/L), which is slightly elevated over normal (100 units/L). The creatinine and conjugated bilirubin levels were 0.5 mg/L and 0 mg/dL respectively, which is similar to normal levels (creatinine=0 mg/L and bilirubin=0 mg/dL). On average, untreated $Fah^{\Delta exon5}$ mice have alanine aminotransferase levels of 1000 units/L, creatinine levels of 1.5 mg/L, and conjugated bilirubin levels of 0.5 mg/dL (Akerley et al. 1998).

Genomic liver DNA was extracted from three repopulated (~50-70% by immunohistochemistry) serial transplant recipients. All three recipient mice had different primary hepatocyte donors, which had been injected with the integrase and FAH-att constructs. Integration sites for the three mice were analyzed by Southern blot after digestion with *Hind*III or *Sca*I, and probed using a portion of the human *FAH* cDNA. The previously known integration site (mpsL1) was in common between all three mice, however several distinct junction fragments were observed in addition. In mouse 1, digested with *Hind*III, two "major " sites of integration (7.7 kb and 6.8kb) and 4-5 additional "minor" sites were identified based upon band intensity (lane 6, Figure 2.4). To confirm these integration sites, a second Southern blot was performed on the same mouse 1 DNA digested with *Sca*I. Again, two major sites were identified (6.0 kb and 4.8 kb) (lane 7, Figure 2.4). DNA from mice 2 and 3 was also digested with *Sca*I (lane 8 and 9, Figure 2.4). Mouse 2 had two major sites of integration (6.0 kb and 6.8 kb) and mouse 3 had three sites of integration (6.0 kb and 6.8 kb, and 8.6 kb). The 6.0 kb band corresponds to the mpsL1 and was shared between all mice. To identify the number of *FAH* gene copies per cell, a third Southern blot was performed using the restriction enzyme *Pst*I, which cuts twice in *FAH* leaving an internal fragment. Mouse 1 showed greater than one copy of *FAH* per diploid genome equivalent, when compared to its matched control (lane 10 and 11, Figure 2.4), while mouse 2 and 3 both showed less than one copy of *FAH* per diploid genome, when compared to their matched control (lane 12, 13, and 14, Figure 2.4).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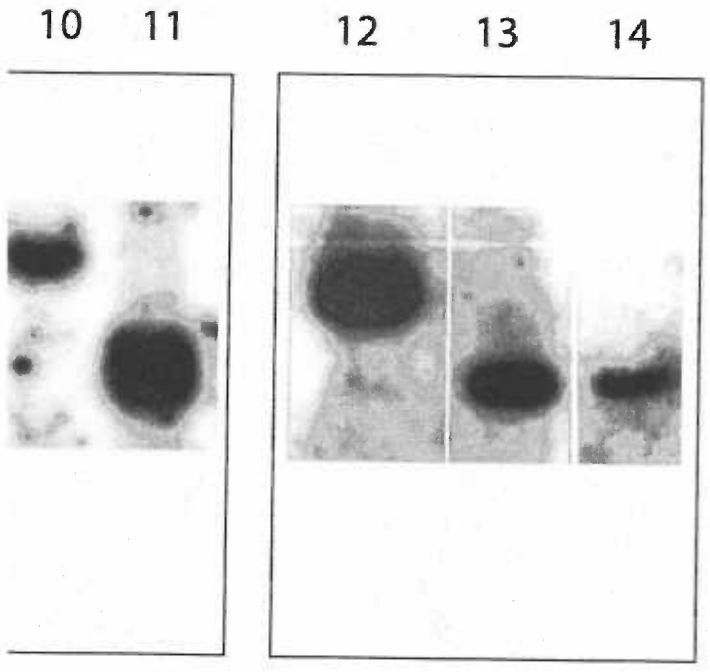
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Figure 2.4. Southern blot analysis of livers mice corrected by ϕC31 integrase. Southern blot of liver genomic DNA from three different mice digested with three enzymes: *Hind*III (lanes 1-6; junction fragments), *Sca*I (lanes 7-9; junction fragments), or *Pst*1 (lanes 10-14; internal fragments) and hybridized with the *FAH* probe. The upper left panel (lanes 1-5) has plasmid copy number controls of 9, 3, 1, 0.3 and 0.1 per diploid genome equivalent. Lane 6, 7, and 11 are from mouse 1, lanes 8 + 13 from mouse 2, lanes 9 + 14 from mouse 3, and lanes 10 + 12 from a single copy control (FAH retrovirus). Mouse 1 displayed two dominant junction fragments seen with both *Hind*III (lane 6) and *Sca*I (lane 7) digestion. Table 3 lists the integration sites and their genomic location. Mice 2 and 3 also had several dominant integration bands. All three mice shared the common mpsL1 integration site (6.0kb) as denoted by the arrow. Lane 11 (mouse 1) has a copy number exceeding one/ diploid genome equivalent, whereas mice 2 and 3 had <1 copy/diploid genome equivalent, consistent with ~ 50-70% repopulation.

A nested inverse PCR approach was used on genomic DNA from Mouse 1 in order to identify the specific integration junctions shown in the *Hind*III Southern blot (lane 6, Fig. 4). Seven different integration sites were identified. The vector-genome junctions and their locations within the genome are listed in Table 2.3. In all seven cases, the switch from the *attB* sequence of the vector to the genomic sequence occurred at or near a TT core, confirming the use of ϕC31 mediated integration. The spaces in the vector sequence leading up to the TT core denote bases that were eliminated by the recombination event. Table 2.3 also predicts the Southern blot band size for each integration site by analysis of the flanking genomic DNA for the next *Hind*III restriction site. As expected, a primary integration site was mpsL1 on chromosome 2 (Olivares et al. 2002). Another major site of integration (as determined by the Southern blots band intensity) was on chromosome 17 within the 3' untranslated region of the Cdkn1a (p21) gene, approximately 900 bp 3' of the stop codon. Cdkn1a, p21, is a transcriptional target of the tumor suppressor p53 and involved in the regulation of cell cycle progression at the G1/S boundary (el-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993). If two gaps are inserted near the middle of the site, then the "pseudo *attP*" site near p21 has 37% homology to the wild type *attP* site, similar to mpsL1 (30%) (Figure 2.5). The 5 other integration sites were not in or near genes (>10,000 bp distance). The presence of all seven integration sites was tested in five additional serially transplanted mice using PCR. In one mouse, 5 out of the 7 integration sites were present, including the p21 site. The other four mice each had two or three of the seven integration sites, but not the p21 site. All five mice utilized the mpsL1 integration site, suggesting that it was the most prevalent site.

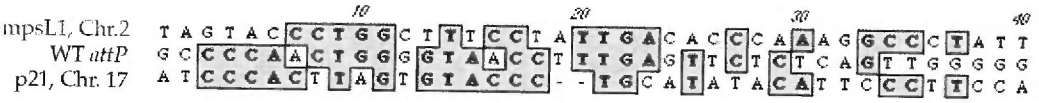

Figure 2.5. Sequence alignment of pseudo *attP* sites and the wild type *attP*.

Wild type *attP* and "pseudo *attP*" sites, mpsL1 and chromosome 17 are shown. MpsL1 and the wild-type *attP* have 30% identity. Chromosome 17 and wild-type *attP* have 37% similarity, if a 2-bp gap is inserted in the pseudo site near the core.

Chromosome Location	Sequence vector-genome junction	Neighboring Gene(s)	Predicted Band
2 (mpsL1)	tcgacgatgtaggtcacggtctcgaagccgcggtgcgggtgccagggcgtgc- AGGGTACTACTCAGATGGTGACAGAGATGACA GCTGCCCACATGAA	-	7.7 kb
7	tcgacgatgtaggtcacggtctcgaagccgcggtgcgggtgccagggcgt------- AGGACATATTCCCAGTGACCCACCAGGGTCCACAG ACAACAGCATGGGTATGGCGCCCAG	-	4.2 kb
10	tcgacgatgtaggtcacggtctcgaagccgcggtgcgggtgccagggcgtgccc--- AGGATCCTTATCTGCTAAGAATAGCTGTGTTTGGT CTGGCTCGGGAGACCCAT	-	6.9 kb
12	tcgacgatgtaggtcacggtctcgaagccgcggtgcgggtgccagggcgt------- TACTCATGGTCTCAAGGTTATGACTCACAACCTGG ACCAATTTTGGGAGGAAGTTGCT	-	5.2 kb
14	tcgacgatgtaggtcacggtctcgaagc---------- GCGGTCCTTTCAGGAGTGTGGTGACTCAAAGTCCT CAGAGTCACCAAATC	Tkt (10 kb 5') Prkcd (11kb 3')	13.3 kb
17	tcgacgatgtaggtcacggtctcgaagccgcggtgcgggtgccagggcgtgccct- GCATATACATTCCCTTCCAGTCCACTGAGCTGTGG GGCAAGTGCCTAGATATG	Cdkn1a (in 3' UTR)	6.8 kb
X	tcgacgatgtaggtcacggtctcgaagccgcggtgcgggtgccagggcgtgcc---- ACACAATGGCATCTTAGAATTCTTTTCAACACATAT AATTTTGCGGTGGACTCAAGAGAGTTACCC	-	3.1 kb

Table 2.3. Junctions of FAH expression vector to genomic DNA.

The vector sequence is in lowercase and the genomic DNA sequence is in uppercase. For each integration site, the corresponding chromosome number and location in relation to neighboring genes is given. Southern blot (Figure 2.4) band size (*Hind*III digest) for each integration are shown.

V. Discussion

In previous studies, ϕC31 integrase has been shown to mediate efficient gene transfer of plasmid DNA into host cells through a recombination event between the attachment sequence *attB* and pseudo *attP* sites present in the host genome (Thyagarajan et al. 2001). This type of recombination event, by requiring significant sequence identity, limits the number of potential integration sites, making the ϕC31 integrase system relatively site specific. In addition, the recombination is unidirectional and integration is stable. These unique characteristics of the ϕC31 integrase system make it an attractive non-viral gene transfer system.

Here we confirmed and extended previously reported properties of ϕC31 integrase mediated gene transfer in murine liver *in vivo* (Olivares et al. 2002). Approximately 3.6% of plasmid-transfected hepatocytes integrated the FAH transgene and expressed the enzyme. This integration frequency is similar to that achieved with the *Sleeping Beauty* transposase system in the same *in vivo* model (Montini et al. 2002). Furthermore, the enhanced site-specificity of integration was confirmed. The mpsL1 site, which is distant from any genes, was by far the most utilized site and was found in all treated mice. However, six additional sites of integration were identified. It is likely that all seven sites represent areas of high FAH expression and/or integration that allow for selective expansion. It is also plausible that other sites of integration did occur initially, but due to minimal expression of FAH and/or placement within the genome the clone was not able to expand. Interestingly, one pseudo *attP* site was in the 3' untranslated region of the p21

gene. This site was detected in two mice, but went undetected in other integrase treated mice. Importantly, however, there is very little sequence conservation between the 3' UTR of the human and murine p21 genes. Therefore the observation made here in mouse does not predict that the human p21 gene will also target for ϕC31 integrase mediated integration.

The *FAH* copy number was determined to be approximately one copy per diploid genome equivalent. However, in one mouse (mouse 1, Figure 2.4), which had the p21 locus as a dominant integration site, the copy number was significantly higher than one. It is therefore possible that the higher copy number was caused by the disruption of the p21 gene. In other samples, where integration into p21 did not occur, there was ≤1 copy of *FAH* per diploid genome equivalent.

We also found that ϕC31 integrase-transfected hepatocytes displayed morphological abnormalities at a higher frequency than controls. This phenomenon was dependent on the amount of integrase expression plasmid present. High levels of hepatocyte dysplasia has not been previously seen with other methods for *in vivo* gene transfer into the liver of $Fah^{\Delta exon5}$ mice, including oncoretroviral vectors, AAV2 and *SB* transposase (Overturf et al. 1996; Montini et al. 2000; Nakai et al. 2003). The abnormal cell morphology could to some extent be ascribed to Fah deficiency itself because dysplastic hepatocytes were seen at low frequencies in controls and are seen at high frequency upon withdrawal of NTBC (Al-Dhalimy et al. 2002; Luijerink et al. 2003). However, it is probable that integrase itself is also responsible for the dysplastic

appearance of hepatocytes. The precise cause of these morphological changes is currently unclear and must be further investigated. A theoretical possibility is that ϕC31 integrase causes some level of chromosomal instability. ϕC31 integrase is a site-specific recombinase as is the unrelated enzyme Cre. It has recently been suggested that Cre-recombinase may mediate "illegitimate recombination" *in vivo*, i.e. create genomic rearrangements even in the absence of a loxP site in the target genome (Schmidt et al. 2000). Since the human and murine genomes contain multiple pseudo *attP* sites (at least 7 in mouse hepatocytes), it is conceivable that ϕC31 integrase expressed at high levels could also mediate recombination between "pseudo *attP*" sites on different chromosomes or even between more distantly related DNA sequences. It is possible that some aspect of integrase behavior, such as putative nicking at pseudo *att* sites, is tolerated in normal cells, but is more detrimental in the perturbed hepatocyte environment of the $Fah^{\Delta exon5}$ mouse model. The microenvironment of the *Fah* mutant liver is abnormal, even in the presence of NTBC, as suggested by the approximate 10 fold-lower than normal transfection efficiency of the hydrodynamic liver delivery method observed here and in previous studies (Montini et al. 2002), the measurable levels of spontaneous hepatocyte dysplasia (Figure 2.3), and the changes in liver gene expression described for this disease setting (Luijerink et al. 2003).

Abnormal cellular morphology has not been reported in previous studies of *in vivo* or *in vitro* ϕC31 integrase gene transfer suggesting that the phenomenon is specific to the $Fah^{\Delta exon5}$ model (Thyagarajan et al. 2001; Olivares et al. 2002; Ortiz-Urda et al. 2002; Ortiz-Urda et al. 2003). For example, ϕC31 integrase-treated human keratinocytes

were biopsied from grafts at 4, 8, 12, and 14 weeks after grafting and analyzed, with the result that the integrase treated cells were indistinguishable from normal cells (Ortiz-Urda et al. 2003). The ϕC31 integrase has been used extensively in mouse embryonic stem (ES) cells to perform genomic manipulations and to generate transgenic mice with no adverse consequences, and ES cells expressing ϕC31 integrase under the PGK promoter have been used to generate lines of mice that contain the ϕC31 integrase gene in every cell (Belteki et al. 2003; Hollis et al. 2003). These mice display normal development and fertility, suggesting that modest levels ϕC31 integrase may have little toxicity. Transgenic *Drosophila* have also been generated by using ϕC31 integrase with no ill effects (Groth et al. 2004). In the present study, FAH expressing hepatocytes were of normal appearance when histology was performed 90 days after injection. This finding indicates that normal hepatocytes are growth-selected or that abnormal hepatocytes revert to a normal morphology with time. In either case, the transient presence of cells with abnormal morphology does not appear to have pathological consequences.

Overall, our findings suggest that ϕC31 integrase is effective for integrating the *FAH* gene into hepatocytes after co-delivery of a plasmid expressing the ϕC31 integrase and a plasmid encoding *FAH* and bearing an *att*B site. Integration was site-specific, with seven integration sites documented, one being predominant. However, the insertion site into the mouse p21 gene serves as a reminder that the human genome may contain "unwanted" pseudo sites that may be used at a lesser frequency. Successful serial transfer of corrected hepatocytes to secondary and tertiary recipients indicated that the integration events were stable and that gene expression was persistent over time and

through multiple rounds of cell division. The elevated frequency of transient hepatocyte dysplasia seen here suggested that there is some level of toxicity associated with integrase expression in the $Fah^{\Delta exon5}$ mouse model. These types of transient morphological abnormalities have been observed to date only in the $Fah^{\Delta exon5}$ mouse setting. Normal, healthy livers without dysplastic cells were observed by 90 days. With careful monitoring for toxicity and for safety of integration sites, the ϕC31 integrase maybe a candidate gene transfer system for clinical use.

VI. Acknowledgements

Dr. Eric Olivares in the laboratory of Dr. Michele Calos at Stanford University designed the integrase plasmids carrying the human FAH gene and also assisted in the identification of integration sites, as shown in Table 2.3. Dr. Milton Finegold and Angela Major at Texas Children's Hospital performed all the Fah immunohistochemistry as shown in Figure 2.2. Tail vein injection of the plasmid DNA was performed by Christina Aguilar in the Grompe Laboratory. This work was supported by the National Institute of Health grants DK048252 (M.G.) and HL68112 (M.P.C.).

Chapter 3

Correction of renal tubular dysfunction by genetic selection

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I. Abstract

Repopulation by transplanted cells can result in effective therapy for several regenerative organs including blood, liver and skin. In contrast, cell therapies for renal diseases are not currently available. Here we developed a new animal model in which chronic injury and regeneration of renal proximal tubules was induced by homogentisic acid (HGA). In these mice, transplanted bone marrow cells could produce significant regeneration of proximal tubular epithelium (up to 50%). The majority of bone marrow-derived epithelial cells were generated by cell fusion, not transdifferentiation. In addition to regeneration by fusion derived epithelial cells, proximal tubular repopulation was also observed by host epithelial cells, which had become genetically resistant to HGA. This data demonstrate that extensive regeneration of the renal proximal tubule compartment can be achieved through genetic selection of functional cells.

II. Introduction

Kidney transplantation is the treatment of choice for many cases of end-state renal failure, which is often caused by genetic disorders of renal tubules such as cystinosis (Gahl et al. 2002) or adult onset polycystic kidney disease (Wilson 2004). While organ transplantation is effective, there is a critical shortage of donor organs (Magee et al. 2004) making cell therapy a desirable alternative (Humes et al. 2004).

Bone marrow cells are capable of giving rise to a broad range of non-hematopoietic cell types *in vivo*, including cardiac muscle (Orlic et al. 2001), skeletal muscle (Ferrari et al. 1998), neurons (Mezey et al. 2000), and liver epithelium (Lagasse et al. 2000). The hematopoietic stem cell (HSC) can serve as the precursor to these different lineages, but controversy exists regarding the mechanism. In the "stem cell plasticity" model, the HSC is capable of differentiation into multiple non-hematopoietic and non-mesodermal cell types (Krause et al. 2001). In the "fusion and reprogramming" model, these unexpected cell types are the result of a fusion between the HSC (or progeny of the HSC) and lineage specific cells (Vassilopoulos et al. 2003; Wang et al. 2003; Willenbring et al. 2004). Regardless of the mechanism, the replacement level by donor cells after bone marrow transplantation (BMT) has been low for most organs and functional correction of relevant disease states has not been achieved. A notable exception is the mouse model of hepatorenal tyrosinemia (HT1) where bone marrow-derived hepatocytes obtained through cell fusion of myelomonocytic cells with hepatocytes, have been shown to correct the liver disease (Lagasse et al. 2000; Wang et

al. 2003; Willenbring et al. 2004). Patients with HT1, caused by a lack of the enzyme fumarylacetoacetate hydrolase (FAH), exhibit both liver injury and renal Fanconi syndrome (Kvittingen 1986). FAH is the last enzyme in the tyrosine degradation pathway and is normally expressed in both hepatocytes and proximal renal tubule epithelium (Labelle et al. 1991; Grompe et al. 1993).

Previously, some observations have suggested that extrarenal cells can contribute at low levels to regenerative responses following acute tubular necrosis (ATN) (Poulsom et al. 2001; Gupta et al. 2002; Kale et al. 2003). To date, however, repair and functional correction of inherited disorders effecting renal tubules have not been shown. Therefore, we wished to determine whether bone marrow-derived cells could correct the renal tubular damage in the murine model of HT1. Renal repopulation was tested under various conditions of stress and chronic injury. In addition, the mechanisms for obtaining bone marrow-derived tubular cells were evaluated.

III. Materials and Methods

Mouse strains and animal husbandry

The following mouse strains were used and handled according to NIH guidelines for animal care and with the approval of the institutional animal care and utilization committee of the Oregon Health & Science University: $Fah^{\Delta exon5}$ (aka. $Fah^{-/-}$ mice) (Grompe et al. 1993), $Fah^{-/-}$,Hgd^{aku}/Hgd^{wt} (*Hgd* heterozygote) (Montagutelli X et al. 1994; Manning et al. 1999) *Rosa26* C57BL/6 (Friedrich et al. 1991), and *Rosa26* 129SV (Friedrich et al. 1991). Both $Fah^{-/-}$ and $Fah^{-/-}$,Hgd^{aku}/Hgd^{wt} mice were treated with water containing 2-(2-nitro-4-trifluoromethylbenzoyl)-1.3-cyclohexanedione (NTBC) at a concentration of 7.5 mg/L (provided by S. Lindstedt, Gothenborg, Sweden)(Grompe et al. 1995). For hematopoietic cell transplantation, syngeneic female *Rosa26* mice were used as donors for male $Fah^{-/-}$ or $Fah^{-/-}$,Hgd^{aku}/Hgd^{wt} mice.

Transplantation

Bone marrow collection and transplantation into $Fah^{-/-}$ or $Fah^{-/-}$,Hgd^{aku}/Hgd^{wt} mice were performed as previously described (Lagasse et al. 2000; Wang et al. 2002). Male $Fah^{-/-}$ or $Fah^{-/-}$,Hgd^{aku}/Hgd^{wt} mice were lethally irradiated twice with a total dose of 1500 Gy, split over a three hour interval. After irradiation, bone marrow cells from donor female *Rosa26* mice were transplanted into the retro-orbital venous plexi of anesthetized male $Fah^{-/-}$ or $Fah^{-/-}$,Hgd^{aku}/Hgd^{wt} mice. Cells were injected at a volume of 100 µl per mouse using insulin syringes (BD Biosciences). For mice receiving sorted KLS or c-Kit

bone marrow, an additional 2 X 10^5 *Fah*$^{-/-}$ congenic adult male bone marrow cells were also transplanted as a radioprotective dose.

Kidney cell isolation

Kidney tissue (350mg) was minced and added to 10 ml of a 4 mg/ml solution of dispase II (Sigma-Aldrich) in DMEM (Invitrogen). The minced tissue and media were transferred to a 50 ml Erlenmeyer flask and incubated for one hour at 37^{0}C with constant gentle stirring using a magnetic stir bar. Following the incubation, the tissue was filtered through a 70 micron nylon cell strainer (BD Biosciences) and washed twice in DMEM plus 10% fetal bovine serum (Invitrogen). The cells were counted using a hemocytometer and approximately 1000 cells were deposited onto glass slides by cytospin centrifugation set at 500 rpm for 5 minutes. The deposited cells were fixed in methanol for 1 minute followed by three washes in acetone for 1 minute each.

Immunohistochemistry/Immunocytochemistry for Fah

For immunohistochemistry, kidney tissue was fixed in 10% phosphate buffered formalin, pH 7.4, dehydrated in 100% ethanol and embedded in paraffin wax at 58°C. Four-micrometer sections were rehydrated and stained with hematoxylin and eosin and with a polyclonal rabbit antibody against rat FAH (kindly provided by Robert Tanguay, University of Laval, Quebec, Canada). The antibody was diluted in PBS, pH 7.4, and applied at a diluted concentration of 1:300,000 at 37°C for 30 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 and methanol. Avidin and biotin pretreatment was used to prevent endogenous staining. The secondary antibody was

biotinylated goat anti-rabbit IgG used at 1:250 dilution (BA-1000: Vector Laboratories). Color development was performed with the AEC or DAB detection kit (DakoCytomation).

For immunocytochemistry, an alkaline-phosphatase-conjugated swine anti-rabbit secondary antibody (DakoCytomation) diluted 1:25 was used. For the detection of antibody-enzyme complexes, the Fast Red Substrate System was used as described by the manufacturer (DakoCytomation).

β-Galactosidase staining

Liver tissues were fixed in 2% formaldehyde and 0.2% glutaraldehyde in phosphate buffered saline (PBS) for 30 minutes. Then the tissues were washed twice with PBS and stained as previously described (MacGregor GR et al. 1987; MacGregor et al. 1987; Wang et al. 2002).

Induction of acute tubular necrosis

Bone marrow transplanted *Fah*$^{\Delta exon5}$ mice were anesthetized and a midline incision was made exposing the left kidney. For unilateral ischemia the left renal pedicle was clamped for 60 minutes. The abdomen was covered with gauze and moistened with 0.9% saline and mice were maintained at 37^{0}C using a warming pad. After one hour, the clamp was removed and reperfusion was confirmed visually and the abdominal incision was closed (Kale et al. 2003).

Staining and purification of HSC

Cells were stained as described (Spangrude et al. 1988; Lagasse et al. 2000). For KLS cell isolation from *Rosa26* 129SV or C57BL/6 mice, the bone marrow cells were incubated with biotinylated monoclonal antibody specific for Sca-1 (PharMingen), allophycocyanin-conjugated 2B8 (c-kit) (PharMingen), and phycoerythrin-conjugated lineage markers (PharMingen), which included RA3-6B2 (B220) for the B-lineage marker; RM2-5 (CD2), GK1.5 (CD4), 53-7.3 (CD5), 53.6.7 (CD8), and 145-2C11 (CD3) for T cell markers; RB6-8C5 (GR-1) and M1/70 (CD11b, Mac-1) for myeloid markers; PK 136 (NK1.1) for natural killer cells; and Ter119 for erythrocytes. Secondary staining was performed for streptavidin-Phared (PharMingen). After the final wash, cells were resuspended in PBS/FCS buffer that contained 1 mg/ml of propidium iodide to discriminate between viable and nonviable cells. Isolation of HSC's was accomplished using a fluorescence-activated cell sorter (FACS™; Becton Dickinson Immunocytometry Systems). Specifically, the FACSVantage SE was configured with argon, krypton, and helium-neon ion. Data parameters were collected in the list mode data file and were analyzed by the software program Flowjo (*www.Treestar.com*). Pure populations of sorted HSCs were resorted directly into Eppendorf tubes by an automated cell deposition unit using counter mode.

Reverse transcription-PCR (RT-PCR)

At the time of sacrifice, approximately 30 mg of kidney tissue from $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice was used for mRNA isolation using the Rneasy Mini Kit (Qiagen).

Ten micrograms of the total mRNA was reverse-transcribed with 0.8 μg oligo (dT) in a volume of 40 μl by using a BRL kit (Life Technologies). Two microliters of the RT reaction was subjected to PCR amplification under the following conditions: 94^0C for 5 min, followed by 30 cycles of 90^0C for 30 sec, 55^0C for 30 sec, and 72^0C for 1 min, and then a final extension step of 72^0C for 10 min. Boehringer Mannheim 10X PCR buffer and 2.5 mM Mg^{2+} (Perkin-Elmer) were used in a 25 μl reaction with Taq DNA Polymerase (Boehringer Mannheim). The reverse primer (nt 1219-1240 in *Hgd*) had the sequence 5'-TCCACCTTGCTTTGCCTCATAG-3', and the forward primer (nt 845-866) had the sequence 5'-CGAGATTTCTTGATTCCCGTTG-3'(Manning et al. 1999).

Western blot analysis

Protein extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane (Millipore). After the transfer, the nonspecific antibody-binding activities of the membranes were blocked in 20% Blokhen II (Aves Labs) diluted in PBS for one hour at room temperature. The primary antibody against Hgd was raised in chickens and used in a 1:5000 dilution in PBS plus 0.1% Tween 20. In addition, protein serum from $Hgd^{aku/aku}$ mice was added to the primary antibody solution at a concentration of 10 μg/ml to diminish the amount of unspecific binding. The primary incubation was performed overnight at 4^0C. A goat anti-chicken IgY secondary antibody conjugated to horse-radish peroxidase (Santa Cruz Biotechnology) was used in a 1:50,000 dilution in PBS plus 0.1% Tween 20 for 45 minutes at room temperature. Extensive washes were performed with PBS plus 0.1% Tween 20. Detection of immunolabeled proteins was done using chemiluminescence kit

(Bio-rad) and Hyperfilm-enhanced chemiluminescence film (Amersham Biosciences).

Measurement of spleen engraftment

A semiquantitative PCR was performed to determine the ratio of wild type to *Fah* mutant alleles in spleen genomic DNA as a measure of percentage engraftment for bone marrow transplanted mice. A three primer competitive PCR was used as previously described (Grompe et al. 1993). To quantitate the relative ratios of *Fah* to wild type, the PCR products were hybridized with a ^{32}P-labeled internal oligonucleotide probe (5'GGCATTATGTTCAGAGGC-3'). The intensities of both *Fah* mutant and wild type signals were quantified by using a Beckman SI Phosphoimager and compared with a standard curve generated by mixing known ratios of DNA from wild type and *Fah* mutant mice (Wang et al. 2003).

Urine amino acid levels

Quantification of urine amino acids was performed using standard ion-exchange chromatography with on-line ninhydrin derivatization (Gupta et al. 2004). Urine was collected daily one week prior to sacrifice of the bone marrow transplanted (BMT) mice. Samples were stored at –80^{0}C prior to analysis.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) following Fah immunohistochemistry was performed as previously described (Jiang et al. 2004). Sections were incubated with 1M sodium thiocyanate solution for 10 minutes at 80^{0}C, washed in phosphate buffered

saline (PBS), and incubated in pepsin solution (0.4 g pepsin in 100 ml of 0.1 M Hcl) for 10 minutes at 37^{0}C. Following digestion, the slides were washed in PBS and fixed in a 4% paraformaldehyde solution for 2 minutes. Following fixation, the cells were washed in PBS and then air-dried. Three microliters of Cy3-labelled Y-chromosome paint (ID Labs Inc) was mixed with 12 μl of hybridization buffer and warmed to 37^{0}C. Fifteen microliters was applied to the center of the slide, a cover slip applied, and the slide was heated to 65^{0}C for 10 minutes and incubated overnight at 37^{0}C. The next day the slides underwent three washes in 50% formamide solution at 37^{0}C, a stringency wash solution at 37^{0}C, a detergent wash solution at 37^{0}C, and then PBS at room temperature. The slides were mounted in DAPI (ID Labs Inc.) and examined.

FISH following immunocytochemistry for Fah was performed as previously described (Wang et al. 2003). Slides were washed in 2 X SSC at 37^{0}C for 30 minutes and then treated with 10% pepsin diluted in 40 ml of 0.01 M HCl at 37^{0}C for 20 minutes (Sigma-Aldrich). The slides were washed in PBS, fixed in formaldehyde, and dehydrated in ethanol. For hybridization the slides were denature in 70% formamide solution at 72^{0}C for 4 minutes while the paints for mouse X (FITC) and Y (Cy3) chromosomes (Vysis) were mixed, denatured at 75^{0}C for 10 minutes, and preannealed at 37^{0}C for 30 minutes. The probes were then added onto slides and hybridization was performed at 37^{0}C overnight. The next day the slides were washed in 2 X SSC at 37^{0}C for 5 minutes and then 2 X SSC/0.1% NP-40 (Sigma-Aldrich) at room temperature for 1 min. The cells were counterstained with DAPI (Vysis) and observed using a Nikon Eclipse photoscope.

IV. Results

Renal tubule repopulation in $Fah^{-/-}$ mice

Bone marrow transplantations were performed in 30 lethally irradiated $Fah^{-/-}$ 129SV mice. All mice received 1-10 x 10^6 unfractionated bone marrow from a *Rosa26* 129SV donor. Mice were on NTBC at the time of transplantation and were kept on the protective drug for 3 weeks after BMT to allow for hematopoietic engraftment (Figure 3.1). After discontinuation of the drug, the animals were sacrificed between 6-9 months and the liver and kidney tissue was analyzed by Fah immunohistochemistry. As previously reported, the emergence of functional Fah positive hepatocytes in this cohort of animals was observed (Lagasse et al. 2000). In wild type mice, Fah is expressed in proximal renal tubular cells during postnatal life and therefore is an excellent marker for that cell type (Figure 3.2). However, only 3 single Fah positive proximal tubule cells (one positive cell in three separate animals) were found in 150 sections screened (5 sections each in 30 BMT mice) (Figure 3.2). There was no consistent pattern regarding radiation dose, time after transplantation, or cell dose in the three animals. Therefore, engraftment of renal tubular epithelial cells from the donor following bone marrow transplantation was rare. Furthermore, only single cells were seen suggesting that cell division had not occurred. In HT1, renal tubular dysfunction improves after correction of the liver disease (Grompe et al. 1993) and it is likely that sufficient injury to induce repopulation of the tubules was not present in the standard HT1 mouse model.

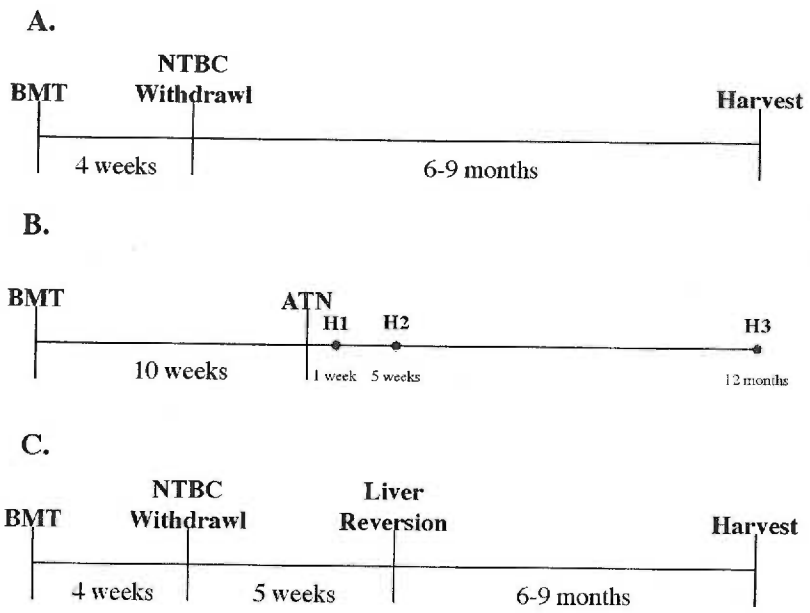

Figure 3.1. Experimental time line.

Time line for assessing kidney repopulation in **(A)** BMT *Fah*$^{-/-}$ mice **(B)** BMT *Fah*$^{-/-}$ mice following ATN **(C)** BMT *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice. BMT=bone marrow transplantation, ATN=acute tubular necrosis, H1-H3=harvest time points

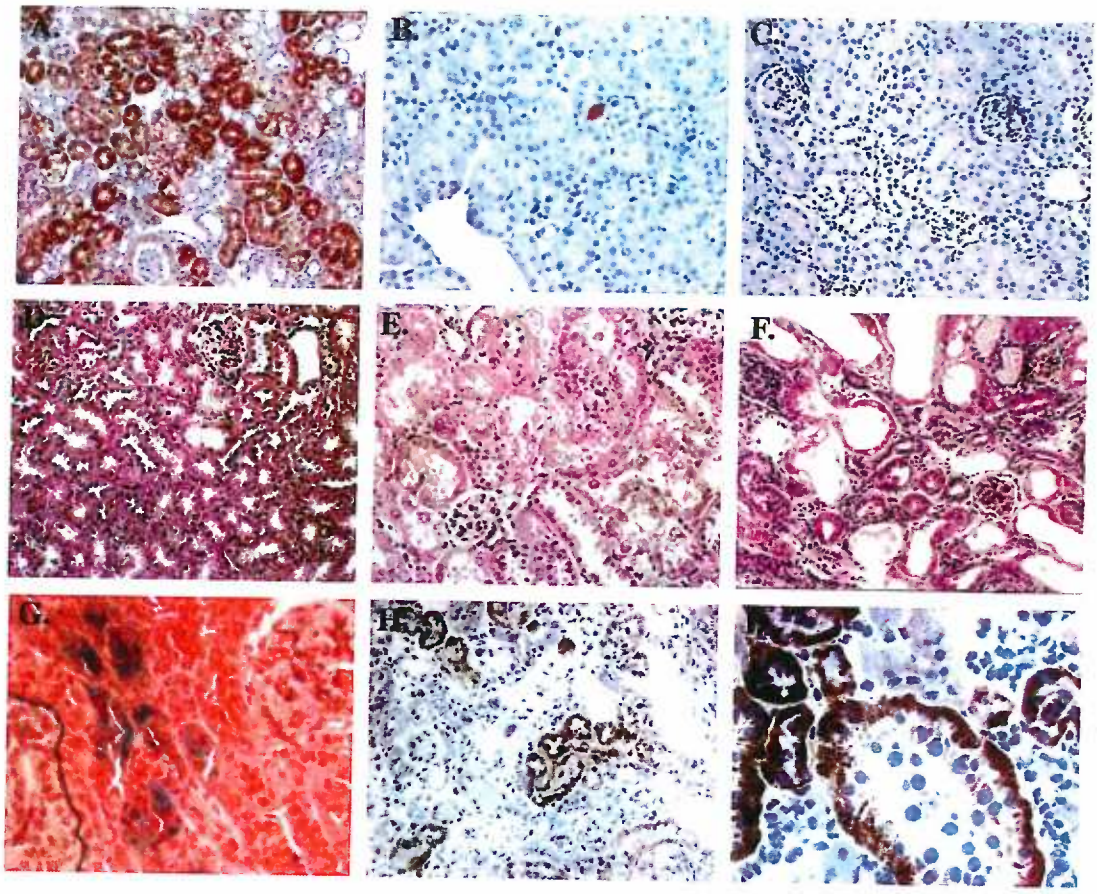

Figure 3.2. Histological analysis.

(A-C) Fah immunohistochemistry in kidney tissue. **(A)** Wild type mouse with Fah positive proximal tubules stained in brown. **(B)** BMT *Fah*$^{-/-}$ mouse after nine months off NTBC. Single Fah+ epithelial cells were present. **(C)** BMT *Fah*$^{-/-}$ mouse harvested 5 weeks after induction of ATN. No Fah+ cells were present. **(D-F)** H&E staining in kidney tissue. **(D)** Wild type mouse with healthy renal morphology. **(E)** *Fah*$^{-/-}$ mouse off NTBC for 4 weeks. Tissue shows evidence of sloughing of the injured epithelial cells, nuclear enlargement and focal cytoplasmic vacuolization of the epithelial lining. **(F)** *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mouse five weeks after liver reversion. Damage to the proximal tubules is more severe than *Fah*$^{-/-}$ mouse (panel E) with greater dilation and more extensive

vacuolization. **(G)** β-galactosidase staining (blue) in kidney tissue from BMT *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} after 9 months of selection for tubule repopulation. **(H-I)** Fah immunohistochemistry in kidney tissue from the same BMT *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} as panel G after 9 months of selection. Many proximal tubules are stained positively for Fah (brown). Panel I is the same tissue section as Panel H under higher magnification. BMT=bone marrow transplantation, ATN=acute tubular necrosis

Renal tubule repopulation in Fah$^{-/-}$ *mice following induction of acute tubular necrosis*

Previously, others have reported that hematopoietic cells could contribute to repopulation of ischemically injured renal tubules (Gupta et al. 2002). To test whether this also occurs in HT1 mice, nine *Fah*$^{-/-}$ 129 mice underwent bone marrow transplantation from a *Rosa26* 129SV donor. At ten weeks post transplantation, unilateral tubular necrosis was induced by clamping the renal vein and artery for 60 minutes. Three mice were sacrificed at each time point: one week, five weeks, and one year following induction of acute tubular necrosis (Figure 3.1). Immunohistochemistry for Fah expression in both the injured and the contralateral control kidney was performed at each time point. Surprisingly, no Fah positive cells were seen in the injured kidney at any of the time points (Figure 3.2). Therefore, acute tubular necrosis was also not sufficient to induce renal repopulation in the 129SV HT1 mouse model.

Fah$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt}*: A new model for chronic renal injury*

Because the mild renal disease associated with simple Fah deficiency was not sufficient to stimulate repopulation, the model was enhanced to induce constant chronic

renal tubular damage. *Fah*$^{-/-}$ mice that are also heterozygous for a mutation in the gene for homogentisic acid dioxygenase (*Hgd*) an enzyme upstream from Fah in the tyrosine degradation pathway, have been shown to undergo loss of heterozygosity (LOH) at the *Hgd* locus in hepatocytes (Manning et al. 1999). The loss of the wild type *Hgd* allele results in strong selection for these hepatocytes and in liver repopulation by revertant cells. In addition, the tyrosine degradation pathway is blocked at the level of *Hgd* inducing systemic accumulation of HGA and chronic renal injury, as schematically depicted in Figure 3.3. Figure 3.2 shows H&E staining in healthy kidney tissue, in kidney tissue from *Fah*$^{-/-}$ mice off NTBC, and in *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice off NTBC that had undergone hepatic LOH of *Hgd*. The renal damage in *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice after LOH (Figure 3.2 F) was significantly more severe than *Fah*$^{-/-}$ mice (Figure 3.2 E).

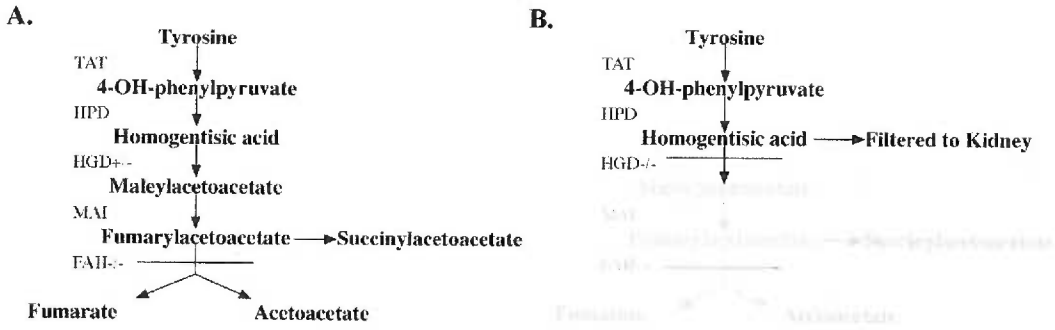

Figure 3.3. Tyrosine degradation pathway.

(A) Schematic depiction of the tyrosine degradation pathway in *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mouse **(B)** Schematic depiction of the tyrosine degradation pathway in *Hgd+/-Fah-/-* mouse after the liver has undergone LOH of *Hgd* allele. TAT=tyrosine aminotransferase, HPD=4-hydroxyphenylpyruvate dioxygenase, HGD=homogentisate 1,2 –dioxygenase, MAI=maleylacetoacetate isomerase

Kidney repopulation in Fah$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} *mice*

Lethally irradiated male *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} C57BL/6 mice were bone marrow transplanted with either 1 X 10^6 whole bone marrow cells, 50,000 c-Kit+ cells (enriched for hematopoietic stem cells), 50,000 c-Kit- cells (depleted of hematopoietic stem cells), or 50 KLS (c-Kit+, Lin-, Sca-1+) cells from a female *Rosa*26 C57BL/6 donor. After transplantation, the mice were kept on NTBC for three weeks to allow for hematopoietic engraftment and then NTBC was removed in order to stimulate LOH of the wild-type *Hgd* allele in hepatocytes. A total of 17 transplanted mice survived LOH of the wild type *Hgd* allele and liver repopulation with *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{aku} hepatocytes. These mice

were sacrificed 6-9 months after stopping NTBC treatment and kidney Fah immunohistochemistry was performed (Figure 3.1). Proximal tubule repopulation with Fah positive cells occurred in 8 out of 12 mice (66%) transplanted with whole bone marrow, c-Kit+ cells, or KLS cells (Table 3.1, Figure 3.2). No Fah positive tubules were seen in the five mice transplanted with c-Kit- cells. The percent repopulation ranged from 50% in the best mouse, transplanted with whole bone marrow, to only one positive tubule in the least repopulated mouse, transplanted with 50 KLS cells. Importantly, Fah positive cells were always observed in clusters, not as single cells. The origin of the bone marrow derived Fah positive proximal tubule cells was confirmed by positive β-galactosidase staining (Figure 3.2). In addition, all eight positive mice had measurable levels (between 0.5% and <99%) of hematopoietic reconstitution by wild type donor cells (Table 3.1). Therefore, Fah positive renal tubular cells obtained from whole bone marrow, or specifically KLS cells, were capable of repopulating the damaged tubular epithelium when constant, chronic renal injury was induced by HGA.

Donor Cell	Mice + for Fah	Selection period	% Spleen Engraftment	Kidney Repopulation
c-Kit + 50,000 cells n=5	3 60%	7 months	2%	30%
		8 months	0.5%	2+ tubules
		8 months	1%	1+ tubule
c-Kit – 50,000 cells n=5	0	6-9 months	None	None
KLS 50 cells n=3	2 66%	6 months	>10%	8+ tubules
		7 months	>10%	5+ tubules
Whole BM 2 X 10^6 cells n=4	3 75%	9 months	>90%	50%
		9 months	>90%	25%
		8 months	>90%	10%

Table 3.1. Detection of donor derived cells after bone marrow transplantation. Seventeen *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice underwent BMT with whole bone marrow cell, c-Kit+ cells, c-Kit- cells, or KLS cells. Only three donor cell types (whole bone marrow, c-Kit+ cells, KLS cells) lead to repopulation of the proximal tubule with Fah positive cells in approximately 2/3 of the transplanted mice. Twenty five different tissue sections were evaluated for each mouse. The 1+, 2+, 5+, 8+, refers to the number of positive renal tubules identified in the 25 sections. In addition, mice within these three groups had hematopoietic reconstitution by the donor derived cells as indicated in the percent of spleen engraftment. *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice transplanted with c-kit- cells did not show evidence of hematopoietic reconstitution, nor any Fah positive tubules.

Bone marrow-derived renal tubule cells obtained by cell fusion

In order to determine whether bone marrow-derived tubular epithelium was the product of cell fusion (Terada et al. 2002), female *Rosa26* C57BL/6 bone marrow was

transplanted into male *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} C57Bl/6 mice. The presence of the Y-chromosome (host marker) in Fah positive (donor marker) tubules would indicate fusion. Single cell suspensions were prepared by protease digestion from the kidneys of bone marrow transplanted *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice. The cells were then stained for Fah followed by interphase FISH for the X and Y chromosome. All ten of the Fah positive cells scored contained the Y chromosome, suggesting that they had arisen by fusion (Figure 3.4). To further strengthen the cytogenetic evidence for fusion, kidney sections from whole bone marrow transplanted *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice were also stained for Fah followed by FISH for the Y chromosome. The majority of nuclei from Fah positive epithelial cells displayed a clear Y-FISH signal (Figure 3.4). Therefore the analysis of both single cells and tissue sections both demonstrated that at least 50% of the bone marrow derived renal tubule cells were generated by fusion. In the tissue section analysis, the a few Fah+ cells without the Y chromosome found in the repopulated tubule could have been generated by direct transdifferentiation. On the other hand, Y-negative cells could also be the result of fusion followed by reduction division and loss of the Y-chromosome (Wang et al. 2003). Additionally, the absent Y-chromosome could simply be due to the limitations of tissue section analysis because the Y-chromosome signal is not captured in 100% of male nuclei (Poulsom et al. 2001).

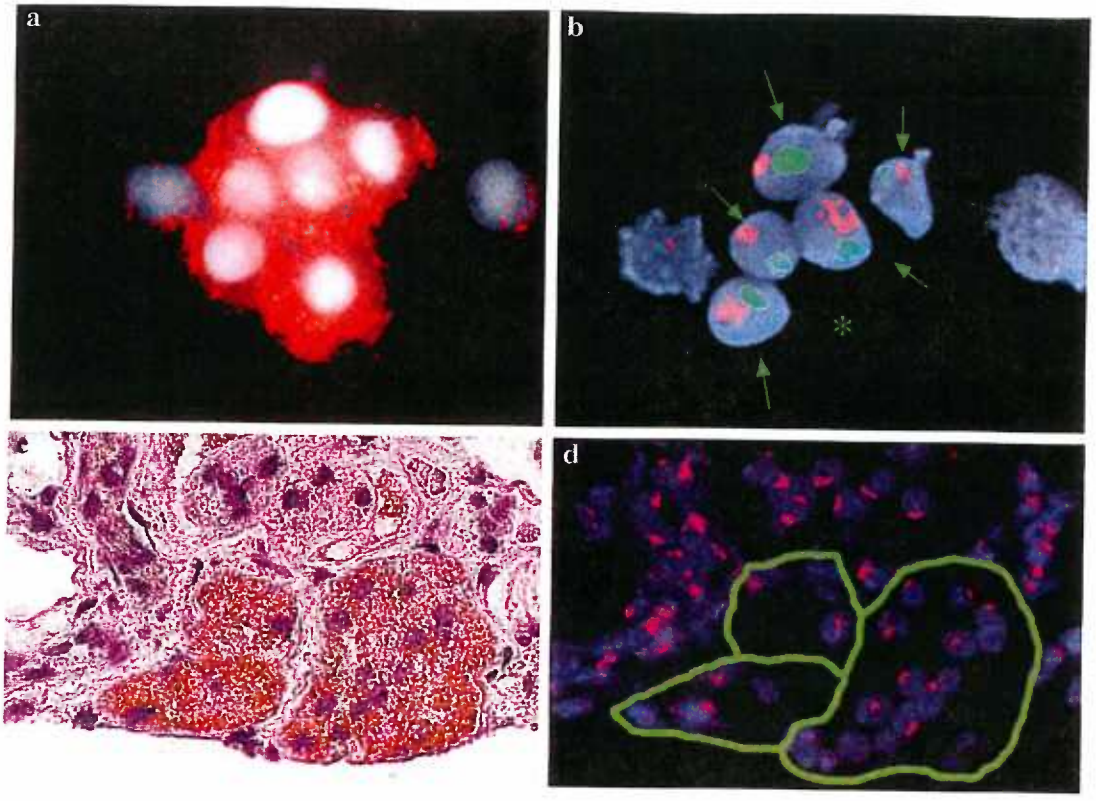

Figure 3.4. Evidence for fusion.

Bone marrow transplantations were performed in male *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice using a wild-type female donor. **(A)** Fah immunohistochemistry on cultured bone marrow derived renal tubule cells from a BMT (1 X 10^6 whole bone marrow) *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mouse after 9 months of selection. **(B)** Sequential interphase FISH for presence of X and Y chromosome (X chromosomes are green, Y chromosomes are pink). *=Fah+ cell lost due to cell movement during FISH procedure. The green arrows point to Fah+ cells that contain the X and Y chromosome. **(C)** Fah immunohistochemistry on tissue sections from the same BMT *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mouse as panel A. **(D)** FISH for presence of Y chromosome in the same tissue section as panel C (Y chromosomes are pink).

BMT=bone marrow transplantation

Functional correction of HT1 renal disease

To determine whether bone marrow derived renal tubule cells corrected the renal disease in *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice, the overall kidney morphology was assessed histologically along with the ability of the tubules to reabsorb amino acids from the urine. *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice that have undergone liver reversion had extensive damage to the renal epithelium and elevated urine amino acid levels in comparison to *Fah*$^{-/-}$ mice or wild type mice (Figure 3.2, Table 3.2). Healthy wild type mice have an average urine serine level of 0.4 +/- 0.2 μmol/mg of creatinine while Both *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} and *Fah*$^{-/-}$ mice off NTBC have significantly elevated urine serine levels, 9.1 +/- 4.2 μmol/mg of creatinine and 12.0 +/- 6.9 μmol/mg of creatinine respectively. All 17 bone marrow transplanted *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice were analyzed for kidney morphology and urine serine levels (Table 3.2). Interestingly, functional analysis segregated the mice into four distinct groups. Group 1 mice (n=2) had abundant Fah positive proximal tubules, good H&E morphology, and no renal Fanconi syndrome (≤1.0 μmol of serine/mg of creatinine present in the urine). Group 2 mice (n=4) had few Fah positive proximal tubules, but overall the kidney morphology was poor and renal Fanconi syndrome was present (>1.0 μmol of serine/mg of creatinine in the urine). Group 3 mice (n=4) had no Fah positive tubules, poor kidney morphology, and >1.0 μmol of serine/mg of creatinine in the urine. Group 4 mice (n=7) had no Fah positive tubules, good histological morphology, and <1.0 μmol of serine/mg of creatinine in the urine.

Group	Mouse Number	Donor Cell	FAH+ Tubules	Kidney Morphology	Urine Serine Levels (μmol/mg)
1	1	c-Kit +	30%	good	1.0
	2	Whole BM	50%	good	0.4
2	3	c-Kit +	2+ tubules	injury	2.6
	4	c-Kit +	1+ tubule	injury	1.4
	5	Whole BM	25%	injury	2.7
	6	Whole BM	10%	injury	3.8
3	7	c-Kit +	-	injury	3.8
	8	c-Kit -	-	injury	1.4
	9	c-Kit -	-	injury	6.6
	10	c-Kit -	-	injury	2.6
4	11	c-Kit +	-	good	0.2
	12	c-Kit -	-	good	0.4
	13	c-Kit -	-	good	0.1
	14	KLS	8+ tubules	good	0.2
	15	KLS	5+ tubules	good	0.3
	16	KLS	-	good	0.2
	17	Whole BM	-	good	0.2

Table 3.2. Functional correction of renal disease by bone marrow derived renal tubule epithelium.

Seventeen *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} mice underwent BMT with whole bone marrow cell, c-Kit+ cells, c-Kit- cells, or KLS cells. At the time of harvest, two conditions of functional correction were measured, kidney morphology and urine amino acid levels. The mice segregated into four groups. Group 1 mice have Fah positive tubules, good morphology, and ≤1 μmol of serine/mg of creatinine. Group 2 mice have Fah positive tubules, renal injury, and ≥1 μmol of serine/mg of creatinine. Group 3 mice have no Fah positive tubules, renal injury and ≥1 μmol of serine/mg of creatinine. Group 4 mice have no Fah

positive tubules, good morphology, and ≤1 μmol of serine/mg of creatinine. The average urine serine amino acid level for wild type mice is 0.4 +/- 0.2 μmol/mg of creatinine and the average urine serine amino acid level $Fah^{-/-}$,Hgd^{aku}/Hgd^{wt} mice off NTBC is 9.1 +/- 4.2 μmol/mg of creatinine.

Loss of heterozygosity in renal tubule cells

The findings in groups 1, 2, and 3 could be explained by the hypothesis that renal function correlates with the degree of repopulation by Fah positive tubular cells, However, Group 4 mice were unusual because no Fah positive tubules were present but the kidney appeared nonetheless healthy and the tubules were functioning correctly by reabsorbing amino acids from the urine. Given this result, it was hypothesized that the renal tubule cells under selective pressure may undergo loss of heterozygosity at the *Hgd* locus, similar to the liver, and thereby become resistant to HGA injury. To test this hypothesis, aliquots of renal tissue from Group 4 were analyzed by RT-PCR for expression of the wild type *Hgd* allele. The wild-type allele was no longer detectable in 3/3 mice analyzed from group 4 (Figure 3.5). Interestingly, the expression levels of the wild-type allele was also reduced (but not absent) in some mice from Group 3 (Figure 3.5) suggesting that *Hgd* LOH had also occurred in these mice. Hgd protein levels in mice from Group 3 and 4 were undetectable (Figure 3.5). In Group 3 mice, *Hgd* loss may have occurred in too small a portion of cells and/or too late in the selection process to correct tubular dysfunction.

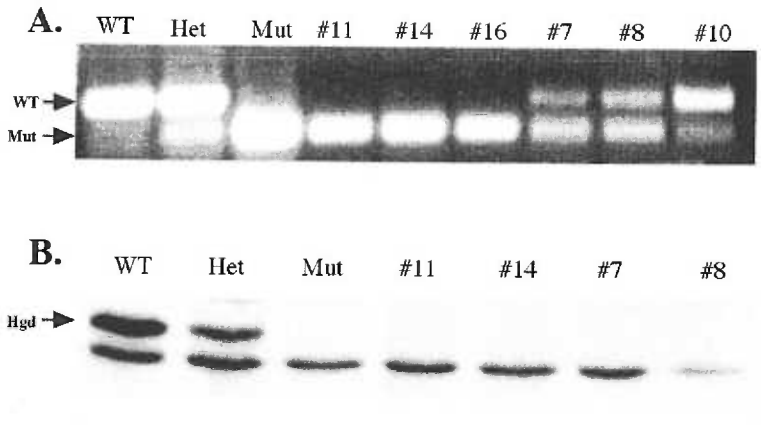

Figure 3.5. Loss of heterozygosity.

(A) RT-PCR on kidney tissue from BMT *Fah*$^{-/-}$,*Hgd*^{aku}/*Hgd*^{wt} following 9 months of selection. Three mice (11, 14, 16) have undergone near complete loss of wild type *Hgd* allele while the other three mice (7, 8, 10) still maintained the wild type *Hgd* allele **(B)** Western blot analysis of Hgd protein from kidney tissue of BMT *Hgd*+/-*Fah*-/- mice following selection. All four mice have lost expression of Hgd. BMT=bone marrow transplantation.

V. Discussion

Repopulation of adult organ systems by healthy cells occurs in situations where there is a selective growth advantage. Spontaneous repopulation in genetic diseases occurs whenever a mutation causes cell autonomous damage in a regenerative tissue. If the genetic mutation is lost or reverted, the revertant cell has a survival advantage and can repopulate. For example, in the autosomal recessive immune deficiency disease, adenosine deaminase deficiency (ADA), revertant T-cells can out-compete mutant cells leading to partial restoration of immune function (Hirschhorn et al. 1996). In high turnover tissues such as skin, examples of repopulation by revertant skin stem cells have also been described (Jonkman et al. 1997). In addition, there are many examples of X-linked diseases in which bias of X-inactivation suggests that cells expressing the wild type allele have a selective advantage (Van den Veyver 2001). Finally, significant and clinically relevant repopulation has been seen in the liver, particularly in patients and mice with hereditary tyrosinemia type 1 (Kvittingen et al. 1994; Manning et al. 1999).

The strong in vivo selection observed in these clinical scenarios has resulted in application of the same principal in gene and cell therapy pre-clinical trials. Positive selection of genetically normal cells has been utilized in the hematopoietic (Aiuti et al. 2002; Galimi et al. 2002), skin (Ortiz-Urda et al. 2002; Ortiz-Urda et al. 2003), and liver systems (Overturf et al. 1996; Overturf et al. 1997; Overturf et al. 1998). Organ repopulation driven by genetic selection has the major advantage of being able to use fairly inefficient methods for cell correction. Over time, the low correction efficiency is

compensated by selective outgrowth of the healthy cells. To date, genetic *in vivo* selection has not been reported for the kidney. In addition, successful reports of kidney gene therapy has been few, most likely due to the tight basement membrane barriers and the lack of in vivo selection (Moullier et al. 1994; Zhu et al. 1996; Lai et al. 1997; Lipkowitz et al. 1999). Several reports have observed the presence of donor-derived cells in the renal tubular epithelium after bone marrow transplantation and in rodents (Poulsom et al. 2001; Kale et al. 2003). Tissue injury such as ischemia could produce significant numbers of apparently epithelial donor cells (Poulsom et al. 2001; Kale et al. 2003), but unambiguous markers of tubular epithelium were not used in these studies. In mice, Fah is an excellent marker of proximal tubular epithelium because it is highly expressed in only this cell type of the kidney (Labelle et al. 1991). We utilized the HTI mouse in order confirm whether induction of ATN could lead to renal tubule repopulation by donor derived cells. However, we did not see the emergence of donor-derived renal tubular epithelial cells, even when evaluated under multiple different conditions. This difference between our results and those of others could be explained by experimental variables such as mouse strains used, or could potentially be due to the misidentification of donor hematopoietic cells as renal epithelium.

Significant renal proximal tubule repopulation by bone marrow-derived cells was not seen in the murine HTI model. Only very rare Fah positive cells were present and it was not possible to be certain that these donor cells were renal tubular epithelium. Because the renal disease is secondary to liver disease in HTI, we tested continuous exposure to HGA as a chronic stress factor of the proximal tubules. The large amounts of

HGA produced by revertant hepatocytes in $Fah^{-/-},Hgd^{aku}/Hgd^{wt}$ mice caused chronic tubular injury (Manning et al. 1999) and in this milieu, two different kinds of repopulation phenomenon by genetic selection were observed. First, a significant percentage of bone marrow transplanted $Fah^{-/-},Hgd^{aku}/Hgd^{wt}$ mice had multiple clusters of Fah positive renal tubules. Repopulation of the tubule with donor-derived cells occurred in mice transplanted with whole bone marrow and in mice transplanted with cells expressing hematopoietic stem cell markers (KLS and c-Kit+ cells). In each case, the entire tubule expressed Fah suggesting clonal repopulation at the level of individual nephrons. Second, histologically and functionally normal kidneys were seen in mice that had no Fah+ tubules. In these animals, reduced or absent expression of the wild type copy of *Hgd* was observed. In the liver, we had previously reported a strong selective growth advantage of *Fah* mutant hepatocytes, which had also become mutant for *Hgd* (Manning et al. 1999). The absence of Hgd expression observed in the kidney in multiple animals suggests that LOH at the *Hgd* locus also occurs in renal tubular epithelial cells resulting in repopulation of nephrons by revertant cells. It is possible that both mechanisms, renal tubule repopulation by bone marrow-derived cells or by endogenous revertant cells, can occur simultaneously to improve renal tubule function in the adapted HT1 mouse model. Taken together, these results demonstrate that extensive repopulation of the proximal tubular compartment can be achieved by genetic selection and may form the basis of *in vivo* selection strategies for kidney cell and gene therapy.

In the liver, cell fusion between myelomonocytic cells and hepatocytes is the dominant source of bone marrow-derived hepatocytes (Willenbring et al. 2004). Cell

fusion has not previously been reported nor vigorously examined in the kidney. In our setting of prolonged tubular damage through HGA exposure, we were able to demonstrate by two independent techniques that a significant fraction of the Fah+ tubular cells are also derived by cell-cell fusion. This observation implies that hematopoietic nuclei can be reprogrammed to express renal tubular genes after fusion. Moreover, the presence of multiple Fah positive cells within proximal tubules suggests that the resulting synkaryons can divide after reprogramming. In contrast to the liver, we currently do not have any information on which hematopoietic cell type is responsible for fusion. The cytogenetic complement of the bone marrow-derived epithelial cells is also not yet known and it is therefore uncertain whether these cells undergo mitotic reduction divisions similar to the bone marrow-derived hepatocytes (Wang et al. 2003). Our experiments were not designed to exclude the possibility of transdifferentiation of hematopoietic cells as a potential second mechanism. However, the high degree of concordance of Fah expression and presence of the Y chromosome suggests that stem cell plasticity – if it indeed occurs in this tissue - is the more rare event.

In summary, the findings reported here provide proof-of-principle that hematopoietic cells could have utility as gene delivery vehicles in the kidney. Stable gene transfer with viral vectors has been notoriously difficult in this organ, in part due to poor accessibility of the epithelium. Many renal diseases are characterized by inflammation and infiltration of hematopoietic cells into the parenchyma. If fusion can be controlled and made reasonably efficient, this could be a way to overcome the delivery problem and introduce functional genetic material into cells of this tissue. In addition,

the *Fah*$^{-/-}$ *Hgd*$^{+/-}$ model of chronic renal injury provides a novel *in vivo* test system for functional renal tubular cells derived from any source. In this system, implantation of even a single genetically resistant proximal tubular cell will result in readily detectable clonal repopulation of the entire tubule.

VI. Acknowledgements

Muhsen Al-Dhalimy in the Grompe laboratory performed the initial experiments to determine whether bone marrow cells were capable to giving rise to renal tubular epithelium. Dr. Milton Finegold and Angela Major at Texas Children's Hospital performed all the Fah immunohistochemistry shown in Figure 3.2 and panel C of Figure 3.4. Dr. Yassmine Akkari and Yumi Torimaru in the laboratory of Dr. Susan Olson performed the FISH in culture renal cells (Figure 3.4 panel B) and Dr. Shuguang Jiang in the laboratory of Dr. William Fleming performed the FISH in tissue sections (Figure 3.4 panel D). Henry Senephansiri in the laboratory of Dr. Michael Gibson performed the amino acid analysis reported in Table 3.2. Dr. Holger Willenbring assisted with technical and experimental advice. This work was supported by the National Institutes of Health Grant (NIDDK DK-51592) to M.G and (HL-69133) to W. H.

Chapter 4

Summary and Conclusions

Hereditary tyrosinemia type I (HTI) is clinically the most severe disease that is associated with a defect in the tyrosine catabolic pathway. HTI patients lack a functional copy of the last enzyme in the pathway, fumarylacetoacetate hydrolase (FAH). As a result, HTI patients develop severe liver disease, renal tubular dysfunction, and have a high risk of acquiring hepatocellular carcinoma (HCC) (Mitchell et al. 1999). Typically, HTI patients are treated with the drug, NTBC, which inhibits the production of the toxic substrate of FAH, fumarylacetoacetate (FAA) (Lindstedt et al. 1992; Holme et al. 1998). However, patients can still develop HCC, even with extensive NTBC treatment (Dionisi-Vici et al. 1997; van Spronsen et al. 2005). Currently, the only known cure for HTI is liver transplantation (Mohan et al. 1999). Yet, liver transplantation carries significant mortality and there is often a shortage of donor organs available (Grompe 1999). Therefore, there is a need for alternative treatments for inherited diseases, such as HTI. The first portion of this dissertation demonstrated correction of murine HTI by using a novel, non-viral gene therapy system, ϕ C31 integrase; while the second portion showed correction of the renal tubular dysfunction, associated with HTI, by genetic selection of functional cells.

ϕ C31 Integrase for liver directed gene therapy

In previous studies, ϕC31 integrase has been shown to mediate gene transfer of plasmid DNA into host cells through a recombination event between the attachment sequence *attB* and pseudo *attP* sites present in the host genome (Olivares et al. 2002; Ortiz-Urda et al. 2002). This type of recombination event requires significant sequence identity between the *att* sites, and therefore a limited number of integration sites are

present within the mammalian genome (Olivares et al. 2002). In addition, integration events facilitated by ϕC31 integrase are unidirectional and therefore stable. These unique characteristics make ϕC31 integrase an attractive non-viral gene transfer system.

One aim of this dissertation was to further characterize the ϕC31 integrase system for non-viral liver-directed gene therapy. I demonstrated that ϕC31 integrase mediates integration of human *FAH* into hepatocytes of $Fah^{\Delta exon5}$ mice. In addition, FAH positive hepatocytes were selectively expanded (>70% liver repopulation) to correct the liver disease. Because of this selective growth advantage for integrase-corrected FAH positive hepatocytes, I was able to identify, quantify, and histologically evaluate all the integration events. Two major findings resulted from this study. First, I estimated that approximately 3.6% of the transfected hepatocytes (when corrected for transfection efficiency) underwent an integration event mediated by ϕC31 integrase. In addition, a total of seven different integration sites were identified, which represented >90% of all the ϕC31 integrase mediated integration events. Second, I found that a high proportion of integrase-transfected FAH positive hepatocytes displayed an initial abnormal cellular morphology, which suggested that integrase induces hepatocyte dysplasia. However, this abnormal hepatocyte phenotype was reversed over time. In conclusion, I confirmed the most attractive feature of ϕC31 integrase, site-specific integration, but also presented evidence for potential side effects of integrase expression, specifically hepatocyte dysplasia (Held et al. 2005).

Two months after the publication of our study, a second study was published comparing ϕC31 integrase to the *Sleeping Beauty* (SB) transposase for *in vivo* non-viral liver-directed gene therapy (Ehrhardt et al. 2005). In this study, mice were coinjected with a vector containing the factor IX expression cassette and either ϕC31 integrase- or SB transposase-expression vector. Factor IX levels were measured in these mice for several days after injection and again after induction of liver repopulation by partial hepatectomy. Expression of factor IX levels dropped by 16% or 66%, following partial hepatectomy, in mice that received either ϕC31 integrase or SB transposase, respectively. In concordance with this finding, the study also reported a less pronounced loss of vector genomes after partial hepatectomy in mice that received ϕC31 integrase compared to mice that received SB transposase. Taken together, this report suggested that ϕC31 integrase maintains transgene expression better than SB transposase in mouse hepatocytes. The authors hypothesized that either ϕC31 integrase can mediate higher integration efficiency in mouse hepatocytes than SB transposase, or that the integration frequencies are identical and thereby transgenes delivered by ϕC31 integrase may display a better expression profile.

Our laboratory independently studied both SB transposase (Montini et al. 2002) and ϕC31 integrase ((Held et al. 2005) for their ability to mediate integration into the liver of $Fah^{\Delta exon5}$ mice. Because of the *in vivo* selection mechanism for FAH positive hepatocytes, both papers were able to measure an initial integration frequency for the FAH expression cassette. The integration frequency in hepatocytes was 1/1,374 for ϕC31 integrase-mediated integration, while the integration frequency was slightly lower,

1/3,815, for SB transposase mediated integration. Our results, in accordance with the results from Ehrdardt *et al.*, suggest that ϕC31 integrase can facilitate integration and maintain transgene expression better than SB transposase (Ehrhardt et al. 2005).

The direct comparison study of ϕC31 integrase and SB transposase by Ehrhardt et al., suggested that neither system induces hepatocyte toxicity. Specifically, alanine aminotransferase (SGPT) levels were measured in mice injected with ϕC31 integrase and SB transposase as an indicator of acute toxicity. After the injection, SGPT levels were significantly elevated in both systems, but by day three the levels had returned to normal. This initial increase in expression of SGPT was thought to be due the plasmid injection technique and not due to the expression of ϕC31 integrase or SB transposase (Ehrhardt et al. 2005). However, given the low integration frequency in both systems at day three (approximately <1% of the hepatocytes, as defined in our study), it seems likely that if integrated hepatocytes were expressing high levels of SGPT, dysplastic hepatocytes may go undetected among the background of healthy untransfected cells. In our study, I histologically evaluated all integrase corrected FAH positive hepatocytes for a normal or abnormal morphology and found that nearly 66% of all integrase corrected FAH positive hepatocytes were dysplastic (Held et al. 2005). Therefore, I conclude that integrase does cause an initial hepatocyte dysplasia. Currently, the long-term effect of this initial hepatocyte dysplasia due to integrase expression is unknown.

I hypothesized that the morphological abnormalities of FAH positive hepatocytes transfected with ϕC31 integrase may be due to chromosomal instability. For example,

ϕC31 integrase may be facilitating "illegitimate" recombination events between two pseudo *attP* sites on different chromosomes or even between distantly related DNA sequences. However, regardless of whether chromosomal instability occurred, the hepatocyte dysplasia was transient and nearly complete liver repopulation (>70%) eventually occurred with healthy FAH positive cells. Currently it is unknown whether integrase causes chromosomal instability. Also, it is unknown whether individual abnormal FAH positive hepatocytes revert to normal cell morphology or whether normal FAH positive hepatocytes have a selective advantage and simply outgrow the dysplastic cells over time. To address whether integrase causes cytogenetic abnormalities and whether these abnormalities are corrected over time, several follow-up studies could be performed. First, to look at the early effects of integrase, primary human skin fibroblasts can be transfected with integrase and cytogenetically evaluated after the first mitosis. Second, the long-term effects of integrase expression can be evaluated by transfecting mouse embryonic stem cells (ES) with integrase and an antibiotic resistance gene. Individual clones that express integrase and the resistance gene can be isolated and cytogenetically evaluated. Lastly, in addition to the *in vitro* studies, cytogenetic evaluation of hepatocytes transduced *in vivo* with ϕC31 integrase followed by repopulation in the $Fah^{\Delta exon5}$ mouse, can be performed. These three suggested studies will enable us to evaluate the cytogenetic effects of integrase, *in vivo* and *in vitro,* and after short-term and long-term expression.

ϕC31 integrase is the best non-viral gene therapy system described to date. The stability of transgene expression, relatively high integration frequency, and significant

site specificity uniquely characterize the ϕC31 integrase system. With careful monitoring of toxicity and safety of the integration sites, as deemed necessary by our studies in the $Fah^{\Delta exon5}$ mouse, the ϕC31 integrase system may be a candidate for gene transfer in a clinical setting.

Bone marrow-derived renal tubule epithelium

While liver-directed gene therapy has been very successful in animal models; there are only a few successful reports of gene therapy for kidney diseases (Moullier et al. 1994; Zhu et al. 1996; Lipkowitz et al. 1999). This is mainly due to the architecture of the kidney, which does not allow for the stable introduction of genes. However, cell therapy for renal disease has been more promising (Kale et al. 2003). The second portion of my dissertation demonstrated bone marrow-derived cells could give rise to tubular epithelium and correct a renal disease. This phenomenon only occurred in the presence of chronic renal injury.

Researchers have shown that circulating cell are recruited from the peripheral blood to aid in regeneration and tissue repair (Poulsom et al. 2001). Specifically, bone marrow-derived cells localized to the kidney after acute injury and appeared to give rise to renal tubular epithelium (Kale et al. 2003). In this study by Kale et al., bone marrow from a β-galactosidase positive mouse was transplanted into a wild-type recipient mouse prior to induction of acute tubular necrosis (ATN). After recovery from ATN, the mice were sacrificed and the injured kidney was examine for bone marrow-derived, β-galactosidase positive, renal tubular cells. Surprisingly, a significant numbers of bone

marrow-derived renal tubule cells were reported. In Chapter 3 of this dissertation, I attempted to reproduce these results, but found no evidence for bone marrow-derived renal tubule cells after acute ischemic renal injury. We believe this discrepancy may be due to differences in mouse strains used or due to the misidentification of blood cells as tubule epithelium in the Kale report. In my study, I used Fah as an unambiguous marker of bone marrow-derived renal tubule cells because it is only expressed within this cell type of the kidney and not in blood cells (Mitchell et al. 1999). Typically, renal cells have endogenous β-galactosidase activity, which could also account for the misidentification of donor-derived renal tubule cells, as seen in other reports (Imai et al. 2004). Therefore, it is my conclusion that bone marrow does not give rise to significant numbers of renal tubule cells after induction of acute renal tubule injury.

Bone marrow-derived renal tubule cells were also not observed in our standard HTI mouse model. Previously, it was shown that bone marrow-derived hepatocytes can repopulate the liver of HTI mice (Lagasse et al. 2000). Therefore, it seemed likely that bone marrow cells would also contribute to renal tubule repopulation in the HTI mouse. However, I found no evidence of bone marrow-derived renal tubular epithelium in the kidney of over thirty bone marrow transplanted HTI mice. Interestingly, renal tubular dysfunction actually improves after correction of the liver disease (Grompe et al. 1993). Therefore, I concluded that our standard HTI model lacked sufficient renal injury to induce repopulation of the tubules.

To induce substantial renal injury in the tubules, an adapted HTI mouse model was used. In this model, $Fah^{\Delta exon5}$ mice are heterozygous for *Hgd* ($Fah^{-/-}$,Hgd^{aku}/Hgd^{wt}) and undergo LOH of the wild-type *Hgd* allele in hepatocytes, followed by repopulation of the liver with these genetically modified cells. High amounts of HGA are produced by the alkaponturic hepatocytes ($Fah^{-/-}$,Hgd^{aku}/Hgd^{aku}) , which induces chronic injury and regeneration in the $Fah^{-/-}$,Hgd^{aku}/Hgd^{wt} renal tubular cells. Bone marrow transplantation in these mice ($Fah^{-/-}$,Hgd^{aku}/Hgd^{wt}) yielded clusters of Fah positive renal proximal tubule cells. Thus, the chronic stress induced by HGA was required to generate a growth selection for Fah positive renal epithelial cells. In addition, repopulation of the damaged tubules exceeded 30% in two mice, and functional correction was achieved. These experiments represent proof-of-principle that *in vivo* selection for bone marrow-derived cells is feasible in our modified $Fah^{\Delta exon5}$ mouse (Held et al., *in review*).

In chapter three, I identified cell fusion as the primary mechanism for generating bone marrow-derived renal tubular epithelium. However, the specific hematopoietic cell type responsible for fusing with the renal tubule epithelial cell has not yet been identified and should be the subject of future studies. Nevertheless, regardless of the cell type, the hematopoietic nuclei appear to be reprogrammed after fusion to express renal tubule genes. In addition, multiple Fah positive cells clustered within the same tubule, suggesting that the resulting synkaryons (mononuclear fusion of two cells) can undergo division after reprogramming. Yet, it is unknown whether normal mitotic division occurs, resulting in two cytogenetically normal cells. Again, these questions need to be addressed in further studies.

The findings in chapter three indicate that hematopoietic cells could have utility as gene delivery vehicles for other inherited renal diseases. In response to chronic injury, hematopoietic cells infiltrated the proximal tubules of the kidney in our model system. Other renal diseases are also characterized by inflammation and infiltration of hematopoietic cells into the parenchyma of the kidney. Therefore, it would be interesting to test whether repopulation of the proximal tubules, or other renal tissues, could occur in additional mouse models where there is constant chronic injury.

LOH in the proximal tubule of the kidney

Chapter three reported a second mechanism by which renal function was restored in the adapted HTI mouse model. Under strong selective pressure, individual renal epithelial cells underwent a loss of heterozygosity (LOH) of the wild type *Hgd* allele. Furthermore, these genetically altered cells were able to repopulate the damaged tubule and correct the renal disease. A similar phenomenon was previously reported by us in the liver (Manning et al. 1999). In the liver, the LOH was caused by FAA induced deletions, insertions, and missense mutations (Manning et al. 1999). It would be important to confirm similar mechanisms for the LOH in proximal tubules of the kidney. In addition, sequence analysis of individual cells within repopulated tubule would tell us whether one cell, or multiple cells, underwent LOH.

There are many examples of reversion in genetic diseases affecting tissues capable of regeneration, namely blood and skin. For example, somatic reversion has

been documented in the hematopoietic system, leading to functional correction of genetic diseases such as adenosine deaminase deficiency (ADA) and Fanconi anemia (Hirschhorn et al. 1996; Joenje et al. 2000). In skin diseases, such as generalized atrophic benign epidermolysis bullosa (GABEB), visible patches of clinically normal skin are present among abnormal skin. This is due to a reversion event (Jonkman et al. 1997). In addition, there are many examples of X-linked diseases in which bias of X-inactivation suggests that cells expressing the wild type allele have a selective advantage (Van den Veyver 2001). Significant and clinically relevant repopulation has been seen in the livers of patients and mice with hereditary tyrosinemia type 1 (Kvittingen et al. 1993; Kvittingen et al. 1994; Manning et al. 1999). This dissertation reported that somatic reversion also occurs within the proximal tubular epithelium of the kidney. Reversion followed by repopulation led to functional correction of the renal disease. To my knowledge, this is the first report of somatic reversion occurring in the kidney.

Future studies to combine the features of ϕC31 integrase with in vivo selection for renal tubule cells

I demonstrated that *in vivo* selection for genetically corrected renal tubule epithelium is feasible in our adapted HTI mouse model. Thus, it is important to revisit gene therapy applications for the treatment of renal diseases using our model system. Different gene therapy vectors, both viral and non-viral, should be tested in the kidney along with various injection techniques. Specifically, the ϕC31 integrase system should be given extra consideration for renal gene therapy because it has many advantages over other systems. Because of the strong selective pressure for repopulation of damage

tubules, if a single proximal tubular cell was corrected by using the ϕC31 integrase system, it would result in readily detectable clonal repopulation of the entire tubule. In addition, the renal disease will be cured.

Typically, the treatment of genetic diseases that affect the hematopoietic system, such as X-linked severe combined immune deficiency syndrome (X-SCID), is an allogeneic bone marrow transplantation (Kohn et al. 2003). However, because of the risk for immune rejection, researchers are developing ways to genetically modify autologous bone marrow stem cells for the use in treatment of hematopoietic diseases. Clinical trails using genetically corrected hematopoietic stem cells were previously carried out for the disease, X-SCID. Specifically, HSC were transduced *ex vivo* with a retrovirus carrying a transgene that could restore the immune system in these X-SCID patients (Hacein-Bey-Abina et al. 2002). However, two patients, treated by retroviral correction of HSC, developed leukemia due to viral vector insertion into the LMO2 gene (Kaiser 2003; Kohn et al. 2003). Thus, the study was suspended. I propose that ϕC31 integrase could be used instead of the retrovirus to deliver the transgene *ex vivo*. Because of the integration site specificity and the stability of transgene expression, it would be an ideal non-viral vector for use in this clinical setting. In addition, I think it would be important to consider whether genetically modified autologous bone marrow could also facilitate repopulation of the liver and/or kidney in the presense of chronic injury.

This dissertation described two major results that contribute significantly to the field of gene and stem cell therapy. First, a novel, non-viral gene therapy vector, ϕC31

integrase, is characterized leading to important findings on the integrations sites for the vector, along with information on the potential toxicity of the system. Second, I demonstrated that *in vivo* selection for genetically corrected renal tubule epithelium is feasible in our adapted HTI mouse model. Genetic correction can occur through two mechanisms: by the fusion of bone marrow cells with pre-existing renal tubule cells, or by genotypic alterations of the endogenous tubular epithelium. Together, these findings represent an important step for developing novel therapies for liver and kidney disease. In addition, these studies have potential clinical application, and could be used in place of whole organ transplantation as a treatment for liver and kidney disease.

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