

NOVEL APPLICATION OF GENE THERAPY AND SOMATIC STEM CELLS IN
TREATING METABOLIC LIVER DISORDERS

By

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Current research is progressing toward replacement therapies that use virally transduced stem cells, or other cell types, for transplantations. These new treatments could change the lives of patients with genetic disorders by making their lives more robust and free them from a life-long need for medication.

To envisage possibilities for developing new treatments using stem cells and gene therapy for liver metabolic disorders, we first showed that monocrotaline can inhibit proliferation of resident liver cells, allowing transplanted cells to take over the process of regeneration and repopulate the organ. Using a 30 mg/kg dose of monocrotaline given twice, two weeks apart, we repopulated rat livers with transplanted hepatocytes near the 20% level.

We further tested our hypothesis that transplanted stem cells, initially transduced with the viral vector, can sufficiently regenerate the damaged liver and deliver the missing enzyme to restore normal liver function. We provided a novel application of gene

therapy and somatic stem cells for treating metabolic liver disorders. We tested this hypothesis in mice where we used the gene for alpha-1 antitrypsin (AAT) to transduce liver stem cells, and then transplanted them into monocrotaline-treated and partially hepatectomized animals. Transplanted animals showed stable expression of AAT during the 16 weeks of the experiment. At the end of the experiment, histological examination of tissue revealed that up to 5-10% of total liver cells were transduced. Repopulation level was approximated at 40-50%.

We also tested the hypothesis in Gunn rats, an animal model for Crigler-Najjar Syndrome (CNS) type 1. In those animals, transplanted oval cells were transduced with lentivirus carrying the UGT1A1 transgene that allows conjugating bilirubin. The transplantation was able to rescue treated animals, and their levels of conjugated bilirubin were increased.

Finally, we showed that bone marrow cells could be potentially used for transplantation therapies. In this experiment, we showed that bone marrow cells have the potential to become hepatocytes and oval cells. This observation could lead to possible treatments using patient's own bone marrow cells as a transgene delivery method to treat metabolic liver disorders.

CHAPTER 1 INTRODUCTION

Recent developments in medical research have opened new and exciting possibilities in the treatment of human disorders. At the beginning of the 21st century, our understanding of science has extended human life and prevented early death for many patients. Diseases like polio and small pox have been eradicated. However, although we can help many sick and ailing patients, there are still disorders that are problematic to treat. Some of these disorders require organ transplantation; however, organ transplantation is not a perfect treatment. Donor organs are difficult to obtain, can be rejected, and even if transplantation is successful, life-long immune suppression is required to prevent rejection. This creates a rather difficult situation for patients with genetic disorders, since for many, organ transplant is the only answer for survival. The emergence of stem cells, however, has created new possibilities of moving away from organ transplants and developing new, radically different treatments. Current research progresses toward regenerative medicine in which virally transduced stem cells, or other cell types, could be used for transplantations. These new treatments could change the lives of patients with genetic disorders by making the patients more robust and freeing them from the life-long need for medication.

Patients with liver disorders are fortunate in one aspect since the organ has extraordinary regenerative properties that can be utilized for their recovery. To envisage the possibilities of developing new treatments utilizing stem cells and gene therapy for

liver metabolic disorders, an understanding of the liver's structure, functions and properties are necessary. What follows is a review of literature pertaining toward stem cells, gene therapy, and possible disorders that can be candidates for treatments are outlined herein.

1.1 Liver Structure and Function

The liver is the largest parenchymal organ in the body. In an adult human, the liver weighs approximately 1400 to 1600 g, which represents 2% of the total body weight. In rats, the weight of the liver is 7 to 8 g which accounts for approximately 5% of the body weight.¹ For its large size, the liver is characterized by highly vascular hepatic parenchyma that is supplied with blood from two separate afferent blood supplies. One of them, the hepatic artery provides oxygenated blood, and the other, the portal vein, supplies the liver with venous and nutrient-rich blood from the intestines and pancreas. Parallel to blood vessels, the biliary tree forms excretory ducts that transport bile into the duodenum. Together, the portal vein, hepatic artery and biliary tree form a bundle of vessels termed the portal triads.

The liver parenchymal structure is comprised of hepatic lobules outlined by portal triads. Each lobule is formed by hexagonal column of hepatocytes arranged in cords radiating from the central vein. Each cord of hepatocytes is flanked by sinusoids forming the micro-vascular circulatory system of the liver. Thus, when the blood enters the liver through the veins of portal triads, it can flow through the parenchyma allowing contact with every hepatocyte before draining into the central vein. As blood enriches the hepatocytes, their metabolic products are emptied through the bile canaliculi into canal of Hering, a terminal part of the bile network located in the portal triad.

The cell types that build the liver parenchyma include hepatocytes, bile duct cells, stellate cells, Kupffer cells, vascular endothelial cells, fibroblasts and leukocytes. Out of these, hepatocytes comprise 90% of liver weight and are responsible for most of liver biochemical functions and production of bile. Hepatocytes, as a unit, are characterized by a large, polygonal cell shape (30–40 μm) and high abundance of smooth and rough endoplasmic reticulum.

The hepatic lobule is considered to be a hepatic histological unit; however, it is the acinus that is described as the functional unit of the liver. The acinus consists of hepatocytes that extend perpendicularly from one portal triad to the central vein. There are three acinar zones within the lobule. Zone 1 includes hepatocytes that surround the portal triad; Zone 2 is composed of inter-zonal hepatocytes; and Zone 3 consists of hepatocytes that surround the central vein. In this acinar structure, blood flows through sinusoids from Zone 1 to Zone 3, and the bile moves in the opposite direction from Zone 3 to Zone 1. It is believed that hepatocytes within different acinar zones have different functions and the hepatocytes within Zone 3 are characterized by their increased DNA content (4N to 16N) and larger size. Also, Zone 3 hepatocytes are found to be predominantly bi-nucleated. Hepatocytes within Zone 1 are smaller and usually single nucleated (2N).²

As a large parenchymal organ in the body, the liver performs a multitude of functions. To control homeostasis of the body, liver metabolizes amino acids, lipids, and carbohydrates, and serum proteins. For example, by converting glucose to glycogen as a part of carbohydrate metabolism, the liver can decrease blood level of glucose and, vice

versa, by changing glucagon to glucose it can increase glucose levels. Additionally, the liver plays a role in the deamination of amino acids and the formation of urea.

Due to the liver's intricate vasculature and large size, a large volume of blood is located within at any given time thus making the liver the largest blood storage organ in the body. Processing large volumes of blood makes liver function as a detoxifying organ. Liver enzymes, such as Alcohol Dehydrogenase (ADH), Cytochrome-P(CYP) and isoforms of uridine diphosphoglucuronate glucuronosyltransferase (UGT), allow alteration of chemical composition of many xenobiotics and their removal. In addition to storing blood, the liver stores glycogen, iron, vitamins A, B12, and D. However, the most important function of liver is the production of bile. Bile is important for intestinal absorption of nutrients and elimination of cholesterol. Bile, mostly comprised of conjugated bilirubin, is collected in the liver biliary tree, stored in the gall bladder and eventually drained into the duodenum to act as a detergent.^{3,4}

Bilirubin is a lipophilic linear tetrapyrrole occurring uniquely in mammals. It is produced during heme catabolism when heme oxygenase cleaves the heme ring to form water-soluble biliverdin, which is subsequently reduced by biliverdin reductase to bilirubin.⁵ However, this form of bilirubin is toxic and insoluble, and it must be glucuronidated before being excreted in the bile. The process of glucuronidation takes place in the liver where lipid-soluble unconjugated bilirubin is taken from the blood and conjugated with glucuronic acid in hepatocytes by bilirubin-uridine 5'-diphosphoglucuronate glucuronosyltransferase (UGT1A1). This conjugation produces a water-soluble form of bilirubin that becomes major part of bile.

1.2 Liver Regeneration

The liver has a property to regulate its growth and mass, and the ability to undergo cell division to replace lost tissue or cells. This property developed in response to the extraordinary detoxification capacity of liver that sometimes puts the liver at risk of damage.^{2,6} In the process of removing potentially harmful substances, some of the liver cells may become damaged or killed, and to compensate, the liver undergoes compensatory hyperplasia, a process that allows re-growth of the remaining parts of the liver to compensate for the lost mass.

In the experimental environment, it was noted that if part of the rat liver was removed through surgical resection, a process called partial hepatectomy (PHx) or damaged due to a toxic agent (CCL₄) or viral agent, hepatocytes proliferate in a semi-synchronous manner to compensate for lost tissue.⁶ In standard PHx, where 70% of liver is removed, the liver is able to restore its original mass within 10-14 days.⁷ The extent of this regenerative property was further explored by Simpson and Finckh⁸ who performed five partial hepatectomies, allowing liver re-growth before each surgery. At the end of the last surgery, only 5% of original liver mass remained. In this experiment, liver was able to restore 97% its original mass after the first two hepatectomies, and approximately 75% after the last surgery. During the entire time of the experiment, the liver maintained its micro architecture and function demonstrating its extraordinary capacity to regenerate.

In compensatory hyperplasia, hepatocytes are responsible for the restoration of regenerating liver mass. In experiments conducted on rats, most of the hepatocytes replicated in a semi-synchronous fashion during the first 3 days post PHx.⁹ This replicative process starts with hepatocytes within Zone 1 of hepatic lobule. Those

hepatocytes exhibit the highest rate of proliferation. Hepatocytes within Zone 3 have the lowest rate of proliferation.^{10,11} Newly replicated hepatocytes form clusters that are later invaded by endothelial cells to form cords of hepatocytes, a histological unit within the hepatic lobule.

At the molecular level, before liver regeneration can take place after injury, hepatocytes have to undergo priming. In the normal liver, hepatocytes are in the state of quiescence resting in the G₀ state. After partial hepatectomy, almost immediately hepatocytes simultaneously transition into the G₁ phase. This stage is initiated by transcriptional factors like *c-fos*, *c-myc*, and *c-jun*. Increase in ornithine decarboxylase activity that allows stabilization of messenger RNA influences the later stage of proliferation initiation that starts 1h post PHx and peaks at 4h.¹²

Cytokines are other factors that influence liver regeneration. Within minutes post PHx cytokines like tumor necrosis factor alpha (TNF- α), interleukin 1 (IL1) and IL6 are released from non-parenchymal liver cells. Those cytokines induce liver cells to produce acute-phase proteins like protease inhibitors. IL6 enhances transcription, triggering transition of hepatocytes from G₀ to G₁ phase. Other mitogens affect proliferation of hepatocytes in further stages of liver regeneration. Those include epidermal growth factor (EGF), transforming growth factor alpha (TGF- α), hepatocyte growth factor (HGF), insulin and glucagon.^{12,13} In this stage, hepatocytes enter S phase and are committed to undergo replication. In rats, peak proliferation rates were recorded at 24h post PHx. In mice, the process occurs slower and peak proliferation rates were recorded at 36h post PHx.

1.3 Liver Repopulation by Transplanted Cells

Currently, to treat acquired and genetic metabolic disorders of the liver, orthotopic transplantation of whole liver is performed.¹⁴⁻¹⁶ Liver transplants, however, are restricted in number to a limited availability of donor organs. Additionally, patients with transplants require life-long immune suppression to prevent organ rejection. On top of all this, transplantation carries significant risk of morbidity and mortality. Thus, in the recent studies, possibilities of restoring liver mass by transplanted hepatocytes have been explored.^{17,18}

The first significant results showing the potential of liver restoration by transplanted hepatocytes came from the studies involving the FAH mouse model.^{19,20} In this model, deficiency of the enzyme fumarylacetoacetate hydrolase (FAH) causes mice to develop hepatorenal tyrosinemia type I, which is characterized by accumulation of fumarylacetoacetate (FAA), a product of the one of the last steps in tyrosine metabolic pathway. FAA is toxic to hepatocytes and leads to liver failure.¹⁹ A drug called 2(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexane dione (NTBC) can be administered to block tyrosine catabolism upstream of FAH thus preventing liver failure.²¹

In the studies involving FAH null mice, transplantation of syngeneic wild-type hepatocytes into animals that were withdrawn from NTBC treatment showed liver repopulation by donor cells in range of 95% of total liver mass in six weeks post transplantation.²² It was further shown that in the FAH model, 1000 donor cells are sufficient to restore near-total liver mass within 6 to 8 weeks post transplantation. To further determine which group of hepatocytes is responsible for liver repopulation, an experiment that used fractionated hepatocytes was performed.²³ In this experiment, three

major size fractions of hepatocytes (16, 21 and 27 μm) were transplanted in competition with un-fractionated liver cells characterized by a distinct genetic marker. The data showed that large hepatocytes, which represent approximately 70% of the hepatocyte population, were primarily responsible for liver repopulation. Small, diploid hepatocytes were inferior to the large cells and did not significantly repopulate the livers. In conclusion, the FAH repopulation experiments proved that adult hepatocytes could be used in liver restoration studies and could become invaluable in clinical applications.

Although the FAH model has potential in proving proof of concept for liver repopulation, it is rather limited in clinical application. First, transplantation therapy utilizing this model could only work with patients deficient in FAH. Second, strict selection on wild-type transplanted cells can sometimes result in very aberrant cell behavior. In a recent FAH transplantation models with stem cells, it was shown that cells may fuse, rather than transdifferentiate, to assume a new functional phenotype.²⁴ So far, fusion of transplanted cells with the cells of the recipient has only been shown in the FAH model.²⁴⁻²⁷ For these reasons, it has been crucial to develop a new model without the problems of FAH.

To develop a new model for cell transplantation, it has to be taken in account that complete or near total eradication of the recipient's hepatocytes cannot be applied since the liver requires approximately 20 to 25% of its metabolic function for survival.^{12,28} Also, to facilitate repopulation by transplanted cells, liver regeneration needs to be inhibited. Otherwise, resident hepatocyte will take over during repopulation and transplanted cells will not be allowed to sufficiently reseed the new liver. Laconi et al.²⁹ developed a model by utilizing retrorsine, a pyrrolizidine alkaloid, to block the

proliferation of resident hepatocytes. In this model, retrorsine is metabolized by hepatocytes to secondary metabolites that form adducts with DNA.^{30,31} Damaged DNA is not allowing replication to proceed past the S phase of the cell cycle.^{32,33} The hepatocytes cannot replicate but remain functional until apoptotic cell death, which occurs 3 to 4 months post retrorsine exposure.^{34,35} In this model, retrorsine affected hepatocytes cannot respond to the proliferative stimulus brought on during the regeneration process.³⁴

Laconi pretreated recipient dipeptidyl peptidase IV negative (DPPIV⁻) mutant rats with retrorsine, injured the liver by performing 2/3 PHx, at which time transplanted 2×10^6 DPPIV⁺ normal hepatocytes. Because DPPIV is uniquely expressed by mature hepatocytes on their canalicular domain, it was possible to follow the fate of the transplanted hepatocytes in comparison with the host hepatocytes.²⁹ The results showed that within first two months, 95% of the liver mass was restored in male rats, and 40% to 60% in female rats. During this process, the entire liver structure become remodeled and appeared normal in histological analysis. Liver functions were analyzed by glucose-6-phosphatase activity, glycogen synthesis and storage, and albumin synthesis.^{29,35}

In a similar experiment, Guha et al.³⁶ obtained near total liver repopulation (75% to 80%) after irradiating the recipient's liver with 50 Gy followed by 70% PHx and hepatocyte transplantation. In this experiment, not only was liver repopulation shown, but also liver function by reducing total bilirubin levels in Gunn Rats (an animal model for Crigler-Najjar syndrome). By transplanting syngeneic wild-type hepatocytes to mutant Gunn rats, a cell replacement therapy was conducted providing the recipient animals with the missing enzyme.

Hepatocytes are not the only cells that allow liver repopulation. Fetal rat liver cells (E12-14) transplanted into retrorsine treated and partially hepatectomized recipients allowed up to 10% liver repopulation.³⁷ The transplanted cells contain epithelial cells that are in different stages of lineage progression; some epithelial cells may still behave as progenitor like cells. To further examine this behavior, studies with transplanted hepatic- and pancreatic-derived progenitor cells were conducted.³² These experiments concluded that progenitor cells exist in both the liver and pancreas, and when transplanted they proliferate modestly and differentiate into mature hepatocytes.

In the experiments described above, a cell proliferation blocker (retrorsine or irradiation) could be effectively used in combination with mitotic stimulus to transplanted cells (PHx) for efficient liver repopulation in rats. This observation could prove invaluable in human clinical application. Already, we know that by infusion of hormones, growth factors, and other agents, such as peroxisomal proliferators, we can stimulate human liver to enter the proliferation mode thus replacing PHx. It remains necessary to find an agent to block the proliferation of resident hepatocytes. To this point, most of the used agents are possibly carcinogenic and will not enter clinical trails. However, conducted studies showed a proof of concept that may lead toward human therapy.

1.4 Adult Stem Cells

Stem cells are defined as cells that retain the capacity for self-renewal and are capable of forming at least one specialized cell type. They are initially divided into two categories, totipotent and pluripotent. Totipotent capacity states that the cell can differentiate into a whole organism and its cells. The fertilized ovum is the only cell with such a capacity. A pluripotent cell has the potential to differentiate into several

specialized cell types. For some time, it was believed that pluripotent stem cells could only be derived from embryonic tissue as embryonic stem cells; however, in recent years a new class of stem cells emerged, somatic or adult stem cells. These cells are found in a differentiated adult tissue and contribute to the maintenance or repair of aged or damaged cells. They may differentiate to produce progenitor, precursor, and mature cells in the tissue of their origin.

Somatic stem cells usually comprise a small minority of the total tissue mass, and because of that, they are difficult to identify and isolate. These cells have been found and described in regenerating tissue of liver, epithelium and muscle. Also, adult stem cells have been identified in the brain, which was previously thought to lack regenerative properties. At this time, the most characterized example of adult stem cells is that of the hematopoietic system. In this system, three types of stem cells are known to exist: hematopoietic stem cells, marrow stromal cells, and multipotent adult progenitor cells.

Originally, hematopoietic stem cells (HSC) have been known to be involved in the production and replenishment of the myeloid and lymphoid cells of the blood, and for many years this was believed to be their only function. However, in the recent years, HSCs have been shown to give rise to non-hematopoietic tissues including liver³⁸ and brain.³⁹ This observation opened new frontiers for understanding the potential of these cells and generated a need to isolate them. The isolation of murine HSCs starts with a lineage-depletion step in which all cells expressing lineage-markers (CD11b - macrophages and granulocytes, CD3 - T cells, B220 – B cells, Ter-119 – red blood cells) are removed. Consequently obtained population of cells, referred to as Lin⁻, is enriched 10 to 100 fold for HSCs. Further enrichment of Lin⁻ cells can be obtained by isolating

cells that exclude rhodamine and Hoechst dyes (Hoechst^{lo}rhodamine^{lo}).⁴⁰ Also, population of Kit⁺, Thy1^{lo}, Lin⁻, Sca1⁺ (KTLS) cells can be considered as further enriched for HSCs.⁴¹ In human, separation based on CD34⁺,CD38⁻ is considered sufficient as enrichment for HSCs.^{40,42} In the experimental environment, transplantation of a single cell from HSC enriched population provides long-term hematopoietic reconstitution 20% of the time.⁴³

Marrow stromal cells (MSC) are another cell type that, like HSCs, can be found in the bone marrow. They can be grown in the culture as adherent cells with a finite life span with the ability to differentiate into osteoblasts, chondroblasts, and adipocytes in response to the correct stimuli.⁴⁴ However, the exact potential of these cells is hindered by a variety of different methods that have been used for the isolation and characterization of MSCs. Many different cytokines like fibroblast growth factor 2 (FGF2), FGF4, platelet-derived growth factor-BB (PDGF-BB), and leukemia inhibitory factor (LIF) have been used to expand MSCs. To add to the confusion, there is no specific markers that identify MSC. Markers like Stro1, CD13, α -integrins (CD49a and CD49b), β 1-integrins (CD29), CD44 (hyaluronate), CD71 (transferin), CD90 (Thy1), CD106 (vascular cell adhesion molecule-1 VCAM-1), and CD124 (interleukin-4 receptor) have been reported to be found on MSCs.^{45,46}

Together with MSCs, another population of cells can be isolated from the bone marrow. These cells, unlike MSCs, can be cultured indefinitely in a nutrient-poor medium. They were termed multipotent adult progenitor cells (MAPCs) and were induced to differentiate into cells with endodermal, mesodermal, and ectodermal markers. Consistent with the ability of MAPCs to grow indefinitely and to self renew in vivo, they

express telomerase allowing repair of lost telomere ends.⁴⁷ Presently, it is not clear whether MAPCs are a distinctive population of cells derived from MSCs or cells whose differentiation potential was uniquely developed during in vitro cell culture conditions.

Unlike cells of the hematopoietic system, hepatic stem cells are less characterized. Hepatic oval “stem” cells have been elusive in defining their origin and function.⁴⁸ The existence of oval cells was initially postulated by Wilson et al.⁴⁹ when it claimed that the cells in the distal cholangioles of the bile ducts were responsible for restoration of liver mass after dietary injury. At the same time, a common embryologic origin of bile duct epithelial cells and hepatocytes was traced to hepatoblasts emanating from the endoderm of the ventral foregut.⁵⁰ Based on this observation, it was concluded that there was a relationship between the cells of the cholangioles and hepatocytes as between a precursor and a product.

In 1956, Farber⁵¹ treated experimental rats with various carcinogens, including 2-acetylaminofluorene (2-AAF), and observed proliferation of epithelial cells in the periportal region of the liver. Morphologically, they were approximately 10 um in size and were characterized as cells containing large oval shaped nuclei and a thin surrounding band of cytoplasm. He termed them “oval cells” and ultimately concluded that they were not progenitors of the hepatocytes.⁵² For over twenty years oval cells were not considered stem cells, until studies by Thorgeirsson and coworkers proved otherwise. They showed that under certain circumstances oval cells could become hepatocytes.^{53,54} In their experiments, rats were treated with 2-AAF and then were subjected to two-thirds partial hepatectomy. Under this condition, there is massive proliferation of oval cells in the periportal region. The newly proliferating and differentiating cells were tracked by

preferential uptake of radio labeled thymidine. The expression pattern of bile ductular (CK7, CK19, OV6) and hepatocytic (AFP, albumin) markers by thymidine labeled oval cells that later progressed to hepatocytes suggested a stem cell to product relationship. Further support that oval cells are stem cells comes from studies showing the same pattern of transcription factors (HNF, CEBP) being expressed in the livers treated with 2-AAF/PHx as in cellular differentiation studies.⁵⁵ In addition, other stem cell markers (c-kit, CD-34, flt3 receptor, and LIF) were shown to be up-regulated or activated during oval cell activation procedure.⁵⁶⁻⁵⁹

In the experimental environment, oval cells are characterized by expressing similar markers to hepatocytes and bile duct cells. Those include alpha1 fetoprotein (AFP), cytokeratin 19 (CK19), and γ -glutamyl transpeptidase.⁶⁰ Additionally, markers like Thy1 and OV6 in rat, and Sca1 and A6 in mice, can aid in their identification and characterization.⁶¹⁻⁶³ They can be found in the livers of 2AAAF/PHx treated rats from day 7 to 11 post PHx, and are isolated via either immunolabeling with Thy1 antibody and consequently selected by FACS or MACS,^{61,64} or by discontinuous gradient centrifugation in solution of Histodenz.⁶⁵

The origin of the facultative liver stem cells has been debated since their discovery in 1956; some believe that they exist in very small numbers in the periportal region of the liver lobule, and that they emerge from that hidden niche within canals of Hering upon liver damage.^{66,67} Another attractive hypothesis has emerged from recent studies is the facultative stem cells of the liver, and perhaps other tissues may reside in the bone marrow and are recruited to damaged areas of other organs^{68,69}; once localized in the liver the recruited cells receive the appropriate signals determining their fate with

regard to differentiation.⁷⁰ This hypothesis of a common facultative stem cell is supported by the emerging amount of data demonstrating that stem cells derived from one adult tissue are able to serve as stem cells when transplanted into a different tissue.⁷¹ One example of this phenomenon was reported by Malouf et al in 2001, when stem cells derived from adult rat liver were transplanted into damaged cardiac muscle, where they differentiated into functional myocytes *in vivo*⁷². Remarkable phenotypic plasticity has also been described for cells from adult brain⁷³, skeletal muscle⁷⁴, and neuronal tissue.⁷⁵ In other experiments, bone marrow derived stem cells were capable of becoming hepatic oval cells after engraftment into the liver. These cells eventually differentiate into mature hepatocytes. Initial studies in rodents show the engraftment in low rate,^{69,76} but that can be attributed to either low seeding efficiency, or to the fact that stem cells, with potential to differentiate into hepatocytes, are present in very low frequency within the bone marrow. Additionally, human studies show a higher rate of liver reconstitution after bone marrow transplantation into damaged human liver.⁷⁷

Additional study of oval cell receptors has shown that they express CXCR4, a receptor for stromal derived factor 1- α (SDF-1 α).⁷⁸ This receptor is also expressed by HSCs, and the fact that SDF-1 α is expressed in the liver following 2AAF/PHx, it may be possible that an SDF-1 α gradient from the liver plays a role in mobilization of BMSC to the circulation with a homing effect to the injured liver.⁷⁹ Based on this information, it is possible that oval cells, or at least a small population of oval cells, could originate in the BM.

1.5 Adult Stem Cell Plasticity and Cell Fusion

Plasticity of stem cells can be defined as ability of a cell to cross lineage barriers and to adopt the gene expression profile and functional phenotype of cells specific to other tissues. Until recently, the theory for stem cell research professed a linear model of hierarchy, whereby naive stem cells exposed to certain growth factors and/or cytokines will progressively acquire specific intrinsic factors and differentiate into a hierarchy of progeny/progenitor cells. These progenitor cells are thought to be stem-like themselves, but restricted in the number of differentiation options (pathways) available to them.^{80,81} However, recent discoveries have begun to expose weaknesses of this theory. The use of both transgenic and knockout mouse models in conjunction with a better understanding from molecular analyses of HSCs and their progeny has led to the realization that these stem cells are very dynamic. Factors that govern the differentiation potential of tissue-derived stem cells are not understood completely, and so there is a widening interest in the isolation, characterization, and therapeutic potential of stem cells that reside in adult tissues for the purpose of tissue repair and regeneration.

Most of the experiments with bone marrow stem cells (BMSCs) are conducted in the environment where those cells and their progeny can be identified. This is usually accomplished by transplanting BMSCs from a donor to a recipient that is genotypically or phenotypically different. The most commonly used approach is based on sex-mismatched transplantation where the male cells are identified by Y chromosome after being transplanted into female recipient. The advantage of this system is that Y chromosome is present in every intact donor-derived cell and can be easily visualized by FISH or whole chromosomal paints. Unfortunately, only whole cells can be used for

proper identification since the cut tissue sections may exclude parts of the cell that could contain the marker. In addition, thick or overlay tissue may produce false positive results.⁸² Also, nonspecific binding of a probe may be a problematic. The same problems may be apparent when using immunohistochemistry or immunofluorescence. In those approaches it has to be confirmed that that the cell labeling is not caused by nonspecific binding or artifacts. Another approach to prevent some of the problems is to use normal mice with genetic polymorphism that can be detected in all daughter cells. In addition, use of mutant animals like rats that are DPPIV-, which is uniquely expressed by mature hepatocytes, allows quick recognition of BMSC plasticity in the liver.

Bone marrow stem cell plasticity demonstrated as engraftment of BM cells in the liver as hepatocytes has been best shown by Lagasse et al.³⁸ In his study, the isolated HSCs (KTLS sorted cell) transplanted to the FAH mouse were capable of reconstituting the regenerating liver up to 40%. The same study shown that as little as 50 KTLS sorted cells transplanted into lethally irradiated FAH animals that were taken off NTBC showed significant reconstitution potential and rescued the animal thus providing a renewable supply of functional hepatocytes. The major point of this study is that BM derived hepatocytes were functional.

Plasticity of bone marrow cells is not only limited to the liver and hepatocytes. In many different studies transplanted bone marrow cells were found within cardiac muscle,⁸³ skeletal muscle,⁷⁴ central nervous system,^{84,85} kidneys,^{86,87} pancreas,⁸⁸⁻⁹⁰ lung,^{43,91} skin,⁹² and gastrointestinal tract.⁹³ In most of these studies, their origin was based on Y chromosome donor marker. Also, it has to be noted that a tissue injury was

used to create favorable condition that induce homing and differentiation. It is possible that local apoptosis or necrosis enables engraftment of circulating stem cells.

The mechanism of BMSCs becoming other cells types is currently unknown. One possibility suggests that those cells transdifferentiate. Transdifferentiation refers to the ability of one committed cell type to change its gene expression pattern and become a completely different cell type. Transdifferentiation can occur via three different pathways. The first pathway suggests that cells not committed to become blood and highly pluripotent may exist within BM and can differentiate into a variety of different cell types when needed. Alternatively, the second pathway suggests that it is a committed HSC that can transdifferentiate. The third pathway suggests that HSC can dedifferentiate becoming a more undifferentiated stem cell, and then mature down an alternative pathway.⁹⁴

An alternative mechanism for BMSCs to become different cell types is cell fusion. The mechanism of cell fusion states that a BM-derived cell fuses with a nonhematopoietic cell to form a heterokaryon, thus converting the gene expression pattern of the original BM cell to the one it fused with. The debate concerning fusion started in 2002, when two papers by Terada et al. and Ying et al. questioned whether transdifferentiation occurs at all. Authors of both papers based the argument on their data that shows either embryonic or adult stem cells grown together in presence of LIF and IL-3 fusing to create tetraploid cells.^{25,95} Additional support to cell fusion came in 2003 when two papers in *Nature* described bone marrow cells fusing with hepatocytes producing new phenotypically different liver cells.^{96,97}

In the light of the above experiments, the question arises if cell fusion is accountable for all lineage changes previously observed, and if cell fusion has been misinterpreted as transdifferentiation. It is possible that some of the observed transdifferentiation was in fact cell fusion that changed the cell phenotype. However, it is most likely that fusion cannot explain all cases of transdifferentiation. Chromosomal analysis of BM-derived cells in lung, muscle,⁹⁸ and kidney⁹⁹ show that those cells are 2N. The data suggest that those cells did not result from fusion. Studies where pancreatic β cells are derived from stop-lox-GFP BM cells transplanted to Cre recombinase carrying recipient mice further support that fusion is not the mechanism behind BMSC transdifferentiation.⁸⁸ However, other studies have shown otherwise in the case of severely injured liver.^{26,96} In those studies, BMSCs were transplanted into FAH mice. In the animals that survived the NTBC withdrawal and had their livers repopulated by donor-derived cells, it was found that, additionally to donor markers, they also expressed markers of the recipient cells thus suggesting that fusion was responsible for transdifferentiation.

With data showing both of the mechanisms, fusion and transdifferentiation, the dilemma is still in question. It is possible that the phenomenon of cell fusion is driven artificially by the stringent approach used in the experiments or that cell fusion is a natural useful process that coexists together with cell transdifferentiation. If the resulting cells are healthy and functional, cell fusion may generate cells of great physiologic significance that could be used in clinical application for either cell or gene therapy. The only problem with fusion may result if fused cells carry a high potential for malignant transformation. In that case, these cells would lose their clinical value. However, more

research is needed to verify this possibility, and at this point, the use of BMSCs for clinical applications should be investigated.

1.6 Gene Therapy

Gene therapy is a new and exciting treatment that aims to correct the disease process by either restoring or modifying cellular functions through the introduction of nucleic acid, either DNA or RNA, into the targeted cell. Currently, most gene therapy studies focus on the replacement of a disease-causing gene with a normal gene accomplished by a carrier molecule (a vector) that targets patient's cells. The most common type of vector is a genetically altered virus with the capability to carry a therapeutic gene. Out of all of the virus families known, only a few are suitable for that role.

The first is the Adenovirus (AV) from the *Adenoviridae* family. AV is an icosahedral non-enveloped dsDNA virus that, in human, causes mild respiratory illness (conjunctivitis). The defective and non-pathogenic form is widely used for gene therapy *in vivo* and is in clinical trials for cancer and Cystic Fibrosis (CF).¹⁰⁰ To generate a safe and defective form of AV, the E1 gene, normally used for viral replication and gene expression, is replaced with the therapeutic gene. Such viruses can only be propagated in cell line stably expressing products of E1 in *trans* (293 cells). Adenoviruses are the most efficient of all classes of vectors at mediating transgene expression in target cells. Their advantage is in the ability to infect a wide variety of dividing and non-dividing cells. Additionally, the relatively large size of the genome offers almost no limit on the size of the therapeutic gene that can be inserted. Unfortunately, using AV is not without problems. Current AV, following infection, expresses low levels of viral antigens that

result in a low level of DNA replication. Also, the adenovirus genome is maintained episomally and can only mediate the transgene expression for a short time (transient expression).¹⁰¹ In addition, infection with AV often generates a potent inflammatory response evoked by the expression of wild-type viral genes in the vector. As a result, the immune system of treated individual quickly kills infecting virus resulting in loss of therapeutic gene expression 1-2 weeks after injection.^{100,102} Current studies involve generation of new adenoviral vectors that lack wild-type genes and are thus less immunogenic. Also, they are able to maintain sustained expression of the transgene in a variety of tissues in vivo raising the possibility of using them for therapies requiring continuous transgene expression.¹⁰³

The second suitable vector is the Adeno-Associated Virus (AAV) from the *Parvoviridae* family. AAVs are one of the smallest viruses known. They are icosahedral ssDNA viruses that require co-infection with a helper virus such as AV for productive replication. There is no pathology associated with AAV infection in humans making this virus suitable for gene therapy. Additionally, wild-type AAV has the capability to stably integrate into a specific locus on chromosome 19. Unfortunately, to generate a recombinant virus, all viral genes (*rep* and *cap*) must be removed to make space for the therapeutic gene and only flanking LTRs, essential for viral replication and integration, are retained.^{104,105} Consequently, generated recombinant viruses not only lose their specificity for integration, but also, most of them do not integrate at all and are episomally maintained within the infected cell. Another disadvantage of working with AAV is the small size of the viral genome that restricts use of therapeutic genes to no more than 4.8 kb. However, even with all these problems, AAV still appears as an

excellent gene transfer agent and has been extensively used in transduction of hepatocytes,¹⁰⁶ muscle cells,¹⁰⁷ hematopoietic stem cells¹⁰⁸ and other cell types.

Currently, AAV vectors have been used in clinical gene-therapy trials for hemophilia B^{109,110}, cystic fibrosis^{111,112}, central nervous system disorders¹¹³, and alpha-1 antitrypsin deficiency syndrome¹¹⁴⁻¹¹⁶. Most of these studies have used vectors with serotype 2, the first serotype characterized. However, different serotypes may prove to be more useful since there is high prevalence of neutralizing antibodies against serotype 2 in humans following natural infection with the wild-type virus.

Retroviruses are the third group of viruses with a potential for gene therapy.

Retroviruses are small RNA viruses that replicate through a DNA intermediate. The most important advantage of retroviral vectors is their ability to transform their single stranded RNA genome into a double stranded DNA molecule that stably integrates into the targeted cell genome. Because of this property, retroviruses are best candidates for gene therapy that relies on permanent modification of host cell nuclear genome. Currently, the most commonly used retroviruses in clinical applications are ones based on the Moloney murine leukemia virus (MMLV). Unfortunately, the major disadvantage of using MMLV is the fact that the virus can only integrate during cell division so it is more suited for cells that are mitotically active.¹¹⁷ To face this dilemma, a new system has been developed. It is based on the HIV virus (lentivirus) and is capable of infecting and stably transducing not only dividing but also quiescent, non-dividing cells.^{118,119} The system uses three different vectors that are transfected together into a cell line to produce the replication defective virus. This new system has been extensively modified to remove any risks that could be associated with using an HIV-based virus. Currently, the potential

risk of homologous recombination, and creating a fully capable HIV virus is virtually non-existent. The only risk involved with using this virus is its enormous infection efficiency, which creates potential health hazards to those that handle it.

In the above list of possible viruses that could be used as vectors for gene therapy, Adenoviruses are the most immunogenic and capable of inducing both cytotoxic T-lymphocyte (CTL) as well as humoral responses. In the human trial of this vector in 1999 for OTC deficiency¹²⁰, a massive systemic inflammatory response induced to the injected vector was responsible for a patient's death. This study demonstrated the possible risks involved with gene therapy, and that different patients have remarkably different inflammatory and immune responses to the same dose of adenoviral vectors. The other two types of vectors are less inflammatory and immunogenic than adenoviruses. AAV and Lentiviral vectors do not generate a CTL response against viral proteins. However, the transgene expression product can still induce the T-cell response.¹²¹ Being less immunogenic, AAV and Lentiviruses are not without a major problem. Both of these viruses have the capability to integrate randomly into active genes thus generating possibility of vector insertion-induced oncogenesis. Originally, it was generally accepted that insertion-induced oncogenesis is a very rare event, and it was never observed in over 500 patients that were enrolled in more than 100 clinical trials involving the use of oncoretroviral vectors.¹²² However, the two cases of leukemia in X-SCID children changed this belief.¹²³ rAAV also integrates in transcriptionally active genes of the host following liver-targeted delivery in mice.¹⁰⁶ However, this integration is rare, and is estimated to be 5%.¹²⁴ It seems that the integration of rAAV is facilitated by existing

chromosomal breaks since the vector lacks the integration machinery and integrases. Current safety implications of this rare integration are unclear.

The last applicable options for gene transfers are non-viral vectors. One of them is the direct introduction of the therapeutic gene into the target cell. Unfortunately, this method has a limited application since it can only be used with certain cell types and requires large amounts of DNA. Another non-viral method is use of cationic liposomes, an artificial lipid sphere with an aqueous core, which can carry a gene of interest across the cell's membrane. The above two techniques are easy to perform and are quite efficient; however, the transferred gene never integrates into the cell's chromosome thus highly limiting its expression. Additionally, DNA transferred in the above fashion cannot be cloned during subsequent mitotic divisions.

To date, most successful applications of viruses in gene therapy involve AAV and retroviruses. They are used for a possible treatment of many acquired disorders, including cancer, vascular disease, degenerative neurological conditions and infectious diseases (HIV acquired AIDS). However in most cases, the studies did not progress beyond the Phase I stage of assessing safety. In studies involving cancer, two different approaches have been used. The first approach is to enhance cellular immunity by increasing production of immunomodulatory agents, like interleukin-2 (IL-2)¹²⁵ or granulocyte-macrophage colony-stimulating factor (GM-CSF)¹²⁶. The other is to replace or repair genetic mutation within p53 or pRB (retinoblastoma) genes that are responsible for normal cell cycle and growth.¹²⁷ Hemophilia is another disorder that received a lot of attention from gene therapy studies. The disorders clinical manifestations are attributed to the lack of a single protein that circulates in very small amounts in the plasma. In pre-

clinical studies, sustained therapeutic expression of factors VIII and IX has been achieved in the levels showing limited efficiency and no toxicity.¹¹⁰ Of all the vectors that were evaluated for hemophilia B gene therapy, rAAV appears to be most promising.^{109,128}

Almost all gene therapy trials involving bone marrow cells used retroviruses.¹²² Inability of infecting non dividing, quiescent cells prevented use of AV and AAV vectors. The most known trials involved correction of monogenetic disorders, including adenosine deaminase deficiency (ADA) SCID, chronic granulomatous disease and Gaucher's disease. All of them used *ex vivo* retrovirally transduced hematopoietic stem cells (HSC) as vehicle for gene therapy. In case of ADA SCID, treated patients (children) were able to develop a functional immune system after infusion with HSCs carrying γ_c chain cytokine receptor. 12 out of 14 treated children achieved immune reconstitution that is superior to that observed after haplo-identical allogeneic HSC transplantation.¹²⁹

Most of the disorders that invoked current gene therapy trials and research result from a mutation within a single gene that leads to either inactivity of an enzyme or total loss of its production. All research treatments try to replace the mutated gene with a correct copy that would result in correction of the disorder. However, not all genes can be corrected as a result of limited capacity for the vectors used. Current candidates for trials require small size of gene that needs to be corrected, and a single phenotypical function of that gene is preferred. That brings us to two disorders that are the best candidates for gene therapy. Both of the disorders originate from a mutation that results in the inability to produce an enzyme that is required for an essential function in the body. In addition, the product that is missing is normally produced by liver hepatocytes. The two disorders are alpha-1 antitrypsin (AAT) deficiency and Crigler-Najjar syndrome (CNS) type 1.

1.7 Alpha-1 Antitrypsin Deficiency

Alpha-1 antitrypsin deficiency is an autosomal-recessive inherited disease that leads to panacinar emphysema of the lung and cirrhosis of the liver. Emphysema develops when the network of elastin fibers in the lung parenchyma is destroyed, resulting in over-distention of distal airspaces, destruction of alveolar architecture, air-trapping, and impaired gas exchange. Abnormalities that increase the activity of protease or decrease available anti-protease increase the likelihood of developing emphysema. The pulmonary disease pathology associated with AAT deficiency is similar to cigarette smoking-related emphysema. Additionally to lung damage, AAT deficiency has been associated with neonatal hepatitis with cholestatic jaundice in 10 to 20% of infants born with the disease. A very small proportion (1-2%) of children with AAT deficiency die from cirrhosis in childhood.

AAT, the most prominent endogenous serine proteinase inhibitor, is normally synthesized by the liver and circulates throughout the body and provides important protection against proteases such as neutrophil elastase. Abnormal production of AAT leads to increased neutrophil elastase activity resulting in a prolonged, recurrent digestion of the extracellular matrix of the lung.¹³⁰ Mutations, a major cause of AAT abnormalities, are designated by their protease inhibitor (Pi) types determined by isoelectric focusing (IEF) gel electrophoresis. Clinically, the most prevalent type of AAT deficiency is classified as Pi Z. This variant has two amino acid substitutions, when compared with normal AAT that possibly lead to misfolding and cellular accumulation of the protein due to inability of transporting it out of the Golgi. Pi Z phenotype is characterized by lower levels of the circulating protein that are 10-15% of the normal. Another variant designed

as Pi S is characterized only by slightly lower AAT levels than normal which are not considered as life threatening. Null mutants are less common; however, they are also prone to emphysema.

Current treatment for AAT deficiency uses protein replacement therapy. The therapy consists of weekly injections of Prolastin®, a concentrated preparation of human 1-proteinase inhibitor (AAT) manufactured by Bayer Corporation. Prolastin® is prepared from pooled human plasma that has been screened for the hepatitis B surface antigen and for HIV. However, this type of treatment is very laborious and requires a life-long administration under the supervision of medical personnel. Additional problems with this treatment come from the fact that when using human plasma there is a theoretical concern of contamination with infectious diseases. More radical treatments for AAT deficiency include lung transplantation for patients with end-stage lung disease, and liver transplantation for patients with end-stage liver disease.

AAT seems to be an attractive target for gene therapy. The phenotypical abnormality in this disorder could be corrected by delivering the normal human AAT gene to the cells of deficient individuals. Additionally, the target cells could be introduced anywhere in the body, as long as sufficient AAT is able to reach the lower respiratory tract. Currently, the best method to treat the abnormality is to intravenously inject once a week plasma-derived, purified AAT, at a dose of 60 mg/kg.^{131,132} Studies using this regimen have shown that it is sufficient to maintain serum levels of AAT above a theoretical protective threshold of 11 μ M throughout the course of treatment. Unfortunately, this type of treatment is very laborious and requires a life-long administration under the supervision of medical personnel. Additional problems with this

treatment come from the fact that when using human plasma there is a theoretical concern of contamination with infectious diseases such as hepatitis or HIV.

1.8 Crigler-Najjar Syndrome (CNS) Type 1

Crigler-Najjar syndrome (CNS) type 1 is a rare fatal genetic disorder characterized by accumulation of bilirubin in the blood plasma due to UGT1A1 deficiency causing persistent unconjugated hyperbilirubinemia.^{133,134} The deficiency results from a loss-of-function point mutation within the coding gene of that enzyme. The lack of bilirubin glucuronidation leads to inefficient biliary excretion of bilirubin and accumulation of unconjugated bilirubin in plasma.

In early stages, CNS manifests itself by jaundice that over time will develop into kernicterus, a potentially life-threatening neurological condition caused by accumulation of bilirubin in the brain that eventually damages central nervous system (bilirubin encephalopathy). Without treatment, majority of patients develop kernicterus and die in the first 18 months of their life. Patients that survive beyond puberty usually succumbed to bilirubin encephalopathy.^{135,136} Other major causes of life-long disabilities in this syndrome include choreoathetosis, hearing problems, and mental retardation.¹³⁷

At present, two available CNS treatments are not definitive. The first is phototherapy. The treatment starts during infancy and is needed on an ongoing basis throughout life. Unfortunately after four years, phototherapy becomes less successful due to thickened skin, which blocks the light. The second and the best option for CNS patients is liver transplantation. Liver transplants, however, are restricted in number to a limited availability of donor organs. Additionally, patients with transplants require life-long immune suppression to prevent organ rejection.

In 1998, Laconi et al.^{29,34,35} developed an efficient method for hepatocyte transplantation in which retrorsine is used as a pre-treatment that inhibits recipient cell mitosis and gives a proliferative advantage to subsequently transplanted hepatocytes. Using this approach, Laconi was able to repopulate 98% of the liver within four months post transplantation. Using a similar technique, in which proliferation of hepatocytes was inhibited due to DNA damage as a result of irradiation, Guha et al.¹³⁸ successfully treated CNS in an animal model, the Gunn rat. Gunn rats are characterized with the same symptoms as CNS related to the inactivity of UGT1A1 gene due to the deletion of a single guanosine base, which results in a frame-shift and a premature stop codon.¹³⁹ Unfortunately, the use of hepatocytes in CNS treatment creates some problems. For example, hepatocyte transplantation offers no advantage over liver transplantation, for both treatments require life-long immune suppression. Moreover, clinical application is limited by the availability of human hepatocytes.

1.9 Conclusion

The ability of the liver to regenerate its mass to compensate for damage is a very important property that can be utilized for the development and improvement of different types of treatments for liver disorders. Use of stem cells (either liver oval cells, bone marrow cells, or hepatocytes) in a cell transplantation therapy could be envisaged that would allow to either replace the damaged cells with new donor derived cells, or to deliver a potential therapeutic gene that could transform a mutated cell into a more normal cell producing a missing protein. In that type of therapy, transplanted cells, normal or transduced with a virus, would repopulate damaged liver that is unable to use its own cells to regenerate, and then function as normal undamaged organ.

CHAPTER 2
MONOCROTALINE, AN ALTERNATIVE TO RETRORSINE BASED
HEPATOCTE TRANSPLANTATION IN RODENTS

2.1 Summary

Retrorsine has been used extensively to inhibit proliferation of resident hepatocytes in various transplantation models. Here, we report a successful alternative to currently unavailable retrorsine that can be used in cellular transplantation models. Based on structural and molecular similarities, we investigate the use of monocrotaline (MCT) in cell transplantation studies in rodents. In this study, monocrotaline was given to rats intraperitoneally in two injections two weeks apart. Two weeks after the final injection, a partial hepatectomy followed by splenic hepatocyte transplantation was performed. The results indicate that monocrotaline, at two doses of 30 mg/kg, highly enhances liver repopulation by donor hepatocytes following partial hepatectomy and produces $15.3\% \pm 4.9$ liver repopulation within the first 6 weeks following transplantation. Additionally, we tested the effectiveness of monocrotaline in a murine model. Using two injections of 50 mg/kg each, given two weeks apart, hepatocyte proliferation in the native liver was inhibited and subsequent oval cell transplants engrafted at $18\% \pm 21.3$ after 16 weeks post transplantation. In conclusion, monocrotaline can be used as an effective selective pressure for donor hepatocytes in cell transplantation to the liver in rodents.

2.2 Introduction

Pyrrrolizidine alkaloids (PA) represent a group of structurally similar toxins that are common secondary metabolites in many species of flowering plants. PAs have been

reported to possess potent anti-mitotic and hepatotoxic activity³⁰ related to the α,β -unsaturated structure of the necic acid esters and C1-C2 unsaturation of the necine base (Figure 2-1A).³¹ To exert their biological action, PAs require metabolic activation. They are metabolically converted by the liver P450 system to produce reactive, highly toxic dehydroalkaloid (DHA) intermediates. These DHA intermediates have been shown to specifically inhibit late S or early G2 phase of the cell cycle.³³

PAs are grouped according to four major structural classes: macrocycles, open diesters, monoesters, and pyrrolizidine base. A member of the macrocycle class, retrorsine, has been extensively used in hepatocyte transplantation protocols developed by Laconi et al.²⁹ In these protocols, retrorsine is used as a pre-treatment that inhibits recipient hepatocyte mitosis and gives a proliferative advantage to the transplanted hepatocytes. This method has proven to be highly successful and leads to near-complete replacement of the recipient liver within 6 months post-transplantation. Unfortunately, retrorsine is currently not commercially available. This limitation has forced researchers to look for an alternative to retrorsine that possesses the same characteristic properties.

Among pyrrolizidine alkaloids, monocrotaline (MCT) is closely related to retrorsine. Structurally, monocrotaline belongs to the same class of macrocycles as retrorsine (Figure 2-1B), and contains the same α,β -unsaturation in the necic acid ester and C1-C2 unsaturation of the necine base that renders retrorsine hepatotoxic. (1,2) Further evidence of similarity is supported by an animal study by Chesney et al.¹⁴⁰ showing MCT arresting mitosis in hepatic tissue in treated rats. These facts led us to hypothesize that monocrotaline could be a potential candidate for cell transplantation protocols. The current studies were designed to test this hypothesis.

2.3 Materials and Methods

2.3.1 Animals

Dipeptidyl peptidase IV deficient (DPPIV⁻) F344 breeding animals were originally obtained from the Albert Einstein College of Medicine via a generous gift from Dr. Sanjeev Gupta. These animals were in-house bred and maintained on standard laboratory chow and daily cycles of alternating 12 hours of light and dark. Normal male F344 rats were purchased from Charles River Laboratories and were used as donor animals. C57BL/6 and Tg(GFPU)5Nagy/J male mice were purchased from Jackson Laboratory. All rats and mice were used at 10 weeks of age. All animal work was conducted under protocols approved by the University of Florida Animal Care and Usage Committee.

2.3.2 Monocrotaline Treatment

MCT was purchased from Sigma Aldrich (St. Louis, MO). To prepare a solution of MCT at 50 mg/ml, 2 ml of saline solution (pH 6.0) was slightly acidified (pH 3.0) with 2N HCl (approximately 200 μ l), and 500 mg of MCT was added with stirring until completely dissolved. Additional saline was added to increase the total volume to 10 ml and the pH of final solution was adjusted to 6.7 with 5N NaOH. MCT solution was freshly prepared for each set of experiments.

The standard MCT treatment for all experiments in rats and mice consisted of 2 intraperitoneal injections of the same MCT concentration performed 2 weeks apart. The MCT concentrations used for the treatments varied accordingly with the experimental design and ranged from 25 to 75 mg/kg of animal body weight. After the final injection, rats and mice were housed for two more weeks before further studies were conducted.

2.3.3 Dose Response in Rats and Mice

DPPIV⁻ female rats were divided into experimental groups of 3 animals each and placed on the MCT protocol. Animals in each group received two doses of 25, 30, 37, 50, and 75 mg/kg of MCT. Dosages were determined based on an LD₅₀ of 110 mg/kg for MCT from a previous pilot study (unpublished). Two weeks after the second MCT injection, PHx was performed with 50 mg/kg of 5-bromo-2-deoxyuridine (BrdU) injected 22 hours later. Two hours post BrdU injection, rats were sacrificed and collected tissue was divided, with half being placed in Optimal Cutting Temperature (O.C.T.) medium (Tissue-Tek, Sakura) for frozen sectioning, and the other half placed in 10% phosphate-buffered formalin for paraffin embedding.

C57BL/6 female mice were divided into three groups of 3 animals each. Of the groups, two were placed on the MCT protocol and received two doses of either 50 or 75 mg/kg. The third group was not MCT treated and was used as a control. Two weeks post the second injection mice underwent 2/3 PHx surgery. They were euthanised 34 hours later (BrdU injection was given 2 hours before euthanasia). Collected tissue was divided, with half being placed in Optimal Cutting Temperature (O.C.T.) medium (Tissue-Tek, Sakura) for frozen sectioning, and the other half placed in 10% phosphate-buffered formalin for paraffin embedding.

2.3.4 Hepatocyte Isolation and Transplantation in Rats

Donor hepatocytes were isolated from male F-344 DPPIV⁺ rats. Hepatocyte isolation was performed using a standard two-step collagenase perfusion.¹⁴¹ Cell viability was determined to be >80% as established by Trypan blue dye exclusion. After isolation, hepatocytes were resuspended in S&M solution (500mg KCl, 8.3g NaCl, 2.4

HEPES, 190mg NaOH, makes 1 liter, pH 7.4, filter sterilized) at the appropriate concentration to give approximately 1×10^7 viable cells per ml.

Transplantation of male DPPIV⁺ hepatocytes into MCT treated female DPPIV⁻ rats was performed following a procedure similar to that described by Laconi et al.²⁹ Briefly, rats were subjected to 2/3 partial hepatectomy (PHx) under general anesthesia⁷, at which time 200 μ l of hepatocyte suspension (approximately 2×10^6 viable cells) was injected into the spleen using a 25 gauge needle. To aid in the coagulation process, an absorbable hemostat (Medical Inc. Arlington, Texas) was applied at the injection site. Post-surgery, animals were placed back in general housing until they were sacrificed in 2-week intervals. From each animal, a sample of liver, lung and kidney was collected and subsequently divided with half being placed in O.C.T. medium for frozen sectioning and the other half placed in 10% phosphate buffered formalin for paraffin embedding.

2.3.5 Mouse Oval Cell Isolation and Transplantation

The activation, isolation and purification of oval cells is described by Petersen et al.⁶¹ For isolation, briefly, after initial two step collagenase perfusion, a portion (approximately half of the total 2×10^8 cells) of the NPC fraction was enriched using magnetic activated cell sorting with Sca-1 antibody conjugated to magnetic beads according to the manufacturer's recommendations (Miltenyi Biotech, Inc. Auburn, CA).

For oval cell transplantation, mice were subjected to 2/3 partial hepatectomy (PHx) under general anesthesia, at which time 100 μ l of oval cells suspension of approximately 2×10^6 viable cells was injected into the spleen using a 26-gauge needle. To aid in the coagulation process, an absorbable hemostat (Medical Inc. Arlington, Texas) was applied at the injection site. Post-surgery, animals were placed on a heating

pad until they recovered from anesthesia and then were placed in general housing until they were sacrificed at predetermined intervals. From each animal, a sample of liver, lung and kidney was collected and subsequently divided with half being placed in O.C.T. medium for frozen sectioning and the other half placed in 10% phosphate buffered formalin for paraffin embedding and 18% sucrose treating.

2.3.6 Immunohistochemistry and Enzyme Assay

All histochemical stainings were performed according to previously described protocols. DPPIV staining procedure was performed as described by Dabeva et al.³² Glycogen staining was based on Periodic Acid Schiff (PAS) stain purchased from Richard Allan Scientific (Kalamazoo, MI), and Glucose-6-phosphatase staining procedure was performed as described by Lillie.¹⁴² Hematoxylin and Eosin staining was performed according to standard procedures.

Histochemical detection of BrdU was carried out by incubating 4 μm thick formalin-fixed, paraffin-embedded sections of rat liver (MCT/PHx protocol) with a mouse anti-BrdU primary antibody at 1:50 dilution from DAKO laboratories (Carpinteria, CA). Secondary antibody detection was performed using the LSAB-Link detection kit (DAKO) as per the manufacturer's instructions.

To directly visualize GFP expression, liver tissue was fixed in 10% phosphate buffered formalin overnight and then placed in 18% sucrose at 4°C for 24 hours. After the sucrose treatment, tissue was O.C.T. embedded and frozen.

2.3.7 Cell Counting and Statistical Analysis

For measuring hepatocyte proliferation and donor hepatocyte engraftment, a minimum of three whole 6 μm sections per liver were analyzed. The inter lobe variability

of proliferation and engraftment necessitates viewing each section in entirety. To determine the level of liver repopulation by transplanted cells, the area of donor cells (patches) on whole liver section micrographs was calculated using Image-Pro Plus: The Proven Solution, version 4.1.0.0 software and compared to the whole area of that section. Standard deviation and statistical significance were determined with the statistical software included in Microsoft Excel.

To investigate the effectiveness of hepatocyte repopulation in MCT treated rats, DPPIV staining was performed and the positive cells were counted. Counting all DPPIV⁺ cells on a whole liver section and then averaging it by the total number of clusters in that section determined the number of hepatocytes per cluster. Similar procedure was applied to determine BrdU labeling index.

2.4 Results

2.4.1 Monocrotaline Dose Response in Rats

Rats received doses of MCT ranging from 25 mg/kg to 75mg/kg. The hepatocyte proliferation index for each animal at the time of peak proliferation (24h) following PHx was determined by BrdU incorporation into S-phase DNA. A positive correlation was found between MCT dose and inhibition of hepatocyte proliferation (Table 2-1, Figure 2-2). At 25 mg/kg we observed on average $5.4\% \pm 1.4$ of dividing hepatocytes as compared to normal untreated animals in which the average number of BrdU indexed cells was defined as 100%. In a similar fashion 30, 37, 50 mg/kg lowered proliferation to 2.9, 2.8, and 2.2%, respectively. At 75 mg/kg, proliferative capacity was not detectable. In addition, histological examination from all MCT treated groups showed sinusoidal

dilation and moderate disorganization of hepatic lobular structure that progressed with increased MCT doses (Table 2-1).

2.4.2 Hepatocyte Transplantation in Rats

In the hepatocyte transplantation protocol, the DPPIV⁻ rat¹⁴³ was employed as the recipient in order to distinguish DPPIV⁺ donor hepatocytes. Based upon the dose response findings, doses of 30, 37 and 50 mg/kg were chosen for the cell transplantation studies.

Utilizing the protocol as described in the materials and methods section, hepatocytes were transplanted into MCT/PHx treated animals. Observations at 4 days were similar to those seen by Laconi²⁹; in the animals treated with 50 mg/kg dose, small groups of transplanted hepatocytes were visible in the recipient livers (data not shown). However, 2 weeks after transplantation, DPPIV⁺ cells appeared in clusters containing on average 20 ± 10.6 cells per cluster as observed in two-dimensional tissue sections (Figure 2-3A). After 4 weeks post-transplantation, the clusters of donor hepatocytes increased in size, averaging approximately 100 ± 65.1 cells (Figure 2-3B). 5 weeks after transplantation, the last time point of the conducted experiment, the DPPIV⁺ cell clusters increased in size and some had become confluent (Figure 2-3C and 2-3F). At this time point, an average of $16.4\% \pm 18.8$ of the recipient liver was repopulated with donor cells. In one case, nearly 44% of the liver was repopulated with donor cells (shown in the whole liver section montage in Figure 2-3F). No significant growth of transplanted hepatocytes was observed in rats receiving partial hepatectomy but untreated with MCT.

To further validate ability of transplanted hepatocytes to repopulate MCT treated liver and to function as normal hepatocytes, histochemical analyses were employed to

detect hepatocyte biochemical functions. Figure 2-3C-E portrays these results. Serial sections of liver tissue obtained 5 weeks post-transplantation, showed that the transplanted hepatocytes retained their ability to store glycogen (Figure 2-3D) as well as highly express glucose-6-phosphatase (Figure 2-3E).

The other two doses, 30 and 37 mg/kg showed similar results as the above with slight drop in percentage of repopulating cells. Using dose of 30 mg/kg, at 3 weeks post transplantation, DPPIV⁺ cells appeared in clusters containing on average 23 ± 9.7 cells. The number of cells further increased to approximately 90 ± 64.3 cells per cluster at 6 weeks post transplantation. At this time point, an average of $15.4\% \pm 4.9$ of the recipient liver was repopulated (Figure 2-3G).

Unfortunately, the MCT dose of 37 and 50 mg/kg caused moderate to severe pulmonary toxicity similar to interstitial lung disease at time points that extended over 6 weeks post-transplantation. The pulmonary toxicity of high doses of MCT has been observed previously and extensively used to cause pulmonary hypertension-like symptoms in experimental animals.¹⁴⁴

2.4.3 Murine Model

In addition to rats, we tested the effects of MCT in a murine model. Table 2-2 shows the results for the murine dose response experiment. Based on BrdU staining, it appears that both doses of MCT (50 and 75 mg/kg) lead to almost total inhibition of hepatocyte proliferation. Histological examination of all MCT treated groups did not reveal significant sinusoidal dilation or disorganization of hepatic lobular structure as seen in the rat model.

To validate the usefulness of MCT for cell transplantation in mice, a cell transplantation experiment involving the use of donor GFP⁺ oval cells was performed. The MCT dose used for these experiments was 50 mg/kg. All 6 mice were sacrificed 16 weeks post transplantation. Figure 2-4A and 2-4B shows direct visualization of GFP⁺ cells in transplanted non-GFP animals. In this experiment, two animals transplanted with GFP⁺ oval cells showed approximately 40% repopulation. The overall average of repopulation was estimated to be 18% ± 21.3. To further validate the presence of GFP cells in the recipient livers, PCR for specific GFP sequence was carried out (Fig.2-4C). As shown, GFP was present in the transplanted livers indicating engraftment and proliferation of donor cells.

Control mice (not treated with MCT) were transplanted with GFP hepatocytes, and as previously reported¹⁴⁵, our results showed less than one positive donor cell per 200X field (n=3, 36 liver sections examined).

2.5 Discussion

In this study, we demonstrate that monocrotaline can be used as an effective alternative to retrorsine in the cell transplantation model for the liver. BrdU detection was used to ascertain the amount of MCT needed to efficiently arrest cell proliferation. Based on the obtained data, we conclude that a MCT dose in the range between 25 and 50 mg/kg could be used to inhibit the proliferative capacity of hepatocytes in rats. There was no significant difference between these doses, however; the pulmonary toxicity of monocrotaline can be very problematic in rats.

Although 37 and 50 mg/kg of MCT could be used to inhibit the hepatocytes' proliferative capacity, its pulmonary toxicity renders recipient rats unable to survive 7

weeks past the MCT injection. However, as we demonstrate, 50 mg/kg of MCT is very effective in hepatocyte inhibition and can lead up to 44% of liver repopulation within first 4 weeks post transplantation.

Based on the data obtained from our dose-response and hepatocyte transplantation experiments, the final dose was further optimized. We have determined that 30 mg/kg of MCT could be effectively used as an optimal dose. At this concentration, transplanted animals show similar repopulation levels as shown for the higher dose of 50 mg/kg without side effect of interstitial lung damage. It should also be noted that the optimal MCT dose coincides with the dose used for retrorsine.

Additionally, as we have shown, MCT can be effectively used to inhibit hepatocyte proliferation in mice thus allowing them to accept cell transplants. At two 50mg/kg doses of MCT mice did not exhibit any pulmonary distress over a 16-week period. In this model, MCT has been very effective in promoting the proliferation of transplanted cells and will provide researchers with an opportunity to use a second species for cell transplantation models.

In conclusion, monocrotaline appears to be a useful and effective alternative to retrorsine in models of cell transplantation to the liver, especially since retrorsine is no longer commercially available. This alternative is useful for cell transplantation studies with hepatocytes as well as other cell types such as hepatic oval or bone marrow cells. In addition, MCT appears to be as effective in the mouse model as in rats, an attribute retrorsine could never achieve.

Table 2-1 Labeling index and liver repopulation in rats treated with monocrotaline.

MCT dose (mg/kg)	BrdU labeling index of proliferating hepatocytes (%)	Sinusoidal dilations and lobular disorganization	Liver repopulation (%)
0	100	—	<0.5%
25	5.4 ± 1.4	+	NT
30	2.9 ± 1.0	+	15.3 ± 4.9
37	2.8 ± 1.7	++	5.4 ± 1.86
50	2.2 ± 0.7	++	16.4 ± 18.8 ^(*)
75	0	+++	NT

Legend: **NT** = not tested, — none observed, + low, ++ moderate, +++ high.

* Repopulation measured 5 weeks post transplantation

Each value represents the average ± s.d from n=3. Liver repopulation was measured at 6 weeks post transplantation unless otherwise marked.

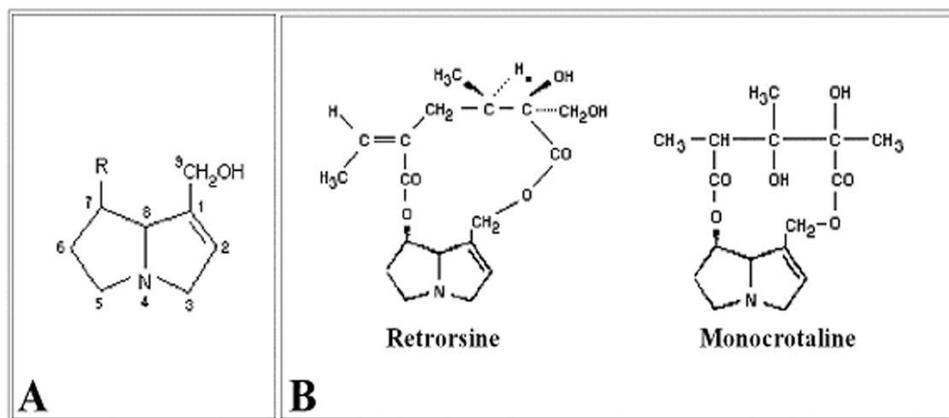


Figure 2-1 Pyrrolizidine alkaloids. (A) The pyrrolizidine nucleus is comprised of two 5-membered rings with a common nitrogen at position 4. C1-C2 unsaturation of this necine base is believed to allow PAs to exert their hepatotoxicity. (B) Molecular structures of retrorsine and monocrotaline.

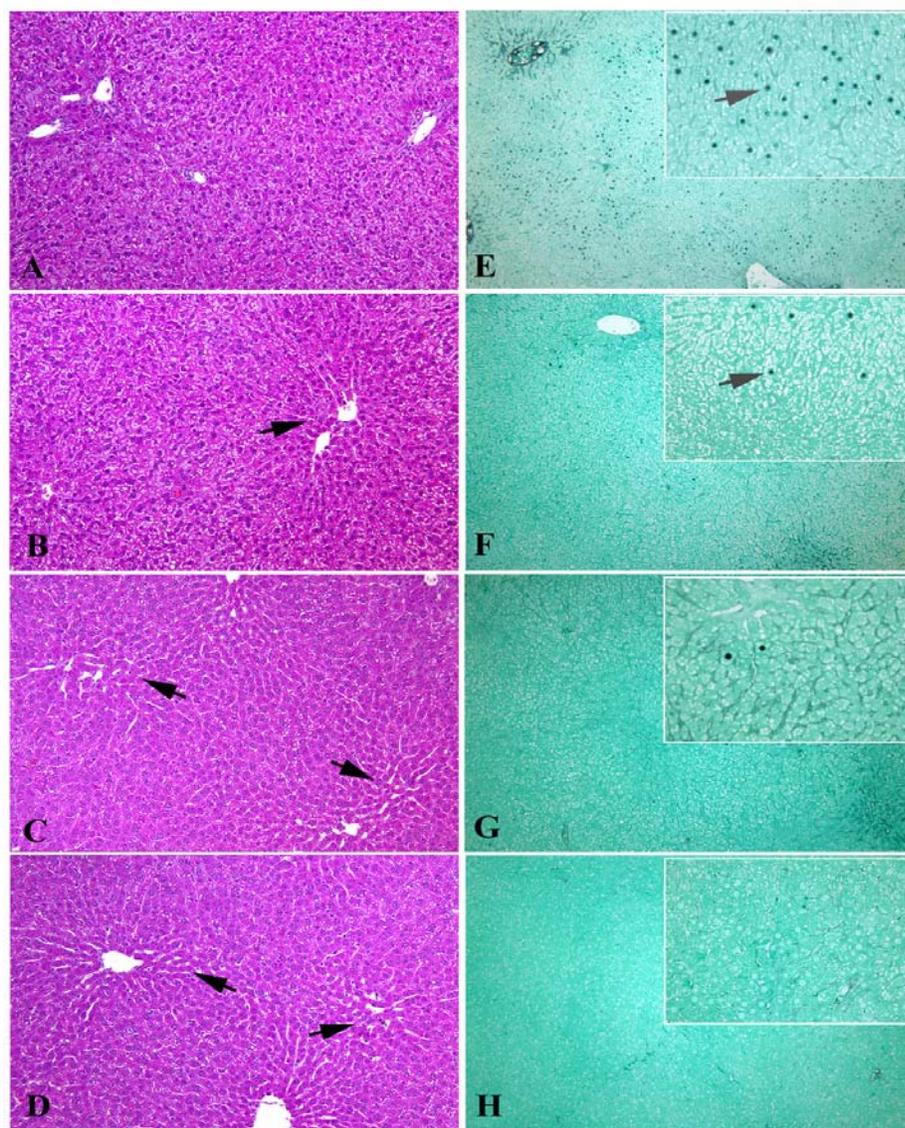


Figure 2-2 Hematoxylin/Eosin and BrdU staining of MCT treated liver. A,E . Untreated liver; B,F. Treated with 2x25mg/kg; C,G. Treated with 2x50 mg/kg; D,H. Treated with 2x75mg/kg. Note progressively increasing disorganization of hepatic lobules and sinusoidal dilations (A-D, arrows) and decreasing number of mitotically active cells with the higher dose of MCT (E-H, arrows).

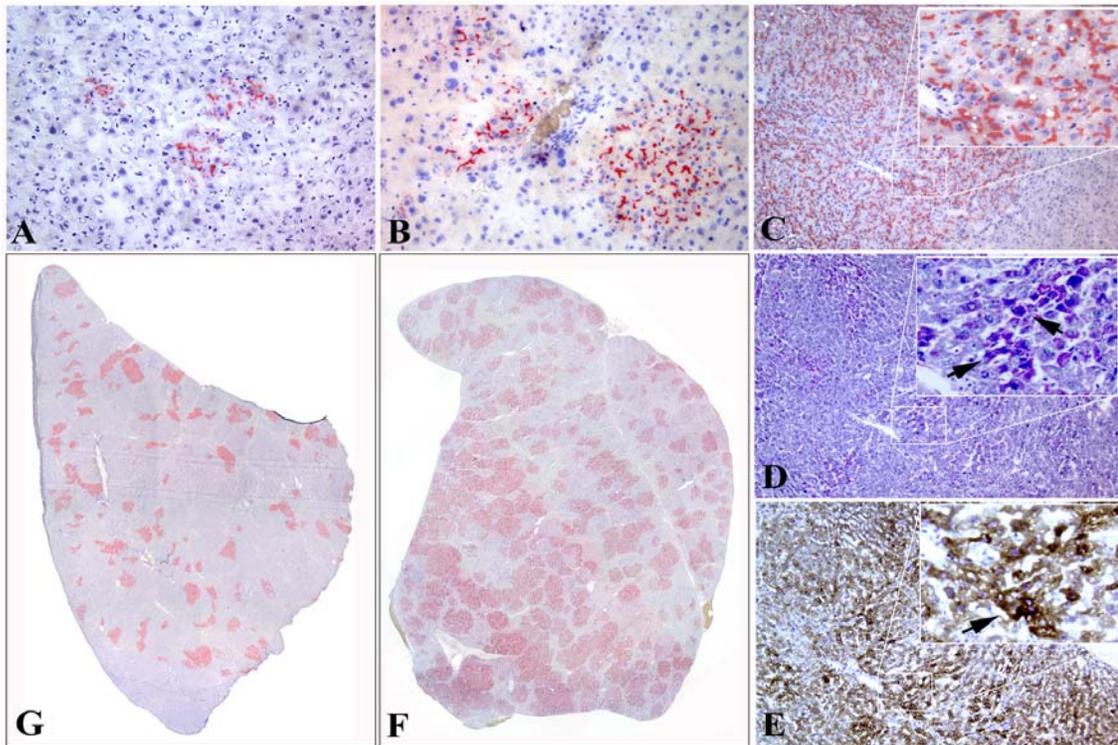


Figure 2-3 Proliferation of transplanted DPPIV⁺ hepatocytes in monocrotaline treated DPPIV knockout rats. Clusters of DPPIV⁺ transplanted cells (A) 2 weeks, (B) 4 weeks, and (C) 6 weeks after transplantation. (C-E) serial sections of cluster of DPPIV⁺ cells in rat 6 weeks post transplantation. (D) Glycogen (arrow), and (E) Glucose-6-phosphatase (arrow) staining showing biochemical functions of transplanted hepatocytes. (F) 6 weeks liver section showing 44% repopulation by transplanted DPPIV⁺ hepatocytes (2x50mg/kg MCT treatment), (G) 6 weeks liver section showing 20% of liver repopulation by transplanted DPPIV⁺ hepatocytes (2x30mg/kg MCT treatment). Original magnification: (A,B) 200 X, (C-E) 100 X, insets 200 X

Table 2-2 Liver repopulation and labeling index in mice treated with monocrotaline.

MCT dose (mg/kg)	BrdU labeling index of proliferating hepatocytes (%)	Sinusoidal dilations and lobular disorganization	Liver repopulation (%)
0	100	—	<0.1%
50	0.8 ± 0.9	—	18 ± 21.3
75	0	—	NT

Legend: **NT** = not tested, — none observed.

Each value represents the average ± s.d from n=3. Liver repopulation measured 16 weeks post transplantation.

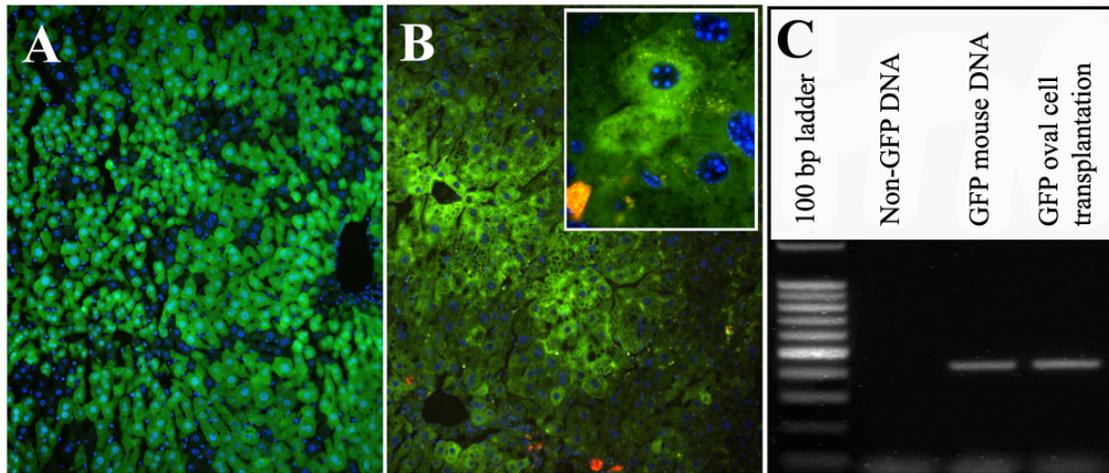


Figure 2-4 Direct visualization of GFP⁺ cell transplanted into non-GFP recipients 3 months post transplantation. A. Positive control, GFP⁺ mouse liver (200 X); B. oval cell transplantation and magnified view of GFP hepatocyte (200 X, inset 1000 X); C. PCR for specific GFP DNA in representative livers of transplanted animals.

CHAPTER 3
EX VIVO TRANSDUCED LIVER PROGENITOR CELLS AS A PLATFORM FOR
GENE THERAPY

3.1 Summary

Allogeneic stem cell-based transplants may be limited by allograft rejection, as is seen with conventional organ transplantation. One way to avert such a response is to use autologous stem cells, but that may carry the risk of recurrence of the original disease, particularly in the context of a genetic defect. We investigated the potential for gene modification of autologous stem cells to avoid both problems, using recombinant adeno-associated virus (rAAV) vector expressing human alpha-1 antitrypsin (hAAT) in murine liver progenitor cells. We showed that rAAV1 was the most efficient vector for liver progenitor cell transduction among 5 different serotypes of rAAV vectors. *Ex vivo* infected GFP⁺ liver progenitor cells from C57BL/6 mice with rAAV1-CB-AAT were transplanted into the liver of MCT treated and partial-hepatectomized C57BL/6 recipients. Utilizing GFP as a donor marker we were able to determine that at 18 weeks post transplantation approximately 40-50% of the regenerated liver was GFP-positive. In addition, transgene expression (serum hAAT) was sustained for the length of the study (18 weeks after transplantation). Immunostaining revealed about 5 to 10% of repopulating liver cells expressing hAAT. This study for the first time demonstrated the feasibility of long-term engraftment and stability of transgene expression from genetically modified liver progenitor cells with a rAAV vector and implies a novel gene therapy approach for treatment of liver diseases, such as AAT deficiency.

3.2 Introduction

Alpha 1-antitrypsin (AAT), a serine proteinase inhibitor, is normally secreted from hepatocytes and circulates in the plasma, protecting lung elastin from degradation by neutrophil elastase and related proteases. Deficiency of AAT can lead to pan-acinar emphysema due to destruction of pulmonary interstitial elastin if serum levels fall below $11\mu\text{M}$ (approximately $800\mu\text{g/ml}$).¹³⁰ In a subset of patients homozygous for the PI*Z mutation, liver disease may also develop, apparently related to accumulation and polymerization of the mutant protein within the endoplasmic reticulum of affected hepatocytes. Strategies of over-expression of wild type AAT for emphysema from AAT deficiency have been attempted and were successful by gene transfer to skeletal muscle,^{146,147} liver,¹⁴⁸ lung and macrophage.^{149,150} Successful gene therapy for AAT deficiency-related liver disease has not been reported. *Ex vivo* gene delivery to liver progenitor cells followed by transplantation may have potential for the treatment of this disease.

The strategy of *ex vivo* gene delivery to stem cells followed by transplantation has been proposed and attempted for the treatment of genetic diseases and acquired diseases. Considering the regenerative ability of stem cells, this strategy has advantages over the conventional gene therapy including avoidance of vector administration into recipient and amplification of transgene expression along with cell proliferation and differentiation.^{151,152} Of all the applications, gene transfer into human hematopoietic stem cells (HSC) remains a promising avenue. The most commonly used vectors are retroviral and lentiviral vectors that have capability to mediate genome integration into HSCs. Recombinant adeno-associated virus (rAAV) vector, a non-pathogenic vector can

mediate efficient long-term transgene expression various cells and tissues without integration. However, it has been shown that rAAV is not efficient for HSC transduction¹⁵³ and that transgene expression declined as cells divided. Recently, rAAV has been used for gene targeting in mesenchymal stem cell (MSC).¹⁵⁴ Here we show that rAAV1 vector transduces liver progenitor cells and mediates long-term transgene expression after transplantation.

3.3 Materials and Methods

3.3.1 AAV Vectors

Plasmid CB-AT, in which hAAT cDNA driven by CMV enhancer and chicken β -actin promoter is between full length AAV2 ITRs, has been previously described.^{148,155} This vector was packaged into five AAV capsids (serotype 1, 2, 3, 4 and 5) as described previously.¹⁵⁶

3.3.2 Liver progenitor cell Isolation

Isolation of hepatic oval cells (liver progenitor cells) was performed as described by Petersen et al.⁶¹ Briefly, to activate the oval cell compartment, C57 BL/6 mice were placed on a diet containing 0.1% Diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridine-dicarboxylate (DDC) for 4 weeks. This diet has been reported to be very effective in producing murine oval cells.¹⁵⁷ Hepatocyte and non-parenchymal cell (NPC) isolation was performed by a 2-step collagenase perfusion. The NPC fraction containing oval cells was separated from hepatocytes by low speed centrifugation (500 rpm). The oval cells were further enriched by magnetic activated cell sorting (MACS) using Sca-1 antibody conjugated to magnetic beads.

3.3.3 Oval cell transplantation

Adult female C57BL/6 mice were injected with Monocrotaline (MCT, 50 mg/kg) twice (2 weeks interval) to inhibit the endogenous liver cell proliferation.¹⁵⁸ Two weeks after the second injection the mice were partially hepatectomized (remove 70% of the liver). During the same surgery, oval cells were transplanted into the remaining liver immediately after the partial hepatectomy. The oval cell transplantation into the remaining liver was performed by intrasplenic injection as described by Shafritz and colleagues.^{159,160} Intra-splenic injection has been reported as a safe and reliable method to transplant hepatocytes and/or oval cells. Approximately 2×10^6 cells in 100 μ l saline were slowly injected into the spleen.

3.3.4 Detection of transplanted cell DNA by genomic PCR

Liver tissue was snap frozen in liquid nitrogen and stored in -80 °C. Liver genomic DNA, was purified using Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Approximately 30 ng of DNA sample was used for each PCR reaction. The primers used for detection of GFP gene were 5'-GCC GCT ACC CCG ACC ACA TGA-3' (forward), and 5'-TCGCGCTTCTCGTTGGGGTCTT-3' (reverse). PCR reactions were performed for 30 cycles using standard three-step procedure with annealing temperature of 60°C.

3.3.5 Microscopy and Immunostaining for hAAT and GFP

In order to detect AAT and GFP protein expressions and co-localization, liver tissues were formalin fixed and embedded in paraffin. The tissue was sectioned at 5 μ m. Immunostaining for hAAT were performed using Rabbit anti-alpha-1-Antitrypsin antibody (RDI, Flanders, NJ) at 1:100 dilution. Staining was detected using ABC-Rb-

HRP and DAB kits (Vector Laboratories, Burlingame, CA). Antigen retrieval was performed using Citrate Retrieval for 25 min. at 95°C. Immunostaining of GFP was performed using rabbit anti-GFP antibody (AbCam Ltd, Cambridge, MA) at 1:200 dilution. Staining was detected using ABC-Elite Rb and Vector Red kits (Vector Laboratories, Burlingame, CA). Antigen retrieval was performed using citrate retrieval for 25 min. at 95 °C.

To co-localization of AAT and GFP on the section by immunostaining, 5 µm sections from formalin fixed paraffin embedded blocks were stained sequentially with anti-GFP and anti-AAT. Briefly, slides were deparaffinized, rehydrated and blocked for endogenous peroxidases before the application of anti-GFP at 1:500 (AbCam Ltd, Cambridge, MA). Detection was achieved with the DAKO rabbit Envision+ system employing enhanced DAB (DakoCytomation, Carpinteria, CA). Antigen retrieval was then performed using DAKO citrate retrieval buffer. This not only enabled the anti-AAT to stain, but also served to inactivate the anti-GFP antibody complex residual from the first round of detection. Anti-AAT was applied to the sections at 1:100 (RDI, Flanders, NJ) and detected with rabbit Envision + reagent followed by AEC as the chromagen.

To directly visualize GFP expression, liver tissue was fixed in 10% phosphate buffered formalin overnight and then placed in 18% sucrose at 20 °C for 24 hours. After the sucrose treatment, tissue was embedded in optimal cutting temperature compound (O.C.T.) and frozen in cold 2-Methylbutane (Fisher Scientific). The tissue was sectioned and stained by DAPI (Vectashield with DAPI, Vector Laboratories, Burlingame, CA). All digital images were taken by Olympus BX51 microscope.

3.4 Results

In order to optimize the transduction efficiency of rAAV vectors to liver progenitor cells, rat liver progenitor cells were infected with five serotypes rAAV vectors. All vectors contained an identical genome, rAAV-CB-hAAT and were packaged into each of 5 different AAV serotype capsids (serotype 1, 2, 3, 4 and 5). Supernatant media were assayed for hAAT using a species-specific ELISA. As shown in Figure 3-1, rAAV1 mediated the earliest and highest hAAT secretion (50-fold higher than that with AAV2, 3 and 5), while hAAT secretion with

Next we sought to test the hypothesis that *ex vivo* transduced murine oval cells, with recombinant adeno-associated virus (rAAV), would serve as a platform for expression and secretion of vector-derived hAAT in a transduction/autologous transplant model. The experimental model is described in Figure 3-2A. In this model system, the donor mice were green fluorescent protein (GFP)-expressing transgenic C57Bl/6-derived. In order to stimulate oval cell proliferation, these mice were treated with a DDC (0.1%) containing diet for 4 weeks. Purified oval cells (GFP+) were infected with rAAV1-CB-AT vector, and transplanted into non-GFP C57Bl/6 recipient mice. These recipients were partially hepatectomized to create liver injury thus enhancing the environment for the proliferation of transplanted oval cells. In order to inhibit the endogenous hepatocyte regeneration, the recipients were also pre-treated with monocrotaline (MCT), which will be taken up by liver and subsequently metabolized.¹⁵⁸ All transplanted animals showed definite evidence of engraftment as evidenced by GFP specific genomic PCR and GFP-fluorescence microscopy (Figure 3-2B and 3-2C). The percentage of GFP positive

hepatocytes overall was estimated at 40-50% by the end of the experiment. No GFP expressing cells were observed in bile duct and spleen.

The serum hAAT levels were then measured serially for 18 weeks after the transplantation of rAAV1-CB-hAAT transduced oval cells. A species-specific hAAT ELISA was used to detect hAAT protein from the transgene expression. The C57Bl/6 mice were specifically chosen for this experiment since many previous studies by our laboratory and others have shown that C57Bl6 mice are naturally tolerant to hAAT, which thus provides a non-immunogenic and easily measured secreted reporter protein.

147,161

Serum hAAT levels varied considerably between individual animals, but all animals showed positive indication of sustained transgene expression throughout the study (Figure 3-3). Notably, levels of hAAT remained elevated at plateau levels of up to 1,200 ng/ml of serum for at least 18 weeks after engraftment. Given that the proliferation of the rAAV transduced cells results in the loss of episomal form of AAV genome and decrease of transgene expression, the sustained transgene expression in this experiment may indicate that the vector DNA is integrated.

Detailed examination of the repopulated livers at 12 to 18 weeks after oval cell transplantation revealed that 5 to 10% of total hepatocytes displayed hAAT expression (Figure 3-4). Importantly, some of the hAAT positive cells showed what appeared to be a clonal pattern indicating that these cluster of cells may be derived from one cell with possibly integrated rAAV genome.

To confirm the above results, we also performed immunostaining to co-localize GFP and AAT protein expression with serial sections. As shown in Figure 3-5 A-D, most

of the AAT-positive cells were also GFP-positive. This technique also showed a clustered pattern indicating that AAT-positive cells were derived from donor cells and that had undergone clonal expansion. Some GFP-negative cells were observed even within the positive control liver section from a GFP-transgenic mouse, suggesting uneven expression. However, staining for GFP with the livers of stem cell transplanted-mice showed that 40-50% of cells were GFP-positive. This result was similar to the observation with fluorescent microscopy.

Finally, we have employed an antigen retrieval technique to double label the GFP and hAAT on the same section. Again, uneven gene expressions were observed. Some cells have high hAAT and low GFP expression, while others have high GFP and low hAAT expressions. Although the sensitivity of the technique was not high enough to double label all co-expressing cells, some cells were clearly positive for both GFP and hAAT (Figure 3-5E and 3-5F).

3.5 Discussion

Recombinant AAV vectors may hold considerable promise for human gene therapy, since they are naturally persistent in human cells and have never been associated with any pathology. In particular, there is great interest in hepatic transduction with rAAV vectors since the liver is such a central organ for secretion of serum proteins, for intermediary metabolism, for detoxification of xenobiotics, and for secretion of bile. While *in vivo* gene transfer to the liver may be practical for certain disease states, others may be associated with ongoing liver damage with loss of hepatocyte mass. In this context, a regenerative genetically modified stem cell approach may be a logical

alternative. This report demonstrates the feasibility of one such strategy, in which murine liver-derived stem cells have been stably transduced with a rAAV1-hAAT vector.

There have been several *ex vivo* gene therapy studies that have utilized mature hepatocytes transduced with retroviral vectors, including a clinical trial in patients with familial hypercholesterolemia.¹⁶²⁻¹⁶⁴ This work differs from those studies in two important respects. First, we used rAAV vectors, which previously had only been used for *in vivo* gene transfer in liver. Second, we utilized a cell population which has many surface markers in common with hematopoietic stem cells (HSC), including CD34 and Sca-1.⁶⁸ Interestingly, hematopoietic stem cells have been relatively resistant to rAAV transduction, while oval cell population was readily transducible in these studies.^{153,165} Although HSC and liver progenitor cells share many common markers; liver progenitor cells also express certain hepatic antigens, such as CK19 and AFP, possibly accounting for their greater permissiveness for rAAV infection.

Previous studies showed that the majority of the rAAV genomes persisted as episomal forms in hepatocytes and mediated stable transgene expression after *in vivo* transduction. However, liver injury i.e. partial hepatectomy resulted in a rapid decline in transgene expression levels and a loss of rAAV genomes due to hepatocyte proliferation and regeneration of the liver mass.^{148,166} Surprisingly, results from the present study showed stable and long-term transgene expression from rAAV transduced liver progenitor cells. The stable AAV transduction was further confirmed by the clonal pattern of transduced cells. Understanding the mechanisms of rAAV integration in these cells remains and will lead the improvement of rAAV application.

In the present study, we employed partial hepatectomy (PHX) to create liver injury and MCT to inhibit the endogenous hepatocyte proliferation. Enhancement of proliferation of the transplanted stem cells will be critical for success in clinical gene therapy and remains to be investigated. However, liver injury is commonly seen in diseases targeted for gene therapy, and hepatocytes in such diseases may be impaired in their regenerative capacity. Therefore, the creation of liver injury and inhibition of endogenous hepatocyte proliferation may not be required for proliferation of transplanted stem cells in the clinical context.

We have evaluated GFP positive cells in multiple sections and animals and estimated that approximately 40-50% of donor cells were involved. Partial hepatectomy removed ~60% of the liver. Theoretically, ~60% of donor cells should be in the fully regenerated liver. Several factors may be taken into account for the difference. First, genes are often expressed unevenly across the liver. Second, some of the donor cells became GFP negative after differentiation. It is possible that the CMV promoter used for GFP in some cells was silenced.¹⁶⁷ Third, some endogenous hepatocytes might also regenerate, although monocrotaline (MCT) was used to inhibit hepatocyte regeneration in the recipients. Nevertheless, majority of the donor cells detected. In this study, we infected oval cell for two hours and freshly transplanted all the cells to the recipients without selection or cloning for hAAT expression. Therefore, we did not expect that all the donor cells would be hAAT-expressing cells. Although only 5-10% long term hAAT expressing cells in the liver was observed by immunostaining, to our knowledge, this is the first report of using rAAV vector to infect liver progenitor (oval) cells and to evaluate these cells by transplantation.

Future studies in this area will focus on increasing the percentage of longer-term transduced cell and transgene expression levels by *ex vivo* cloning or selecting transduced stem cells. Additional studies may evaluate a broader range of newly available AAV capsid variants including rAAV8, which may be even more effective for gene delivery in cells of hepatic origin.¹⁶⁸ Ultimately, the availability of the hepatic stem cell population for human studies remains to be seen. Oval cell isolation requires considerable liver injury, which may be problematic in the clinical setting. However, if some analogous population of cells could be isolated from humans, the potential of this technique for therapeutic application could be very great in genetic diseases that affect the liver.

In summary, this study for the first time demonstrated the feasibility of long-term engraftment and stability of transgene expression from genetically modified liver progenitor cells with a rAAV vector and implies a novel gene therapy approach for treatment of liver diseases, such as AAT deficiency.

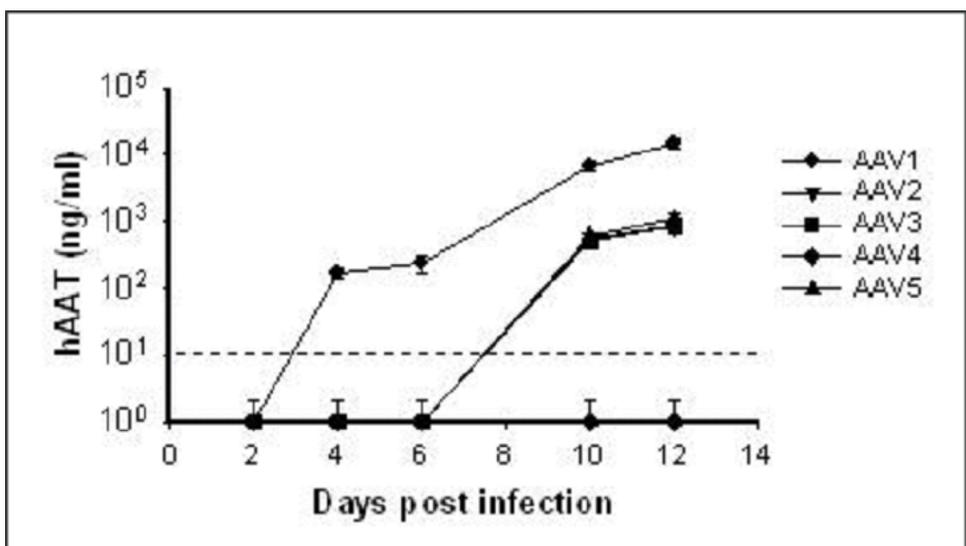


Figure 3-1 Optimization for stem cell transduction efficiency of five serotypes of rAAV-CB-hAAT. Rat oval cell were grown in the 24-well plate (2×10^4 cell/well, $n=3$) and infected with the same dose of each AAV vectors (type 1 to 5, $MOI=3.75 \times 10^4$). hAAT secretion in the culture medium was measured by ELISA. The dashed line indicates the sensitivity of the ELISA

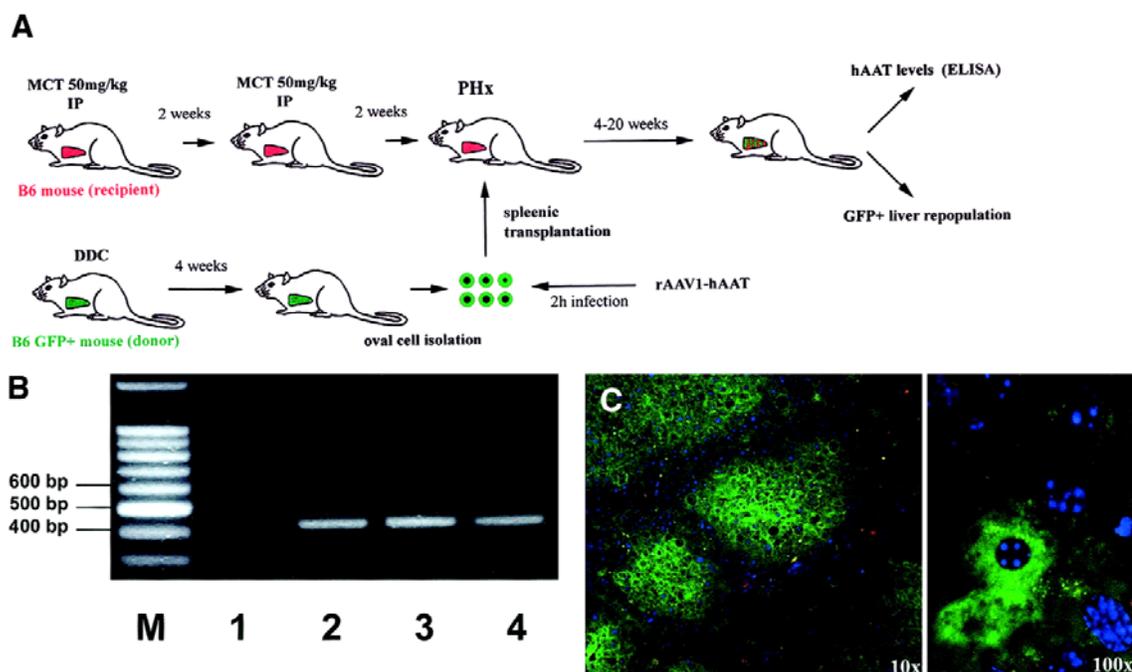


Figure 3-2 Ex vivo transduction and transplantation of oval cells. (A) Outline of the experiment. (B) Detection of transplant DNA by GFP-specific polymerase chain reaction. Lane m, 100-bp marker; lane 1, DNA from non-GFP mouse (recipient) liver; lane 2, DNA from GFP mouse (donor) liver; lane 3, DNA from recipient liver received GFP-oval cell transplantation (20 wk after transplantation); lane 4, DNA from recipient liver received GFP-oval cell transplantation (4 wk after transplantation). (C) Green fluorescent microscopy. Representative images taken from liver sections of transplanted mice at 12 weeks after transplantation. Overall, the percentage of GFP positive (GFP+) hepatocytes was estimated at 40% to 50%.

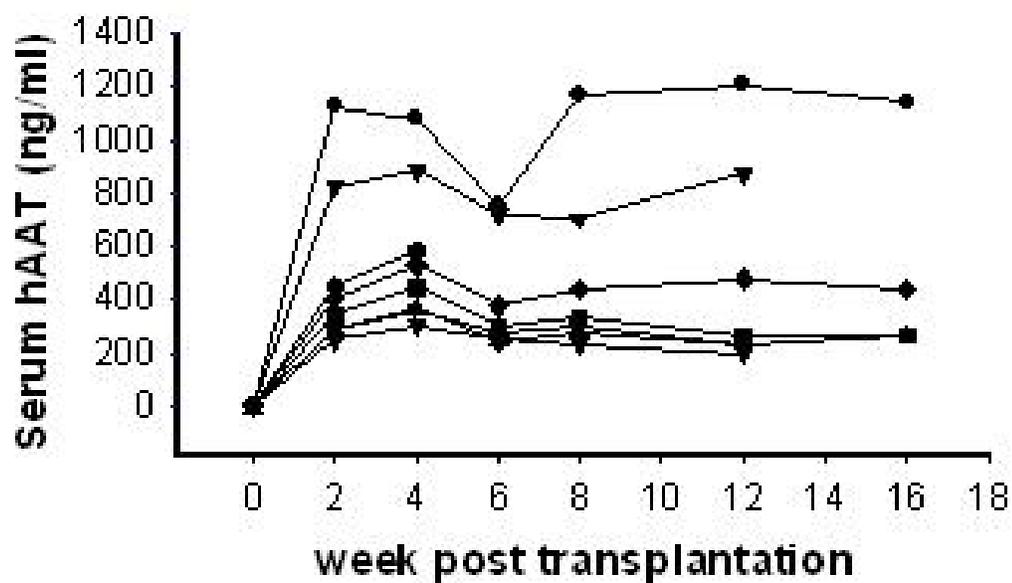


Figure 3-3 Human AAT expressed from engrafted oval cells. The transgene expression was monitored by measuring the serum level of hAAT.

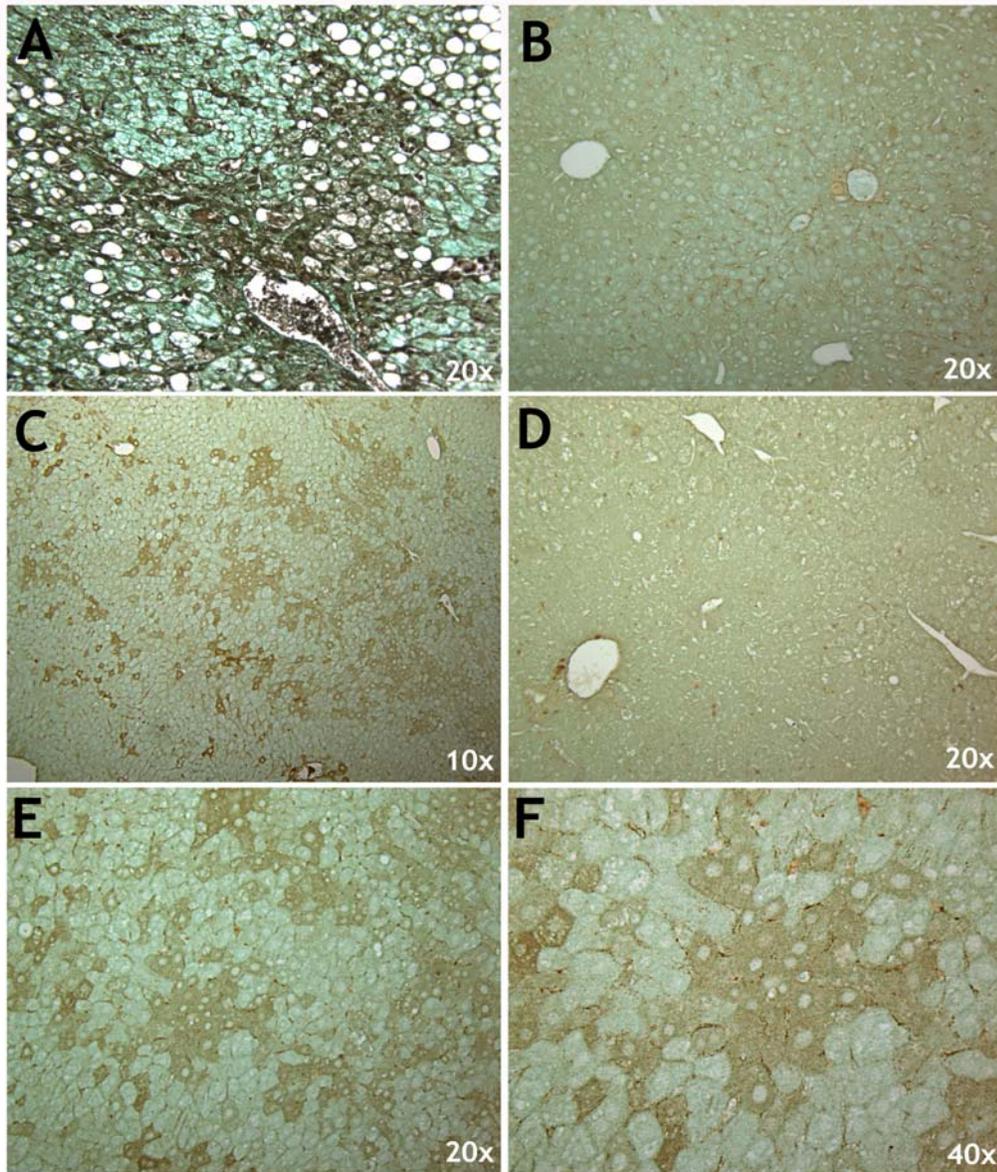


Figure 3-4 Detection of stable transduced cells by immunostaining for hAAT. A, human liver section serves as positive control (brown); B, liver section from an un-transplanted C57BL/6 mouse serves as negative control. C, E, and F are the images from the same liver section of a transplanted mouse liver. D, liver section from the same animal as in C, E and F stained by anti-rabbit IgG serving as a negative control. Note the clonal or clustered pattern of positive cells in C, E and F.

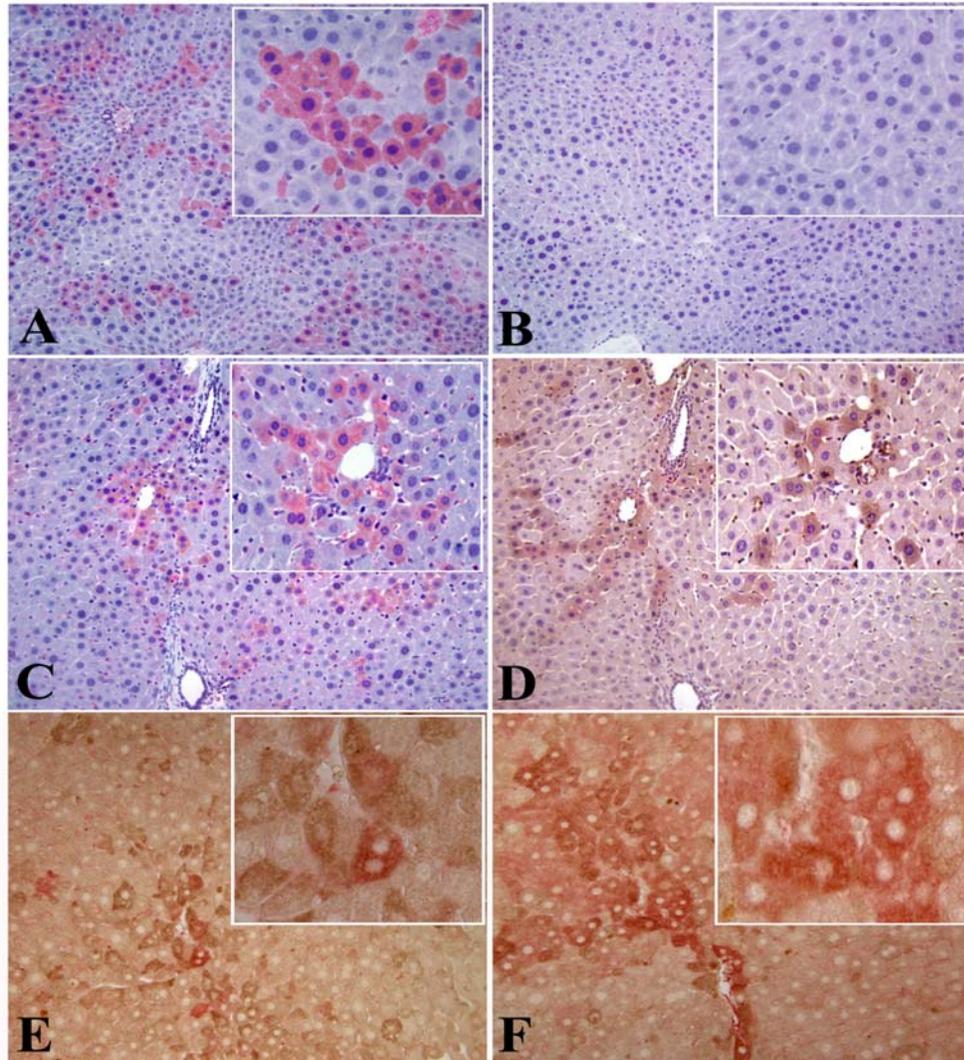


Figure 3-5 Detection of co-expression of hAAT and GFP by immunostaining. A, liver section from GFP transgenic mouse stained for GFP serving as positive control. Note: some cells were GFP-negative suggesting uneven gene expression. B, liver section from C57Bl/6 mouse serving as GFP negative control. C, liver section from oval cell transplanted mouse showing GFP expression. D, liver section adjacent to the section in panel C stained for hAAT. Representative slides were viewed at 20X magnification. The inserts were viewed at 40X magnification. E, and F, transplanted mouse liver was stained for hAAT (brown) and GFP (red) on the same section. The inserts were viewed at 100X magnification. Note, some double stained cells were indicated by arrow.

CHAPTER 4
EX VIVO TRANSDUCED LIVER PROGENITOR CELLS AS TREATMENT FOR
CRIGLER-NAJJAR SYNDROME

4.1 Summary

Crigler Najjar Syndrome (CNS) is a genetic disorder that is characterized by persistent unconjugated hyperbilirubinemia. The disorder is caused by a point mutation in the uridine diphosphate glucuronosyltransferase (UGT) enzyme required for the conjugation and subsequent excretion of bilirubin from the blood. The mutation leads to inactivation of the UGT enzyme causing abnormal accumulation of bilirubin in the blood. Currently, liver transplantation is the best option for treatment of CNS; however, donor organs are limited, and the new liver requires life-long immune suppression.

Due to the above problems, a new treatment for CNS should be devised. In 1999, Petersen et al.⁶⁸ showed that bone marrow cells have the potential to produce hepatic oval cells (liver stem cells) subsequently used in regeneration of the liver after hepatic injury. Applying this information, a CNS treatment that uses liver stem cells and a monocrotaline transplantation protocol, can be envisioned.

Here we show that oval cells can be ex vivo transduced by lentivirus carrying UGT1A1 transgene and subsequently transplanted to allow repopulation of liver. Using this technique, we were able to lower levels of total bilirubin by 50% in Gunn j/j rats, an animal model for CNS.

4.2 Introduction

Crigler-Najjar syndrome (CNS) type 1 is a rare fatal genetic disorder characterized by accumulation of bilirubin in the blood plasma due to UGT1A1 deficiency causing persistent unconjugated hyperbilirubinemia.^{133,134} The deficiency results from a loss-of-function point mutation within the coding gene of that enzyme. The lack of bilirubin glucuronidation leads to inefficient biliary excretion of bilirubin and accumulation of unconjugated bilirubin in plasma.

Single gene mutation in CNS type 1 makes this disease suitable for cell or gene therapy. Initial positive results were reported by Guha et al.³⁶ He successfully treated CNS in an animal model, the Gunn rat, by transplanting normal hepatocytes into recipient animals whose livers were irradiated. Irradiation, as a result, caused arrest of proliferation of resident hepatocytes thus providing replication potential for transplanted cells. In this experiment, treated animals start conjugating bilirubin within a month post treatment. The levels drop to near zero and stabilize 3 months after transplantation. Similar experiment performed by Fox et al.^{18,169} further supported the use of hepatocytes for CNS treatment.

The above technique proved that concept of hepatocyte transplantation, as a treatment for liver metabolic disorders, is feasible. However, the use of hepatocytes in clinical treatments creates some problems. For example, hepatocyte transplantation offers no advantage over liver transplantation, for both treatments require life-long immune suppression. Moreover, clinical application is limited by the availability of human hepatocytes. In addition, specific irradiation of liver is an invasive and complicated procedure that may endanger the live of already ailing patient.

In 1998, Laconi et al.^{29,34,35} developed an efficient method for hepatocyte transplantation in which retrorsine is used as a pre-treatment that inhibits recipient cell mitosis and gives a proliferative advantage to subsequently transplanted hepatocytes. Using his approach, Laconi was able to repopulate 98% of the liver within four months post transplantation. The procedure, which still contains use of hepatocytes, creates a new avenue since liver irradiation is no longer necessary.

Previously we conducted similar experiments with transplantation of hepatocytes and oval cells into monocrotaline treated animals.^{64,158} In those experiments, oval cells were used as an initial step for developing stem cell based gene therapy. With the possibility that oval cell may originate from bone marrow,⁶⁸ those experiments could implicate final use of bone marrow derived stem cells as delivery vehicle for liver gene therapy. Using similar approach, here we describe treatment for CNS that uses *ex vivo* transduced oval cells as a delivery vector of the therapeutic gene to the liver.

4.3 Materials and Methods

4.3.1 Animals

All animals used in experiments, Gunn j/j and Gunn N/N, were purchased from Harlan and used at age of 12 to 14 weeks. During the experiments, they were maintained on standard laboratory chow and daily cycles of alternating 12 hours of light and dark. All animal work was conducted under protocols approved by the University of Florida Animal Care and Usage Committee.

4.3.2 Monocrotaline Treatment

Monocrotaline was purchased from Sigma Aldrich (St. Louis, MO). Solution was prepared as previously described by Witek et al.¹⁵⁸ The standard MCT treatment for all

experiments in rats consisted of 2 intraperitoneal injections of 30mg/kg performed 2 weeks apart. After the final injection, rats were housed for two more weeks before further studies were conducted.

4.3.3 Hepatocyte Isolation

Donor hepatocytes were isolated from male Gunn N/N rats. Hepatocyte isolation was performed using a standard two-step collagenase perfusion. Cell viability was determined to be >80% as established by Trypan blue dye exclusion. After isolation, hepatocytes were resuspended in S&M solution (500mg KCl, 8.3g NaCl, 2.4 HEPES, 190mg NaOH, makes 1 liter, pH 7.4, filter sterilized) at the appropriate concentration to give approximately 1×10^7 viable cells per ml.

4.3.4 Oval Cell Isolation

Gunn N/N male rats were placed on 2AAF/PHx oval cell activation protocol. Nine days post PHx, oval cell isolation was performed using a standard two-step collagenase perfusion. Obtained cells were gradient centrifuged first at 500g and then 1000g. Collected fraction of cells was incubated with Thy1 FITC conjugated antibody (BD, Biosciences) and then with anti-FITC-microbeads (Milteney, Biotec). After incubation, cells were positively selected using MACS sort. Cell viability was determined to be >90% as established by Trypan blue dye exclusion. After isolation, oval cells were resuspended in Iscove's media at the appropriate concentration to give approximately 1×10^7 viable cells per ml.

4.3.5 Cell Transplantation

Transplantation of hepatocytes or oval cells into MCT treated female Gunn j/j rats was performed following a procedure similar to that described by Laconi et al.²⁹ Briefly,

rats were subjected to 2/3 partial hepatectomy (PHx) under general anesthesia, at which time 200 μ l of hepatocyte suspension (approximately 2×10^6 viable cells) was injected into the spleen using a 25 gauge needle. To aid in the coagulation process, an absorbable hemostat (Medical Inc. Arlington, Texas) was applied at the injection site. Post-surgery, animals were placed back in general housing until they were sacrificed in 2-week intervals. From each animal, a sample of liver, lung and kidney was collected and subsequently divided with half being placed in O.C.T. medium for frozen sectioning and the other half placed in 10% phosphate buffered formalin for paraffin embedding.

4.3.6 Virus Preparation and Transduction

Lentiviral particles were prepared according to Chang et al.¹⁷⁰ The titers were estimated at 1×10^9 viral particles per ml. MOI was estimated to be between 100 and 1000. Cells were infected in Iscove's medium for 2h after which they were extensively washed to remove any loose particles of virus. Subsequently cells were transplanted.

4.3.7 Blood Analysis

Blood (250 to 300 μ l) was collected at each time point using simple needle stick from the tail vein. An opaque heparin treated tube was used for blood storage and further processing which included serum separation. Obtained serum was tested for bilirubin content by pathology core labs at University of Florida, Shands hospital.

4.4 Results

The experimental design to test the use of ex vivo transduced oval cells as platform for hyperbilirubinemia treatment consisted of two parts (Figure 4-1). The first part utilized normal male Gunn rat hepatocytes transplanted into MCT pretreated Gunn j/j female animals to establish positive controls as a test for the hypothesis (Figure 4-1A).

The second part consisted of *ex vivo* viral transduction of isolated oval cells from male Gunn j/j, and their subsequent transplantation into Gunn j/j females (Figure 4-1B).

Total bilirubin levels measured in normal animals (Gunn N/N) do not exceed 0.2 mg/dl as compared with jaundiced Gunn j/j rats that averaged at 5.4 mg/dl (Figure 4-2A). In the Gunn j/j animals transplanted with normal hepatocytes, there was no significant drop of total bilirubin until 10 weeks post transplantation (Figure 4-2B). At that time, there was on average a one-fold drop in total bilirubin (Avg. 2.7 mg/dl).

The three-vector system has been utilized in making lentiviral particles (Figure 4-3A). The system consists of using HP cassette containing trans viral proteins that are not packaged within the virus but are necessary for viral production. The second TV vector contains cis elements that are packaged inside the VSV envelope produced from the third expression vector called Envelope. TV vector also contains the transgene of interest with a promoter. For the experiment, a bicistronic transgene expression cassette was designed containing the UGT1A1 gene, and independent eGFP driven from IRES element (Figure 4-3C). The entire cassette expression was under control of CB promoter. The expression cassette was cloned into pTYF-Linker plasmid to create TV vector (Figure 4-3B).

To test whether the UGT1A1 expression cassette was designed properly and is functional, the cassette was cloned into eukaryotic expression vector and used for transfection. NIH3T3 and WB344 cells were transfected with the vector using Lipofectamine 2000. 96 hours later expression of eGFP was detected in both of the cell cultures (Figure 4-4A and 4-4B). Transfection efficiency was determined to be 70% for NIH3T3 cells, and 30% for WB344 cells. After visualization, cells were pelleted and RNA extracted. RT-PCR was performed to test the expression of the UGT1A1 transgene.

Figure 4-5 shows the detection of the transgene in both of the cell lines. An additional construct that used CMV promoter in place of CB was tested with similar results.

Expression of UGT1A1 was not observed in the cell lines or hepatocytes collected from Gunn j/j rats.

After transplantation of lentivirally transduced oval cells, there was no significant change in total bilirubin levels at 16 weeks post transplantation. However, at that time, the direct bilirubin levels increased in treated group of animals (Figure 4-6). Phenotypic observation of transplanted animals revealed that they were healthy and observed levels of jaundice dropped. The untreated animals were characterized by persistent jaundice and increased death rate due to high levels of bilirubin in the blood. Out of four untreated Gunn j/j animals only one (25%) survived 16 weeks duration of the experiment comparing to almost 100% survival in treated animals.

4.5 Discussion

Crigler-Najjar syndrome is genetic disorder, due to its one-gene one-function nature, is a good candidate for gene therapy. Recent advances in hepatocyte transplantation allow liver repopulation by transplanted cells. Unfortunately, hepatocyte transplantation does not offer any advantages over organ transplantation. Here we propose use of hepatic oval cells as a delivery method for the transgene to the liver.

The first part of the experiment tested the proof of a principle that transplanted normal hepatocytes can rescue the Gunn j/j animals by lowering their total bilirubin levels in the blood. The results showed that normal hepatocytes were able to lower bilirubin levels by approximately 50% at 10 weeks post transplantation. The length of time required for bilirubin levels drop was longer than previously reported.^{36,171} This

possibly resulted from use of monocrotaline that may have to be cleared from the liver before normal function of hepatocytes could be fully restored. Also, possibility of inefficient transplantation and animal variations could reduce levels of repopulation thus inefficient conjugation of bilirubin. However, the result shows near one fold decrease of total bilirubin levels in treated animals when compared to untreated group.

The second part of the experiment consisted of *ex vivo* transduced Gunn j/j oval cells with UGT1A1 transgene and their subsequent transplantation into MCT pretreated Gunn j/j rats. Lentivirus used for transduction was tested for expression of its bicistronic construct. Construct used eGFP to aid in identification of transfected cells and possibly to trace transduced cells after their *in vivo* transplantation. Transfection experiments showed expression of eGFP suggesting proper design and function of the construct. Expression of UGT1A1 was tested by RT-PCR. As expected, we observed expression of the transgene in all transduced cell lines. Knowing that our construct was functioning properly, it was packaged into viral particles by Dr. Chang laboratory according to their established procedure.¹⁷⁰

Final experiment tested whether lentivirus can efficiently transduce liver oval cells, and following their transplantation, rescue the treated Gunn j/j animal. After transplantation, the results were monitored by blood collection to test levels of total and direct (conjugated) bilirubin. We detected activity of transgenic UGT1A1 based on levels of direct bilirubin that started to increase 16 weeks post transplantation. At this time all treated animals appeared healthy unlike the untreated rats. Unfortunately, as our data shows, the total bilirubin levels remained high. We hypothesize that this may resulted from inefficient transportation of conjugated bilirubin through the bile canalicular ducts.

It is possible that newly established hepatocytes (that arose from transplanted oval cells) did not form proper connection with existing network of bile canaliculi, and some of conjugated bilirubin enters the blood stream. This may be conformed by looking at normal rat levels of conjugated bilirubin. In these animals bilirubin is quickly conjugated and leaves the blood stream thus levels of conjugated bilirubin in the blood are very low <0.1 mg/dl. Additional support to our hypothesis comes from biliary obstruction were conjugated bilirubin accumulates in the plasma. It may be possible that longer time is necessary for proper canaliculi connection formation. For that, long time experiments are necessary.

Clearly more data is needed to further establish the optimal methods for transduction and transplantation; however, based on obtained results, our data support the concept of using oval cells for transgene delivery to the liver. This technique may not find direct use in human applications, but it may open new possibilities of using adult stem cells for gene delivery.

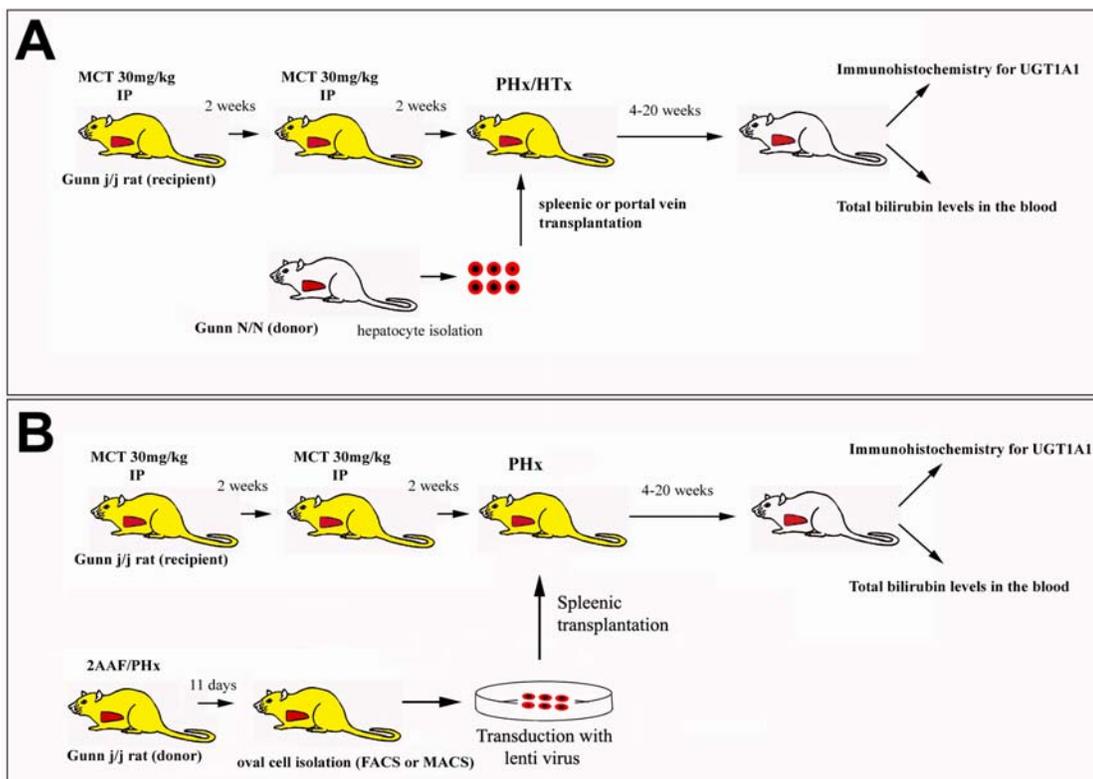


Figure 4-1 Experimental design. A. Hepatocyte transplantation treatment to establish positive controls, B. Ex vivo transduced oval cell transplanted into Gunn rats.

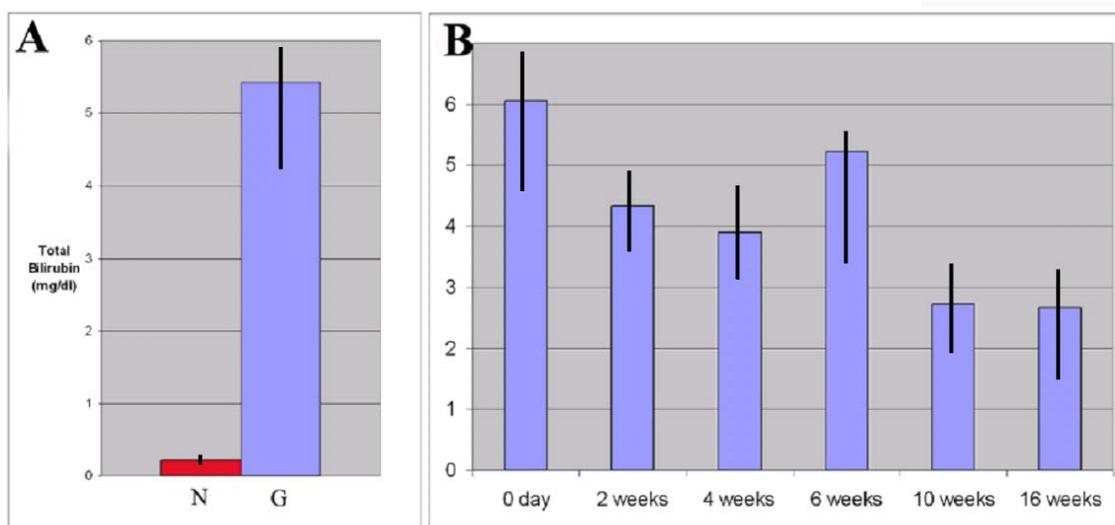


Figure 4-2 Total bilirubin levels after hepatocyte transplantation. A. Average of total bilirubin level measured in normal Gunn N/N (N) and jaundice Gunn j/j (G) rat; B. Levels of total bilirubin in Gunn j/j animals transplanted with Gunn N/N hepatocytes. Note: levels at 16 weeks are approximately 50% lower as compared with initial readings.

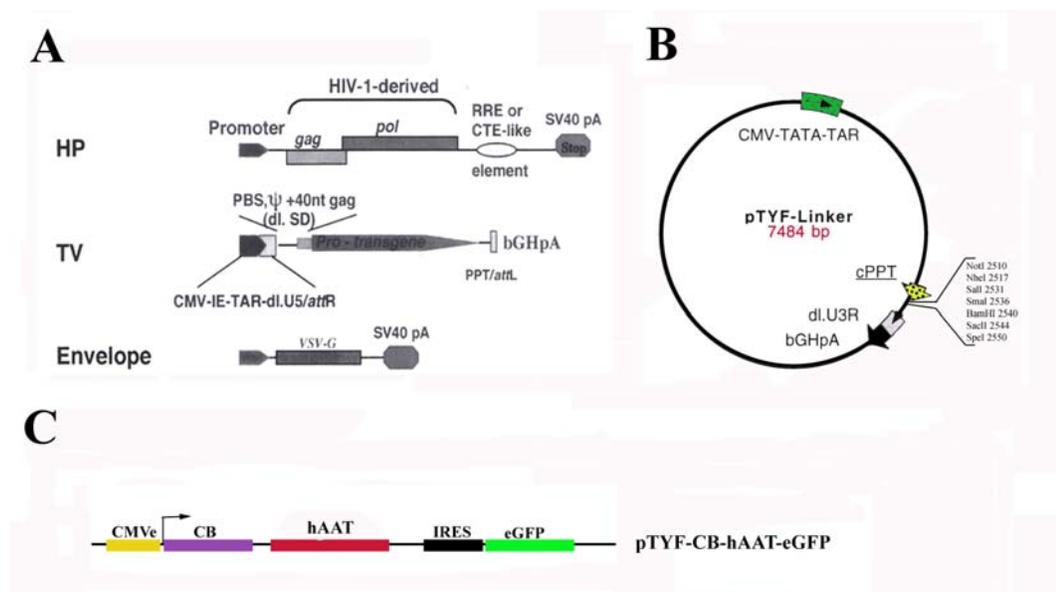


Figure 4-3 Lenti virus derived from HIV-1. A. Three vector system used in making replication defective viral particles. Note. Expression products of plasmid HP are not packaged in the final virus. B. pTYF- Linker plasmid used for cloning UGT1A1 (C) transduction cassette.

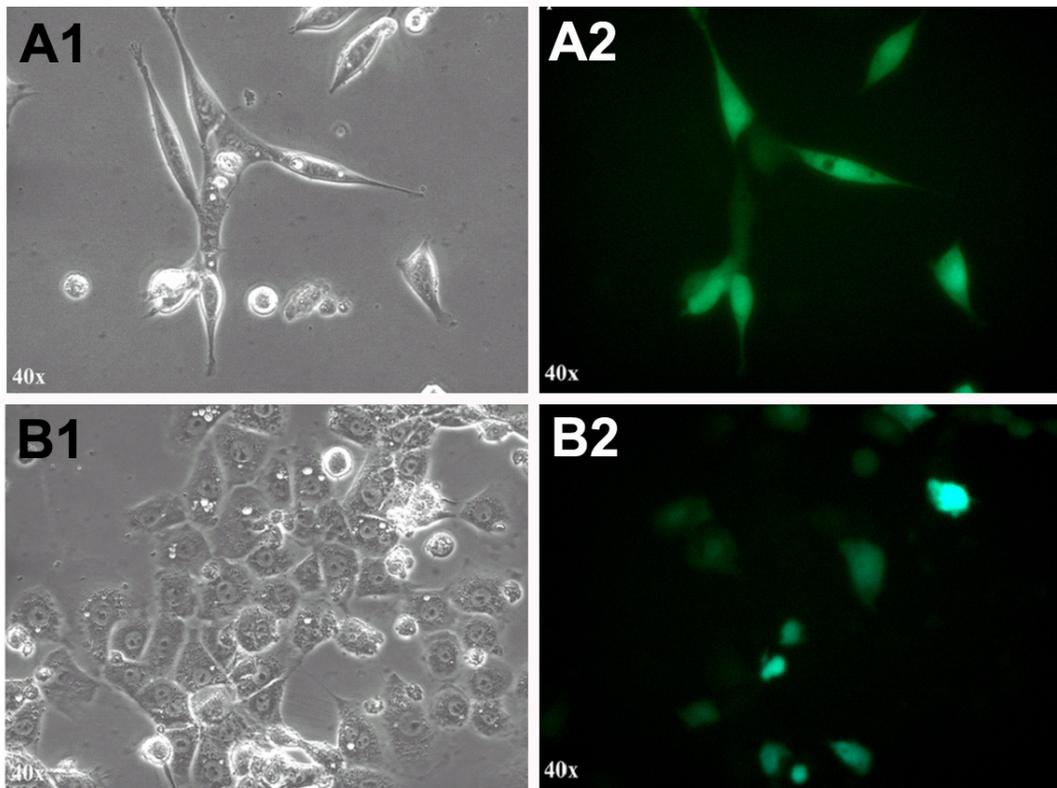


Figure 4-4 Eukaryotic transfection of NIH3T3 (A) and WB344 (B) cells with CB-UGT1A1-IRES-eGFP cassette. Note eGFP expression (green) from transfected cells.

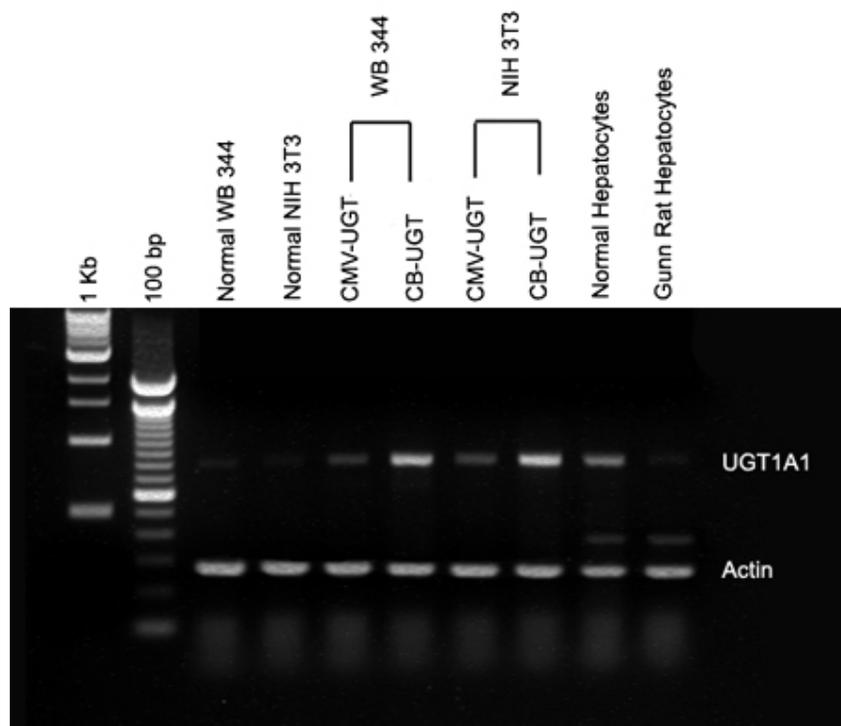


Figure 4-5 RT PCR detection of UGT1A1 expression in transfected cells. Note the expression from transfected cells only and normal hepatocytes. Gunn j/j hepatocytes and un-transfected cell NIH3T3 and WB 344 cells lines lack expression of UGT1A1.

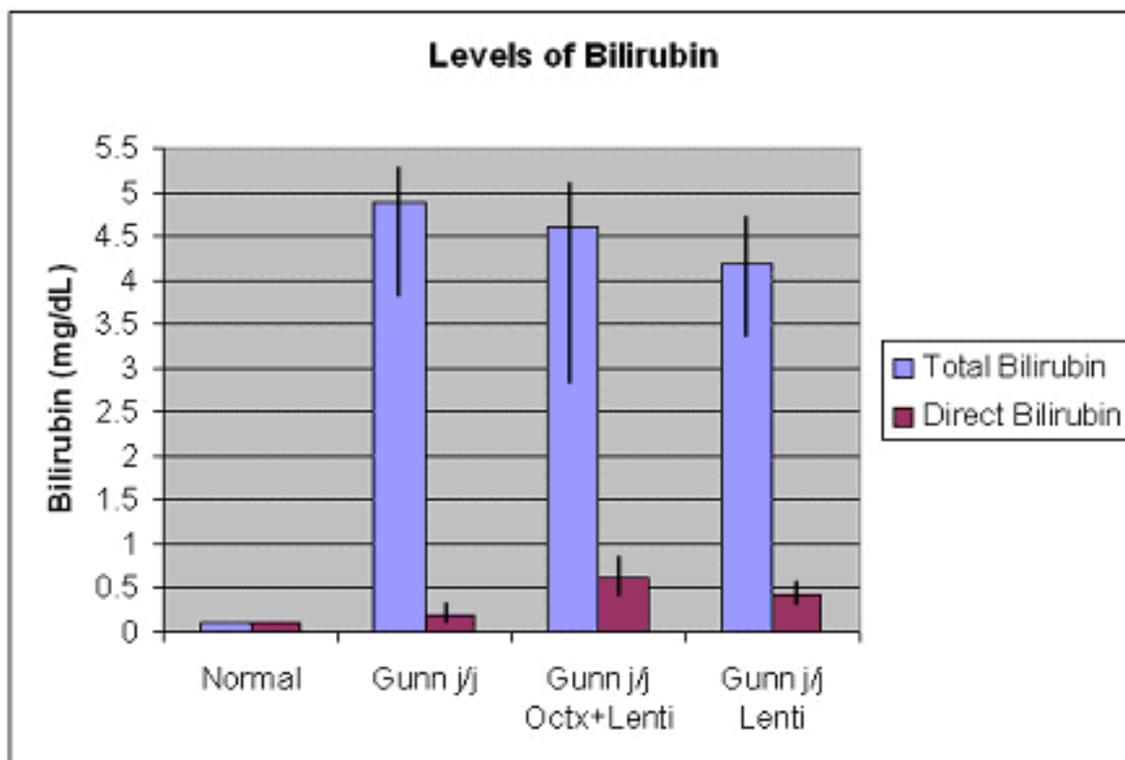


Figure 4-6 Bilirubin levels in rats 16 weeks post transplantation of transduced oval cells. Note increasing levels of direct bilirubin in Gunn j/j animals after transplantation of lentivirally transduced oval cells (Gunn j/j Octx+Lenti) and injection of virus via tail vein (Gunn j/j Lenti) as compared with untreated animal (Gunn j/j). Normal refers to untreated Gunn N/N animal.

CHAPTER 5
MIGRATION OF LIVER PROGENITOR CELLS FROM THE BONE MARROW
DURING THE 2AAF/PHX INDUCED LIVER REGENERATION

5.1 Summary

Recently, several reports pointed to bone marrow (BM) cell ability to differentiate into several tissue types including muscle, heart, liver, pancreas, and lung cells. This led to speculation that BM cells may be the source for the expanding population of adult stem cells found in these organs. Hepatic stem cells, also called oval cells, were reported to follow this pathway. However, a new report suggests that BM cells are not the source of this expanding population of oval cells. To clarify this confusion, we report that utilizing monocrotaline (MCT, a mitotic inhibitory agent) exposure prior to DPPIV⁺ male BM transplantation, an abundance of BM-derived oval cells could be seen proliferating thorough the lobule following 2-acetyl-aminofluorene (2AAF) and partial hepatectomy (PHx) hepatic injury. These cells were isolated via magnetic cell sorting (MACS) and found to express Thy1, OV6, AFP, and CK19 markers known to characterize oval cells. Performing secondary transplant of the BM-derived oval cells into MCT/PHx treated rats, it was found that oval cells continued to differentiate into hepatocytes. The formation of mature hepatocytes appears to be from transdifferentiation and not from fusion based upon X-chromosome in situ hybridization. Finally, we conformed that the expression pattern of CXCR4 and SDF-1 α may play a migratory role in the homing of BM stem.

Taken together, these data clearly shows that a portion of oval cells arise from the BM, and under certain physiological conditions, will differentiate into hepatocytes. This provides an additional source of potentially useful cells in a clinical setting.

5.2 Introduction

In the absence of resident cell proliferation, potential organ regeneration would require the presence of a progenitor cell, which is characterized by the ability to proliferate, self-renewal, and differentiation into cell types of this organ. Reports of these cells have lead to the isolation and purification of hematopoietic stem cells,¹⁷² neural stem cells,¹⁷³ and hepatic oval cells.⁶³ In addition, recent advances has led to speculation that bone marrow (BM) cells could differentiate into muscle,⁷⁴ heart,⁸³ liver,^{38,68} pancreas,⁸⁸ and lung cells,^{174,175} thus creating a pool from which cells could be derived during organ repair or regeneration. In addition, in *in vitro* studies, BM cells have been shown to express hepatic oval cell markers¹⁷⁶ and differentiate into hepatocytes,^{177,178} as well as being able to differentiate into Beta-like cells that produce and secrete insulin.⁹⁰

Hepatic oval cells participate in liver regeneration under certain conditions, and are implicated in hepatic carcinogenesis.^{51,53,179} They are thought to have the ability to clonally expand and posses a bipotential capacity, which allows them to differentiate into both hepatocytes and bile ductular cells. Furthermore, oval cells in culture may be induced to differentiate to pancreatic-like cells.¹⁸⁰ Markers commonly used to assess differentiation and to trace lineages of oval cells include expression of antigenic markers for hepatocytes, bile ducts and oval cells (BSD7, OC2, OC3, OV-1, and OV-6), intermediate filaments, extracellular matrix proteins (CK 19), enzymes and secreted proteins (alpha-fetoprotein, gamma-glutamyl transferase).^{60,181} In addition, it has been

demonstrated that oval cells also express Thy-1, CD-34, c-kit and Flt-3, all of which are known to be hematopoietic stem cells markers^{38,69,77,182} thus suggesting their possible hematopoietic origin.

Studies of hematopoietic stem cells (HSCs) show that they express CXCR4, a receptor for stromal derived factor 1- α (SDF-1 α). CXCR4 has been shown to play a role in the systematic movement of HSCs in the fetal and adult stages of hematopoiesis.^{183,184} This receptor is also expressed by oval cells, and the SDF-1 α is expressed in the liver following 2-acetyl-aminofluorene (2AAF) and partial hepatectomy (PHx), an oval cell activation model.⁷⁸ This supports the possibility that SDF-1 α gradient from the liver plays a role in mobilization of oval cells.⁷⁹

Studies with adult stem cells have showed that they have a capability to differentiate into other specific cell types. The phenomenon of transdifferentiation has been very controversial. Particularly since cell fusion has been reported as an alternative mechanism responsible for cell fate changes.^{185,186} However, many reports support adult stem cell differentiation without fusion.^{88,99,187-189} In addition to cell fusion, reports have stated that the bone marrow progenitors cells are not the source of expanding oval cells in injured livers, adding even more controversy into the adult stem cell research.¹⁹⁰

To shed light on this controversy, the potential of BM cells to become hepatic progenitor cells was examined. The findings of this study shows hepatocytes derived from the bone marrow, as well as hepatic oval cells from the BM source. In addition, BM-derived oval cells were isolated and transplanted into secondary recipients in order to differentiate to hepatocytes. Lastly, we provide evidence that hepatic oval cells might be

migrating from the BM cells via SDF-1 α /CXCR4 interactions, thus playing a role in stem cell driven liver regeneration.

5.3 Material and Methods

5.3.1 Animals

Dipeptidyl Peptidase IV deficient (DPPIV⁻) F344 breeding animals were originally obtained from the Albert Einstein College of Medicine via a generous gift from Dr. Sanjeev Gupta. These animals were in-house bred and maintained on standard laboratory chow and daily cycles of alternating 12 hours of light and dark. They were used at approximately 8-10 weeks of age. Normal male DPPIV⁺ F344 rats (age 8-10 weeks) were purchased from Charles River Laboratories and were used as donor animals. All animal work was conducted under protocols approved by the University of Florida Animal Care and Usage Committee.

5.3.2 Monocrotaline Treatment

Monocrotaline treatment has been conducted accordingly to previously published procedure.¹⁵⁸ Briefly, the initial MCT treatment for rats that were later irradiated and BM transplanted consisted of 2 intraperitoneal injections of 30 and then 10 mg/kg performed 2 weeks apart. The animals that underwent oval cell transplantation were treated using standard MCT protocol that consisted of two injections of 30mg/kg, two weeks apart. After the final injection, rats were housed for two more weeks before further studies were conducted.

5.3.3 Bone Marrow Isolation and Transplantation

DPPIV⁻ F344 female rats (150-170g) were exposed to 900 rads total body γ -irradiation (137 Cesium, JL Shepherd Mark I) administered in 2 doses of 450 rads, 3

hours apart. Bone marrow was isolated from the long bones of DPPIV⁺ F344 male rats. The cells were passed through a 130 μ m cell strainer, collected by centrifugation at 1000g for 5 min, and resuspended in Iscove's media. Subsequently, 5×10^7 freshly isolated BM cells were transplanted via tail vein injection right after second total body irradiation

5.3.4 Oval cell Isolation and Transplantation

Donor oval cells were isolated from female F-344 DPPIV⁻ rats that were MCT treated, BM transplanted and placed on 2AAF/PHx protocol (Figure 5-2B). Isolation was performed using a standard two-step collagenase perfusion.¹⁴¹ Obtained cells were gradient centrifuged at 500g to isolate the larger hepatocytes fraction from the non-parenchymal cell (NPC) fraction, which was later collected at 1000g. The NPC fraction of cells was incubated with Thy1 FITC conjugated antibody and then with anti-FITC-microbeads. After incubation, cells were positively selected using magnetic cell sort (MACS). Cell viability was determined to be >90% as established by Trypan blue dye exclusion. After isolation, oval cells were resuspended in Iscove's media at the appropriate concentration to give approximately 1×10^7 viable cells per mL.

Transplantation of oval cells into MCT treated female DPPIV⁻ rats was performed following a procedure similar to that described by Witek et al.¹⁵⁸ Briefly, rats were subjected to 2/3 partial hepatectomy (PHx) under general anesthesia⁷, at which time 200 μ l of oval cell suspension (approximately 2×10^6 viable cells) was injected into the spleen using a 25 gauge needle. To aid in the coagulation process, an absorbable hemostat (Medical Inc. Arlington, Texas) was applied at the injection site. Post-surgery, animals were placed back in general housing until they were sacrificed in 2-week

intervals. From each animal, a sample of liver, spleen, lung and kidney was collected and subsequently placed in O.C.T. medium for frozen sectioning.

5.3.5 Immunohistochemistry and Enzyme Assay

All histochemical stainings were performed according to previously described protocols. DPPIV staining procedure was performed as described by Dabeva et al.³² Immunostaining for Thy1 (CD90), CK19, CD45, AFP, and OV6 was performed on cytopspined oval cells and frozen sections using standard staining protocol. Briefly, slides were avidin/biotin blocked after which they were incubated with 1^o Ab for 1hr. 2^o Ab followed for 30 min. Detection was performed using Vector ABC kit (Vector Laboratories, Burlingame, CA) and DAB reagent (DakoCytomation, Carpinteria, CA).

5.3.6 Analysis by DNA-PCR and RT-PCR

PCR analysis for the Y chromosome was performed on DNA extracted from transplanted female animals using primers for the SRY gene. For the RT-PCR analysis, total RNA was isolated from the BM cells, hepatocytes, and BM-derived NPCs by RNeasy kit (Qiagen, Valencia, CA). Total of 2 µg RNA was used for each cDNA synthesis. Reverse transcriptase polymerase chain reactions were performed as previously described by Oh et al.¹⁷⁷ Following primers for SDF-1 α were used: 5'-ATG GAC GCC AAG GTC GTC G -3' (sense strand), 5'-CCA CGG AGG TCA GCC TTC CT-3' (antisense strand), which delineated a 333-bp product. For the CXCR-4 primers included 5'-TTC TCA TCC TGG CCT TCA TC-3' (sense strand) and 5'-GGA ACT GGA ACA CCA CCA TC-3' (antisense strand), both of which produced also a 333-bp product. The resulting RT-PCR products were amplified and subjected to electrophoresis in 1.5% agarose gel and stained with ethidium bromide. The purified PCR products were directly

sequenced using an AmpliTaq cycle sequencing kit (Perkin-Elmer Setus, Branchburg, NJ) for genetic confirmation.

5.3.7 In Situ Hybridization

Cytospin slides were fixed for 15 min. each in 4% paraformaldehyde. The rat X-chromosome probes were designed using X-specific sequence of rat amelogenin gene flanked by following sequences: 5'-ACA CCC TTC AGC CTC ATC A-3' (sense), and 5'-GAG AAC AGT GGA GGC AGA G -3'. Final probes were DIG labeled (Roche, Indianapolis, IN), denaturated at 80°C for 5 min and applied to sections at 52°C. The sections were coverslipped and sealed with rubber cement for incubation overnight in a hydrated slide box at 52°C. The next day, the coverslips were carefully removed in preheated 2x SSC buffer, pH 7.0, at 65°C. The sections were washed twice in preheated 50% formamide in 5x SSC buffer for 5 min each at room temperature and were then gently washed twice in preheated 0.1xSSC buffer for 5 min each at 65°C. Color development was performed at room temperature in buffer (Tris 100 mM, NaCl 100 mM and MgCl₂ 50 mM, pH 9.5) containing NBT and BCIP (Roche). Sections were counterstained with nuclear fast red (Vector Lab, Burlingame, CA) and mounted in Cytoseal (Richard-Allan Sci. MI).

5.4 Results

5.4.1 Characterization of Oval Cells

In the standard 2AAF/PHx oval cell activation model, described by Thorgeirsson and coworkers,^{53,54} there is massive proliferation of oval cells in the periportal region (Figure 5-1A and 5-1B). H&E staining of oval cells appear as dark blue patches of cells due to their large nuclei and small amount of cytoplasm surrounding it. Oval cells can be

further characterized by labeling with OV6 and CK19 bile ductular markers (Figure 5-1C and 5-1D). Additional immuno-marker widely utilized in oval cell labeling, Thy1, can be used for their isolation. Thy1, a hematopoietic marker, antibody in conjunction with magnetic cell sorting can isolate oval cells with high purity averaging 3×10^6 cells per animal (days 9-11 post PHx). Cytospins of isolated oval cells show over 95% cells staining for OV6 and Ck19 (Figure 5-1E and 5-1F). These results confirm that 2AAF/PHx model can be effectively used for activation of oval cells in periportal regions during the liver regeneration, and that MACS-Thy1 sorting is an efficient mean of isolating oval cells.

In order to test the hypothesis that oval cells originate from bone marrow, an experimental model was developed (Figure 5-2). In the first stage of the experiment all animals were treated with MCT and then transplanted with BM cells. The second stage consisted of two parts. First part used liver damage by 2/3 PHx alone (Figure 5-2A), and the second part, 2/3 PHx in conjunction with 2AAF (Figure 5-2B). The third stage of experiment utilized secondary transplantation of isolated oval cells from stage two 2AAF/PHx treated animals (Figure 5-2C).

5.4.2 Induced hepatocytes and Oval Cell From BM-Transplanted Rats

MCT pre-treated female DPPIV⁻ rats were lethally irradiated and rescued with whole BM cells from a DPPIV⁺ male rat. Nucleated blood cells of these animals were tested by DNA-PCR to establish that BMTx was successful.¹⁷⁷ Figure 5-3A shows the presence of SRY band in all animals transplanted with male BM.

In the second stage of the experiment, the recipient animals underwent 2/3 PHx followed by 4 weeks of recovery period. At that time, liver tissue analysis for DPPIV

marker revealed scattered patches of DPPIV⁺ hepatocytes (Figure 5-3B,C, and D). The size of patches ranged from single to multiple cells as shown in Figure 5-3E. In addition to DPPIV bile canalicular membrane staining that characterizes adult hepatocytes, the same sections contained cells with diffused cytoplasmic staining (Figure 5-3B white arrows).

5.4.3 Hepatic Oval Cells From the BM

The second group of female recipient rats in stage two of the experiment was treated with 2AAF/PHx to activate oval cell proliferation. DPPIV analysis of tissue collected 3 to 12 weeks post PHx revealed normal oval cell activation with DPPIV⁺ cell mixed in the liver lobule (Figure 5-4A). Additional staining of serial section showed that DPPIV⁺ cells also label for OV6 (Figure 5-4B). Furthermore, double immunohistochemistry for DPPIV (green) and OV6 (red) was performed to verify these cells as donor derived oval cells (Figure 5-4C and 5-4D). As described previously, Thy-1 is immuno-marker readily utilized as sorting tool for oval cells. Using Thy1 Ab and MACS, oval cells were isolated from these animals and cytopspined for analysis. Figure 5-4E-H, show that approximately 50% isolated cells stained for DPPIV, 84% for AFP, 89% for CK19, and 100% for OV6. These results indicate that the donor oval cells in this model are proliferating and carry the normal markers characteristic of resident oval cells.

5.4.4 BM-Derived Hepatic Oval Cells Transplanted Into MCT/PHx Rat Model

Thy1/MACS isolated oval cells (male BM derived DPPIV⁺) were used for transplantation to female DPPIV⁻ rats that were pre-treated with monocrotaline at 2x30mg/kg (Figure 5-2C). 8 weeks post transplantation liver and spleen tissue were collected and analyzed. PCR for the SRY gene showed presence of donor derived cells in

the liver but not in the spleen (Figure 5-5B). DPPIV staining on liver sections revealed scattered small patches of hepatocytes (Figure 5-5 A1-A5, C-H). In most of the cases, DPPIV⁺ hepatocytes were proliferating as shown on representative serial section of a patch (Figure 5-5 A1-A5). As previously reported,²⁹ each patch of DPPIV⁺ cells is clonally derived from a single cell that was localized in the liver during cell transplantation. In addition, we examined Y chromosome expression in recipient livers (Figure 5-5B) We found that transplanted animals with BM-derived hepatic oval cells were expressing Y chromosome SRY gene.

5.4.5 X-chromosome and SDF-1/CXCR-4 in BM-Derived Oval Cells

The X-chromosome expression of BM-derived hepatic oval cells was assessed by *in situ* hybridization. Figure 5-6A and B shows X-chromosome in the nucleus of isolated hepatocytes and BM-oval cells. Approximately 55% of isolated BM-derived oval cells contain one X-chromosome suggesting 1:1 ratio with Y chromosome. These results support DPPIV staining of the same cells that showed about 50% of donor-derived cells (Figure 5-4E-H). Furthermore, we examined SDF-1 and CXCR4 expression in the BM cells, hepatocytes, and BM-derived NPCs. BM cells and BM-derived NPCs were expressing CXCR4, SDF-1 receptor, also hepatocytes expressed SDF-1.

5.5 Discussion

The origin of hepatic oval cells has been debated since their discovery in 1956. Initially they were thought to originate within canals of Hering, but within the last few years the possibility of their bone marrow origin began to surface. Petersen et al.⁶⁸ reported the bone marrow as a potential source of hepatic oval cells. Recently, this data has been questioned by Menthenet et al.¹⁹⁰ who claim that bone marrow progenitors are

not the source of expanding oval cells in injured livers. The data was based on three different hepatic injury models that utilized D-Galactosamine, retrorsine, and 2AAF/PHx to activate oval cell compartment. In their study, they failed to observe any homing of oval cells from bone marrow to the liver. However, the data presented within the current study, shows that their conclusion may not be entirely correct. In the current study, using a combination of monocrotaline treatment along with bone marrow transplantation followed by oval cell activation by 2AAF/PHx, an abundance of oval cells expressing bone marrow markers was observed.

In order to recruit oval cells from bone marrow two requirements need to be fulfilled. First, the liver injury has to be sufficient to activate the oval cell compartment. The 2AAF/PHx induces liver injury that is known to produce a high number of oval cells at days 9 to 11 post partial hepatectomy^{53,54,191} and fulfills this requirement. The second requirement is inhibition of proliferation of all resident cell types that could influence liver regeneration. Monocrotaline and retrorsine, pyrrolizidine alkaloids, fulfill this requirement. In general, pyrrolizidine alkaloids (PA) have been reported to possess potent anti-mitotic and hepatotoxic activity.^{30,31} They are metabolically converted by the P450 system to produce reactive, highly toxic dehydroalkaloid (DHA) intermediates that form adducts with DNA resulting in mitotic arrest in rapidly dividing cells. However, since P450 is not only restricted to the hepatocytes, PA's inhibit proliferation of other cell types including pulmonary and bone marrow cells.¹⁹²

In the current experiments, monocrotaline was injected prior to the bone marrow transplantation in order to minimize its activity on transplanted cells (Figure 5-2). This timing is very critical because this will give a growth advantage to the donor cells. The

difference between the study of Menthenal et al.¹⁹⁰ and the current study is the timing of PA administration. The Menthenal study had their PA dosing after BMTx while the current study had PA dosing prior to BMTx. This observation may explain their inability to find any bone marrow derived oval cells in retrorsine-injured livers. As current results show, by giving monocrotaline injection prior to BMTx, an abundance of bone marrow derived oval cells or hepatocytes can be observed (Figure 5-3 and 5-4).

In addition, secondary transplant of isolated BM derived oval cells showed that these cells are indeed oval cells; they retain their donor characteristics (DPPIV and SRY), and were able to differentiate to hepatocytes (Figure 5-5). The three dimensional growth of transplanted cells supports their ability for clonal expansion, which may be hindered if the BM derived cells fused with MCT-affected hepatocyte.

The final question to what is the origin of hepatic oval cells could be answered by looking at the phenotypical characteristics and the possibility that the oval cell population is heterogeneous.^{193,194} This would suggest that there are two populations of oval cells. One population of cells is endogenously present in the liver and can be located in the canals of Hering.^{66,67} Since, the liver has been functioning as a hematopoietic organ during the first stages of development, it is possible that some of the stem cells become quiescent and are retained in the liver during its progression to maturity. Hence the presence of hematopoietic markers on isolated oval cells. This population of cells would arise if hepatocyte proliferation was compromised (2AAF/PHx treatment) and would be sufficient to allow liver regeneration. Other oval cell activation methods (D-Galactosamine) would produce similar results since they only affect hepatocyte division but have no effect on liver resident oval cells. However, if for some reason the activation

of resident oval cells was blocked, oval cells would home to the liver from the bone marrow, as a second population of oval cells. Under this condition, bone marrow derived oval cells would allow liver regeneration without the influence of resident oval cells.

To assess whether BM cells become oval cells we looked for fusion events. We hypothesized that looking at the distribution of the X-chromosomes would be sufficient to answer the question of fusion. Normal recipient cells contain two X-chromosomes per cell (females) as shown in Figure 5-6A, and male donor cells contain only one X-chromosome (Figure 5-6B). If fusion of these two cells occurred, then cells that have three or more X-chromosomes would be observed. Due to the fact that hepatocytes show different ploidy (2N, 4N, 8N, and 16N), any odd counts of X-chromosome number would represent fusion. Based on data collected, there was not a significant number of cells that would contain fusion genotype (3 cells out of 1400 cell counted, Figure 5-6D) thus BM-derived hepatocytes were coming from transdifferentiation rather than fusion. Additionally, the fact that resident cells were under the influence of monocrotaline, fusion with donor cells would render the newly established cells unable to replicate. In current study, however, the cells are growing in 3D patches supporting their ability to proliferate.

In order for BM cells to become hepatic oval cells in the liver, they have to have homing capability. Our additional studies focused on this property. We found that BM cells and BM-derived hepatic oval cells express CXCR4, a receptor for SDF-1a (Figure 5-6D). Previously reported, this receptor is also expressed by HSC. In addition, SDF-1 is expressed in the liver following 2-acetyl-aminofluorene (2AAF) and partial hepatectomy (PHx), an oval cell activation model. Because SDF-1/CXCR4 are expressed in oval cell

activation, it is possible that an SDF-1 gradient from the liver plays a role in mobilization of bone marrow stem cells (BMSC) to the circulation with homing effect to the injured liver.⁷⁸ Based on this information, it is possible that oval cells, or at least a small population of oval cells, could originate in the BM cells.

Clearly, more data is needed to show whether all oval cells originate from BM; however, the findings of this study suggest that a population of oval cells could represent progeny of BM derived stem cells. Efforts should be made to isolate a highly enriched fraction of BM derived oval cells from the liver and characterize them more closely. A better understanding of the BM to liver pathway could lead to development of clinically useful protocols for the treatment of hepatic disorders.

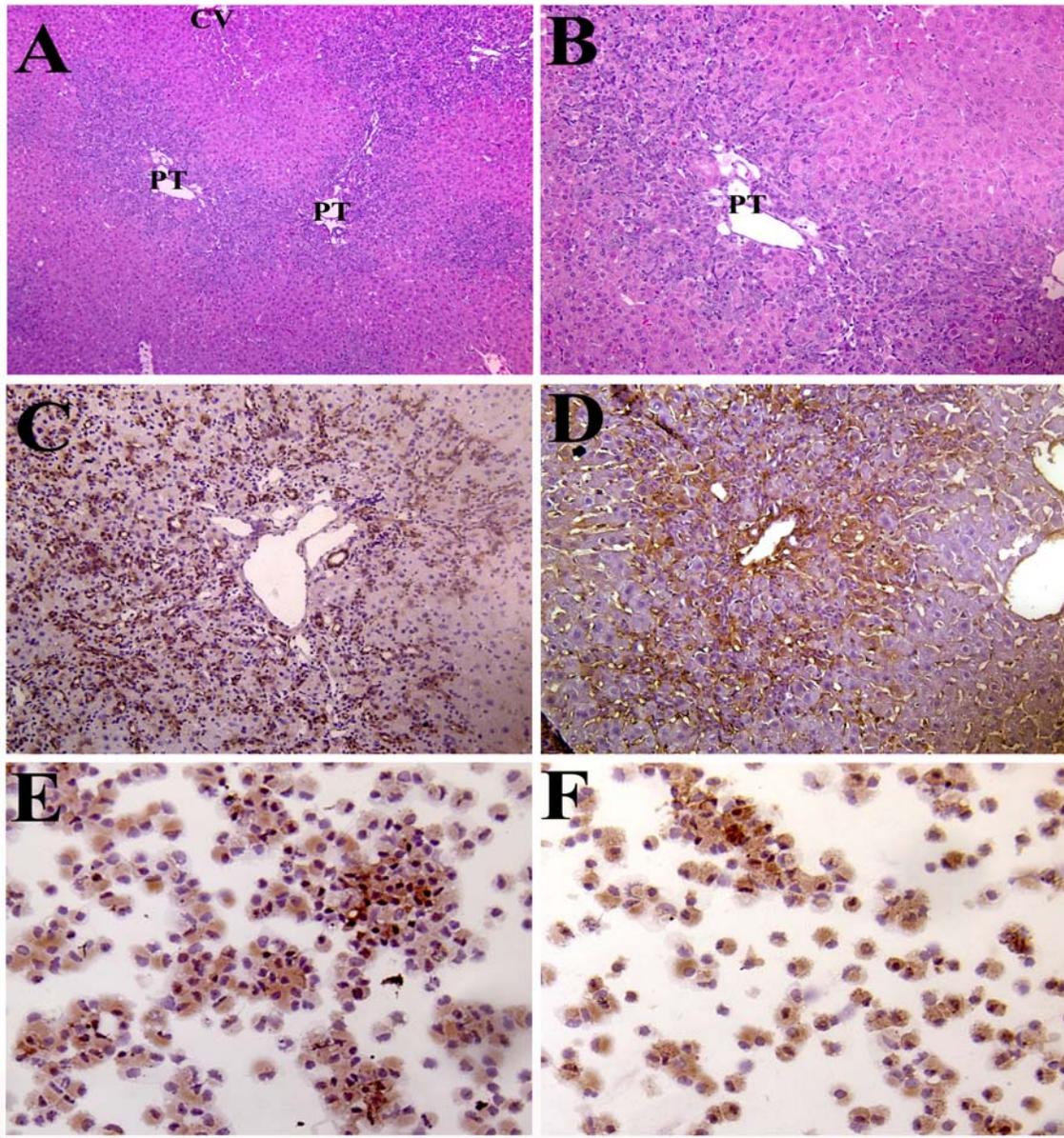


Figure 5-1 Analysis and Oval cell isolation from 2AAF/PHx liver tissue. The liver tissues collected at day 8 after 2AAF/PHx. A and B show H&E at 100 and 200x respectively. Note large patches in the periportal regions stained dark blue that represent activated oval cells (CV-central vein; PT-portal triads). Oval cells were stained with OV6 (C) and CK19 (D), and cytopins of oval cells isolated via Thy1 Ab stained for OV6 (E) and CK19 (F).

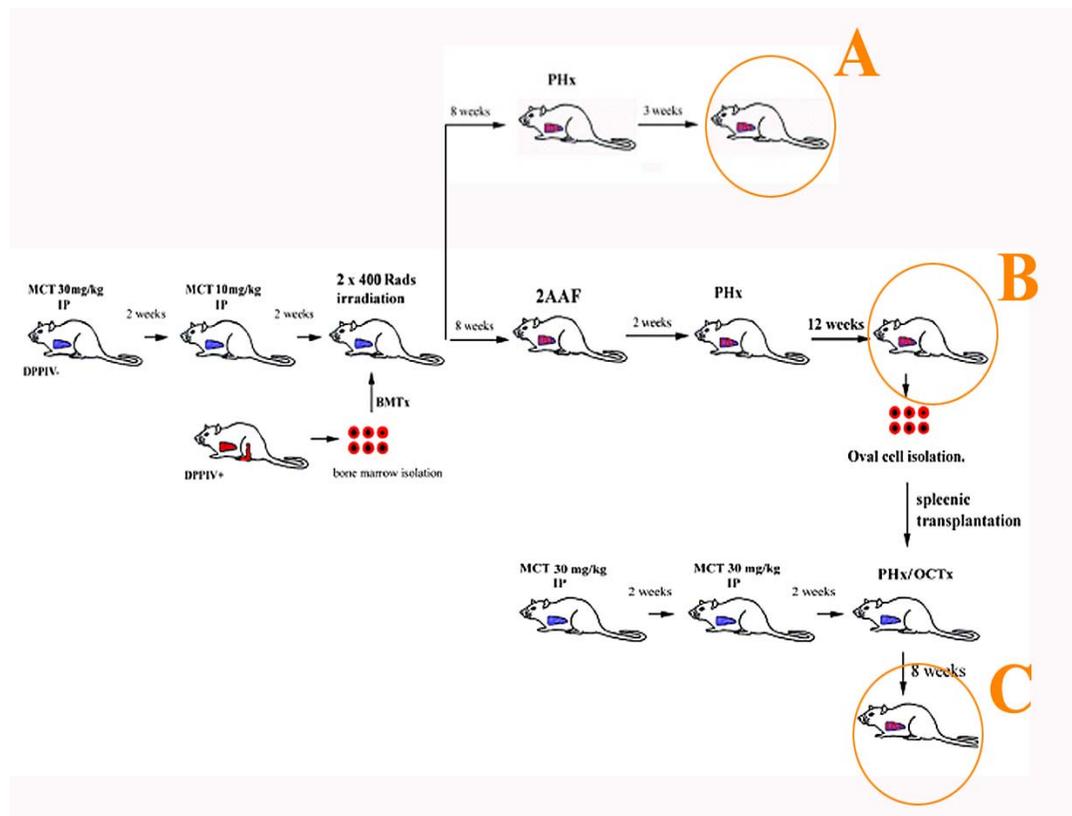


Figure 5-2 Experimental design. Letters designate tissue collection points. A. BM derived hepatocytes; B. BM derived oval cells; C. BM-derived oval cells transplanted into secondary animals.

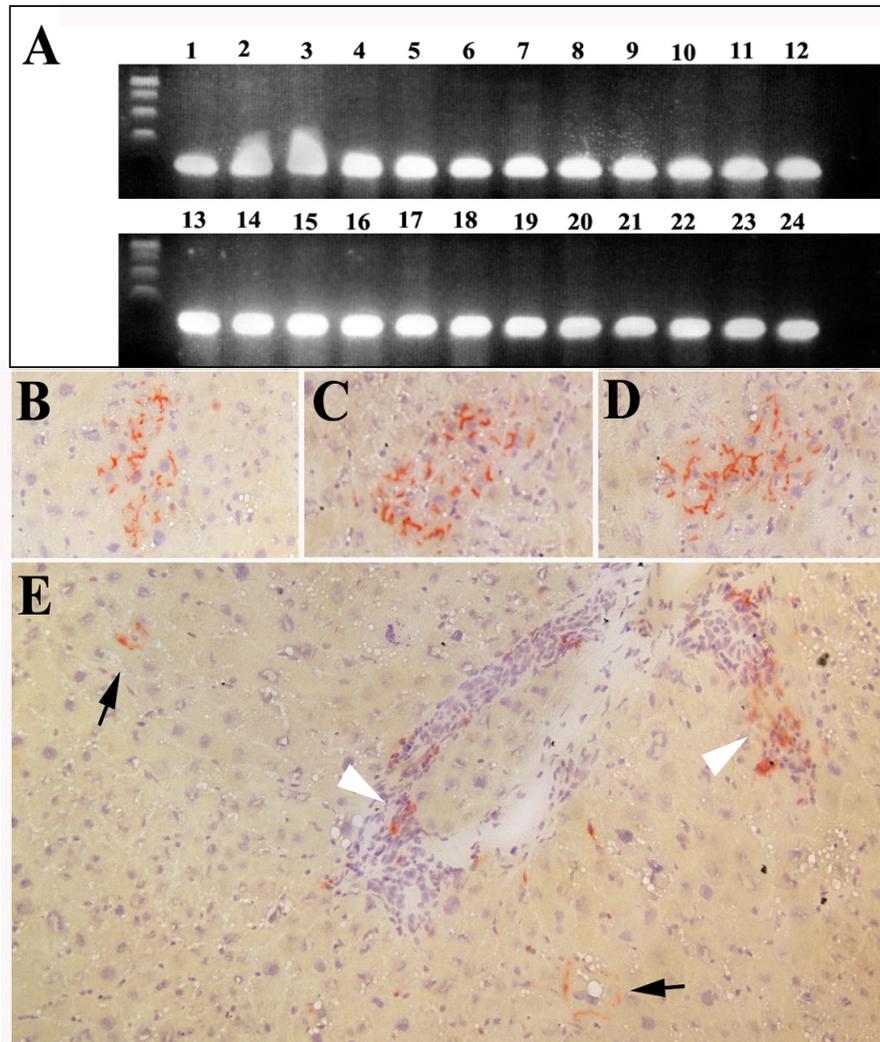


Figure 5-3 Y chromosome detection and analysis of BM-derived hepatocytes. A. SRY gene PCR amplification to verify BMTx. Numbers represent animals used in the experiment; B, C, D. Representative groups of BM derived hepatocytes found in PHx BMTx-ed animals (DPPIV staining); E. A representative section from the same animals showing the presence of scattered DPPIV⁺ hepatocytes (black arrows) and small DPPIV⁺ cells appearing in the portal region (white arrows).

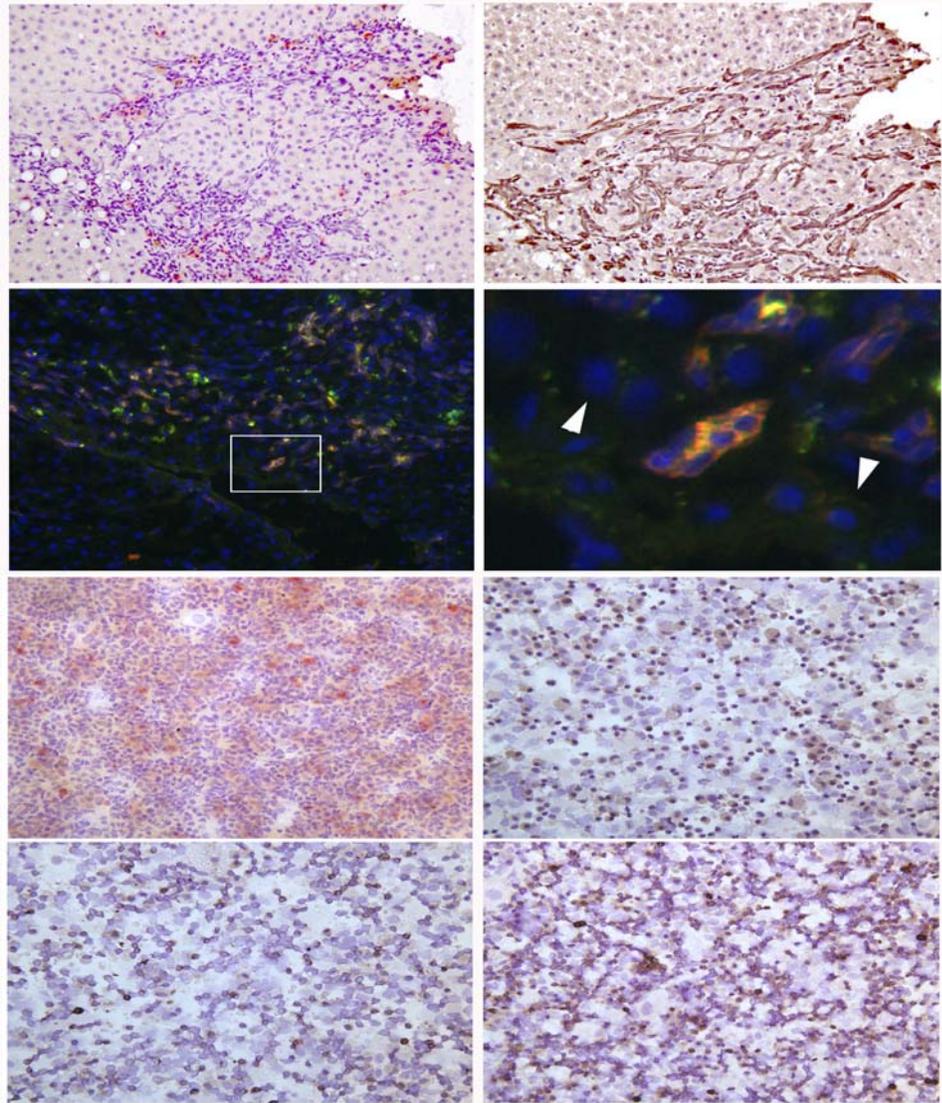


Figure 5-4 Bone marrow derived oval cells. A. DPPIV⁺ (CD26) and B. OV6⁺ serial sections showing overlapping of both of the stains; C. Double immunohistochemistry for DPPIV (green) and OV6 (red). Overlapping results in yellow color visible in higher magnification D. Note size of the hepatocyte nuclei, indicated by white arrows, as compared with stained cells. Isolated by Thy1 oval cells were cytopsined and stained for E. DPPIV; F. AFP; G. CK19; H. OV6.

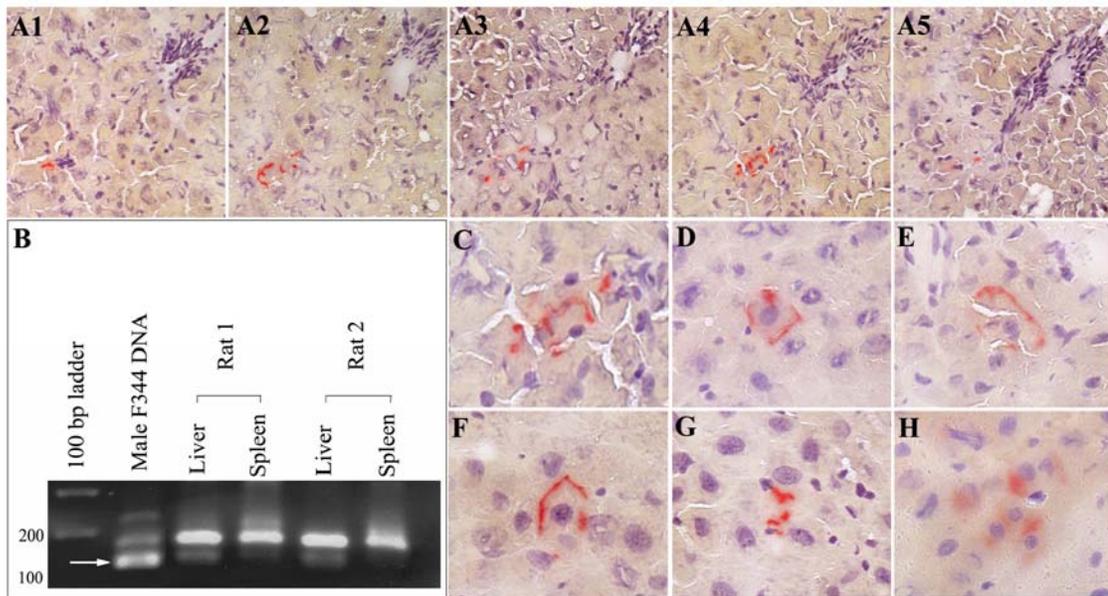


Figure 5-5 Secondary transplant of BM-derived oval cells. A1-A5. Serial sections of representative group of hepatocytes found in transplanted animals. Note that the cells are growing in all directions showing clonal expansion of transplanted cells (DPPIV staining); B. SRY gene PCR amplification showing presence of male markers in representative livers of transplanted animals; C-H. Representative groups of hepatocytes found in the transplanted animals.

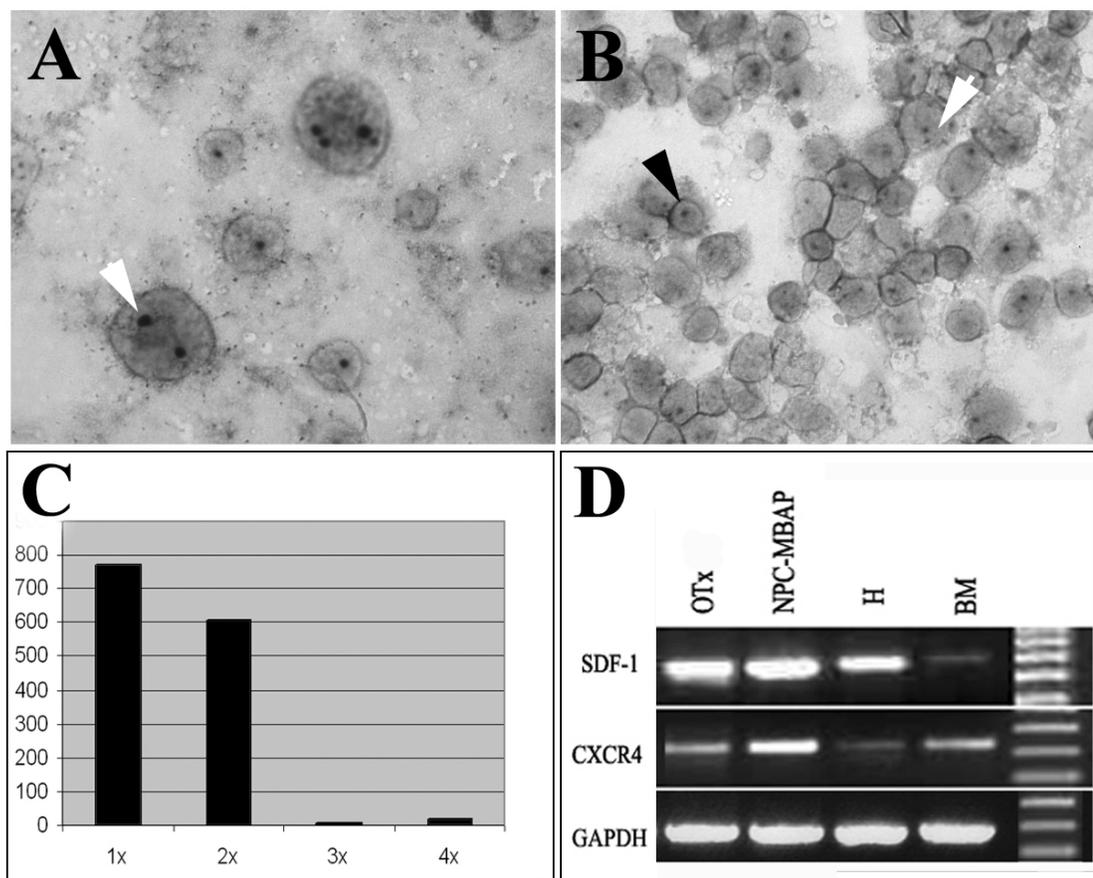


Figure 5-6 In situ hybridization of the X-chromosome and expression of SDF-1/CXCR4 on BM-derived hepatic oval cells. ISH analysis of X-chromosome in BM-derived hepatic oval cells. Hepatocytes isolated from the BM-cells transplanted animal (A), and BM-derived NPC cells (B). C. X-chromosome counts in BM-derived NPC cells. D. RT-PCR analysis of SDF-1/CXCR4 gene in BM cells, hepatocytes, BM-derived NPC cells, and BM-derived NPC cells transplanted liver.

CHAPTER 6
CONCLUSION: CAN CELL TRANSPLANTATION AND GENE THERAPY BE
USED FOR TREATMENT OF LIVER METABOLIC DISORDERS?

Transduced stem cells are becoming a promising new technology that could be utilized in treatment of many liver metabolic disorders. In this work, we show that by preconditioning liver with proliferation inhibitor, transplanted cells can repopulate the liver and influence its metabolic activity. We hypothesized that a novel treatment for liver metabolic disorders can be envisaged by using adult stem cells as carriers for transgene in combined transplantation and gene therapy. To test this hypothesis, we developed a transplantation model, used this model to proof the principle of the treatment for two different liver disorders, and finally showed that bone marrow cells are the origin of hepatic adult stem cells thus they are the best candidates for transplantation-gene therapy.

First, our work describes use of monocrotaline, a pyrrolizidine alkaloid, as proliferation inhibitory agent which used in its optimal dose of two IP injections of 30mg/kg two weeks apart, allows transplanted hepatocytes to repopulate rat liver in up to 20%, six weeks post transplantation. We also showed that transplanted hepatocytes retained their normal physiological behavior by expressing DPPIV, Glucose-6-phosphatase, and storing glycogen. In addition, we showed that oval cells could be transplanted with similar efficiency as hepatocytes and become functional hepatocytes. This model works sufficiently to inhibit proliferation of resident hepatocytes needed for cell transplantation in rats and in mice.

To test our transplantation model using monocrotaline, in following experiments, we used oval cells as a gene-carrying vector after their transduction *ex vivo* by AAV and Lentivirus to treat AAT and CNS. To treat AAT, we used rAAV1 vector, which was the most efficient for liver progenitor cell transduction among 5 different serotypes of rAAV vectors. We *ex vivo* infected liver oval cells from mice with hAAT transgene and subsequently, transplanted the cells into the liver of MCT treated and partial-hepatectomized recipients. We were able to determine that at 18 weeks post transplantation approximately 40-50% of the regenerated liver was donor derived. In addition, transgene expression was sustained for the length of the study. This study for the first time demonstrated the feasibility of long-term engraftment and stability of transgene expression from genetically modified liver progenitor cells with a rAAV vector and implies a novel gene therapy approach for treatment of liver diseases, such as AAT deficiency.

In the second experiment, we showed that oval cells can be *ex vivo* transduced by lentivirus carrying UGT1A1 transgene and subsequently transplanted to allow repopulation of liver. Using this technique, we were able to lower levels of total bilirubin by 50% in Gunn j/j rats, an animal model for CNS.

Finally, we found that using combination of monocrotaline, mitosis inhibitory agent, injected prior to bone marrow transplantation, an abundance of oval cell like cells could be found after their activation through 2AAF/PHx liver injury. Those cells expressed the CD26 marker that was only present on transplanted bone marrow cells. We further demonstrated that those cells were hepatic oval cells and could transdifferentiate to become hepatocytes.

With the possibility that oval cell may originate from bone marrow, as demonstrated by Petersen et al.⁶⁸ and our final data, our transplantation-gene therapy experiments could implicate final use of bone marrow derived stem cells as delivery vehicle for liver gene therapy. This type of therapy would not require life-long immune suppression, and the donor cells could be obtained directly from the patient. With both of these advantages, bone marrow and gene therapy could be efficiently fused together to create novel treatment for many liver metabolic disorders.

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BIOGRAPHICAL SKETCH

Rafal P. Witek was born in Poland, January 11, 1976. He attended Polish high school from 1991 to 1994. During this time, he participated in the Biological Olympiad. In May 1994, Mr. Witek arrived in the United States with purpose of permanent residency. From 1994 to 1995, he attended Clarke County High School, in Georgia. His education continued and from 1996 to 1999, he attended Gainesville Community College, GA. In August 1999, Mr. Witek transferred to the University of Georgia (UGA) where in December, 2000, he obtained his Bachelor of Science degrees in microbiology, and cellular biology. In 2001, Mr. Witek was accepted to the Interdisciplinary Program in Biomedical Sciences at the University of Florida (UF), College of Medicine, where in May 2005 he received degree of Doctor of Philosophy.

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