



Development of the liver during early life and regeneration of the mature liver following injury or surgery are processes that share common mechanisms.

TOP: Cellular events in two early stages of liver development. a) First, the hepatic endoderm (in dark blue) extends into the developing body cavity, between where the heart and intestine will be, forming hepatoblasts (in light red). Signals driving this process emanate from cells of the septum transversum mesenchyme (in white), from the primitive endothelial cells (in dark red), and from within the hepatoblasts themselves. b) Next, the hepatoblasts proliferate and form the "liver bud." The beginnings of the liver architecture are established in this stage. Adapted with permission from Dr. Kenneth Zaret, from *Nature Reviews Genetics* 3: 499-512, 2002, Figure 3.

BOTTOM: Cellular events in liver regeneration in a rat model in which two thirds of the liver is removed. In this model, the left and median lobes of the liver (LL and M) are removed, leaving only the right and caudate lobes (RL and C). Within days, genes activated by cytokines and growth factors (shown in lower graph) boost proliferation while suppressing death (apoptosis) of hepatocytes. By three weeks after surgery, the rat's liver will have regenerated to its original mass. Adapted with permission from Dr. Nelson Fausto, from *Robbins and Cotran Pathologic Basis of Disease*, 7th ed., Figure 3-11, page 101. Copyright 2004. Reprinted with permission from Elsevier.

CHAPTER 3: DEVELOPMENTAL BIOLOGY AND REGENERATION

INTRODUCTION AND BACKGROUND

The human liver has a unique ability to regenerate, which has been recognized since ancient times. Unlike other mammalian organs, the liver, after partial resection or massive injury, can rapidly regenerate and regain its original size, structure and function. Regeneration is a complex and highly ordered process involving multiple intra- and extra-cellular pathways and signals. In many ways, the processes of liver development during embryogenesis and liver regeneration are linked through common events. Knowledge obtained from the analysis of liver development and regeneration has also contributed to understanding of the mechanisms causing developmental defects, acute and chronic liver diseases, and liver carcinogenesis.

Through a series of elegant and precise studies, much is now known about the developmental events associated with commitment of endodermal cells to form the nascent liver (“primordium”); the role of endothelial cells in the formation of the primordium; and the role of transcription factors at various stages of liver development. Other defining features of this process are the expression of alpha-fetoprotein and albumin mRNAs, both markers of commitment to the liver cell lineage, and the replication and differentiation of immature hepatoblasts into hepatocytes or bile duct cells. Interestingly, though the capacity of liver cells to synthesize bile salts develops fairly early, bile salt transport develops later in fetal development and is completed in the weeks after birth. As late as early childhood, further refinement of the liver architecture

takes place, with the final arrangement of hepatocytes into single-cell plates that maximize access to the blood supply.

Liver regeneration is necessary for survival after acute liver injury, chronic hepatic diseases, liver transplantation, and surgical removal of a portion of the liver (partial hepatectomy). Hepatocyte replication underlies the restoration of liver mass in the donor following living donor liver transplantation, as well as the growth of the transplanted liver in the recipient of a living-donor, partial, or split-liver transplant. Within only 3 to 4 weeks, the liver doubles in size in both the donor and recipient. In patients with cirrhosis, hepatocyte replication sustains liver function until relatively late stages of the disease, while recovery from fulminant liver failure is dependent on hepatocyte repopulation of the injured liver.

The capacity of the liver to regenerate relies on a two-tier system of liver cells capable of high degrees of replication on demand, namely, hepatocytes and intra-hepatic progenitor cells, which are also known as oval cells, or ductular cells. Bone marrow cells have been proposed to function as a third tier, producing hepatocytes during liver regeneration and repopulation. However, bone marrow cells are probably relatively inefficient in supporting new liver growth. Cytokines and growth factors serve as important signals to bring about the initiation and progression of hepatocyte replication. Once the regenerative process has restored the liver so that it is proportion-

al to overall body size, growth ceases. The factors that terminate this process are not as well defined as those that promote regeneration, yet they are likely to be important in recovery from liver cell injury and in carcinogenesis.

RECENT RESEARCH ADVANCES

The unique aspects of liver development and regeneration have sparked vigorous research activity in these areas over the years.

Liver and Biliary Tract Development: Significant advances have been made in understanding the tissue interactions that control the specification of the liver and formation of the liver primordium in early embryogenesis. The roles of growth factors such as fibroblast growth factor and bone morphogenetic proteins; of the septum transversum mesenchyme (the mesodermal tissue that contributes cells to the heart, diaphragm, and liver); and of endothelial cells in these processes have been studied in detail using tissue explant cultures and other techniques. Additionally, studies in cell culture and animal models have delineated the role of transcription factors such as HNFs and others (e.g., GATA, Hex, Prox1) in liver formation and hepatocyte differentiation. Finally, data are now available regarding the genetic regulatory mechanisms that control cholangiocyte and bile duct formation.

Sources of Replicating Cells in Liver Regeneration:

In rodents, liver mass is rapidly regained after removal of 70 percent of the liver (classic partial hepatectomy model), such that the organ is completely restored within about 2 weeks. Although mature hepatocytes replicate only once or twice during this process, cell transplantation studies have shown that hepatocytes are capable of replicating multiple times, to the extent that a single liver cell

could, theoretically, reconstitute an entire mouse liver. Despite their seemingly unlimited proliferative capacity, hepatocytes may eventually reach a state of replicative senescence, such as in the late stages of cirrhosis, or may be prevented from responding to a growth stimulus. Under these conditions, as well as in most cases of chronic liver diseases, intra-hepatic progenitor cells help to restore the liver by proliferating and differentiating into hepatocytes. In both humans and animals, recovery from massive acute necrosis (fulminant liver failure) depends on the generation of intra-hepatic progenitor cells, which probably originate from precursor cells (stem cells) located in the biliary ductules (canals of Hering). In experimental animals, coupling of partial hepatectomy with the administration of agents that cause livery injury, such as acetylaminofluorene, galactosamine, or other carcinogens, can lead to massive oval cell proliferation.

In addition to intra-hepatic progenitor cells, bone marrow cells are capable of producing hepatocytes under special culture conditions, and after transplantation *in vivo* in one model of severe liver injury. The number of bone marrow-generated hepatocytes present during liver regeneration and repopulation after injury is small, and often such cells are undetectable. In transplantation experiments, the generation of hepatocytes from hematopoietic stem cells is much slower than that from hepatocyte or oval cell transplantation, and appears to be the consequence of the fusion of bone marrow-progeny cells (possibly macrophages) with yet to be identified liver cells.

Stages of Cell Replication in Regenerating Liver:

The sequence of events leading to hepatocyte replication after partial hepatectomy has been extensively studied, and major advances have been achieved through the use of genetically modified mice. Liver regeneration after partial hepatectomy starts with a cytokine-mediated stage that causes the cells to transition from a resting, non-replicative phase to

a preparatory phase for replication in the cell cycle. This stage, known as priming, is mediated by TNF and other cytokines, and may involve components of the complement system. To complete the cell cycle by moving through the progression stage, primed hepatocytes respond to growth factors, including HGF, which is essential for liver regeneration, TGF- α , and HB-EGF. Among the approximately 100 genes that are activated shortly after partial hepatectomy during the immediate early phase of gene expression, expression of the protooncogenes *c-jun* and *c-myc* appears to be essential for hepatocyte replication. In addition, the activation of transcription factors such as NF- κ B, STAT3, AP-1, C/EBP- β , and Foxm1B by cytokines, growth factors, or hormones provides important signals for hepatocyte survival and proliferation.

Cell cycle progression of hepatocytes in the regenerating liver follows a sequence common to most replicating cells, involving the activation of cyclin dependent kinases and the phosphorylation of members of the RB protein family. Less is understood about the mechanisms of oval cell replication, although this process is known to require signaling through TNF receptor type I, to be enhanced by some growth factors that also stimulate hepatocyte replication, and to depend on interactions with nearby stellate cells. A more complete understanding of liver cell proliferation during regeneration would facilitate clinical efforts to promote regeneration in patients with liver disease, as well as in persons undergoing hepatic resection for tumors, partial hepatectomy to donate part of the liver, or liver transplantation as a recipient.

Thus, important advances have been made in understanding the events, as well as the key cellular and molecular players, that drive liver development and regeneration.

RESEARCH GOALS

The major goals for research on liver development and regeneration are to fully define the molecular and cellular mechanisms underlying these processes in health and disease and to apply these findings to developing improved therapies for liver disease.

Basic Research on Liver Development: Although progress has been made in understanding some of the regulatory mechanisms involved in liver development, a complete picture of this process has yet to emerge.

- *Research Goal:* To more fully define the cellular and molecular events that underlie liver development from the endodermal liver primordium during fetal life (Matrix Cell C2).

Most of the steps involved in the formation of hepatocytes from progenitor cells and of the liver from the primitive outpouchings of the nascent intestine are highly conserved in evolution.

- *Research Goal:* To study these pathways of liver development in animal models, including non-mammalian model organisms such as *Xenopus* and zebrafish (Matrix Cell A3).

Model organisms would be particularly helpful in this endeavor because single genes can be knocked out and the role of new genes identified from genetic screens. Elucidating the mechanisms underlying liver formation would benefit understanding not only of how the liver develops, but also of the growth- and differentiation-related pathways that can be perturbed by disease later in life. For example, understanding developmental mechanisms in the liver will inform studies of the loss of growth control that occur in liver cancer. Improved understanding of liver cell differentiation during organogenesis

could also facilitate the development of accurate cell culture models for pre-clinical studies.

Embryonic stem cells provide an important model in which to define the cellular pathways that lead cells to differentiate into hepatocytes. Studies on embryonic stem cells have suggested that these cells can readily differentiate into visceral endodermal cells that express many of the genes commonly found in liver. However, definite differentiation of embryonic stem cells into hepatocytes has yet to be proven.

- *Research Goal:* To delineate and characterize the cellular and molecular steps that lead from an undifferentiated embryonic stem cell to a mature, differentiated hepatocyte during development through research on embryonic stem cells supported within established policies for NIH funding (Matrix Cell B3).

Interactions of hepatocyte progenitors with other nonparenchymal liver cells are important in this differentiation process, particularly in the development of the complex acinal structure of the liver with its three-dimensional relationships between hepatocytes and cholangiocytes, as well as among endothelial, stellate, and Kupffer cells. Understanding how structural relationships within the liver influence cell differentiation will also contribute to understanding of liver diseases in which the normal liver architecture is disrupted, such as cirrhosis.

- *Research Goal:* To develop an *in vitro* organ culture system and use it to analyze the cellular processes and interactions that occur during liver organogenesis (Matrix Cell C3; see also Chapter 10, B1).

Basic Research on Liver Regeneration: Regeneration of the liver after major resection or massive injury is the consequence of a highly regulated sequence of molecular and cellular events that begins with the cessation of many normal liver cell functions and

the induction of DNA synthesis, cell cycle progression, mitosis, and cell division. Multiple cellular pathways interacting at many levels are involved in regeneration, with many of their endpoints being the control of gene expression.

- *Research Goal:* To delineate the nature and time course of transcriptional regulatory and cellular signaling events that underlie liver regeneration (Matrix Cell A1).

Gene array and proteomic analyses of normal cell types in different stages of hepatic growth are appropriate approaches to achieve this goal.

More is currently known about the pathways that propel regeneration than is known about the pathways that terminate it. The signals that terminate regeneration are likely to play an important role in preventing or limiting carcinogenesis in the liver.

- *Research Goal:* To identify the pathways that terminate the regenerative process as the liver returns to its normal mass (Matrix Cell B2).
- *Research Goal:* To identify how deregulation of genes and pathways involved in normal regeneration may promote hepatocarcinogenesis (Matrix Cell B1; see also Chapter 14, C2).

The complexities of these processes are such that their elucidation would be facilitated by the formation of interdisciplinary teams of investigators, including experts in cell and molecular biology, stem cell biology, cancer biology, genetics, proteomics, bioinformatics, and network theory.

Murine models of partial hepatectomy have been used extensively to study regeneration and are likely to accurately reflect what occurs after partial resection in humans. However, regeneration in the presence of injury, inflammation, and fibrosis—as occurs in cirrhosis and acute liver failure—may involve differ-

ent pathways or growth mechanisms that interact with inflammatory and cell apoptotic pathways. Massive injury may also trigger the activation and proliferation of other compartments of progenitor cells, either in the liver or elsewhere, such as bone marrow.

- *Research Goal:* To develop animal models of liver regeneration in the presence of inflammation, fibrosis, and injury that would enable studies to define the cellular events that are important for regeneration to occur under these conditions (Matrix Cell A2).

While liver regeneration is usually defined as being based upon hepatocyte growth and proliferation, the other cells of the liver must also be duplicated and may play equally important roles in the regenerative process.

- *Research Goal:* To define the role of nonparenchymal liver cells in regeneration, focusing upon Kupffer, endothelial, stellate, and intra-hepatic NK cells (Matrix Cell A3).

Studies could be undertaken to characterize the patterns of gene expression of these cells in normal and regenerating livers.

In addition to the contributions of differentiated liver cells, progenitor stem cells are believed to play an important role in liver regeneration and recovery from injury.

- *Research Goal:* To identify and characterize hepatic stem cells (Matrix Cell A1), particularly to define the role played by different compartments of progenitor cells in different stages and conditions of regeneration.

The signals that trigger activation, migration, division, and differentiation of these progenitor cells are

important to elucidate, as are the signals that terminate regeneration and further DNA synthesis and cell division. These investigations focusing on progenitor cell populations might also consider the plasticity of the known cells in the liver.

Clinical Investigation and Therapy of Liver Disease:

Improved understanding of liver development and regeneration will greatly improve the management of liver disease. For example, greater understanding of the normal process of regeneration may help to define targets to promote regeneration in situations such as hepatic resection or living donor liver donation. Similarly, understanding the role of injury and inflammation may lead to new ways to promote regeneration and healing in acute viral hepatitis and fulminant liver failure. Additionally, despite great advances in defining the mechanisms of liver regeneration in recent years, there has been little application of these findings to clinical situations.

- *Research Goal:* To translate findings from animal or *in vitro* models to therapeutic approaches that can enhance liver regeneration in humans (Matrix Cell C2; see also Chapter 12, B3).

Agents that promote regeneration (e.g., growth factors, cytokines, growth-promoting hormones, or innovative small molecules) would be potentially very helpful in managing conditions such as acute liver failure, as well as following liver resection or transplantation. Evaluation of these agents would be aided by biomarkers or imaging techniques to monitor therapies directed at optimizing regeneration, allowing for rapid assessment of the benefit or harm of various forms of therapy.

- *Research Goal:* To develop biomarkers and imaging methods to assess the degree of regeneration, which would also be of great help in clinical practice (Matrix Cell A2; see also Chapter 13, C2).

- *Research Goal:* To validate these biomarkers in clinical situations such as acute liver failure or living donor liver transplantation (Matrix Cell B2).

Another important clinical application for research findings on liver stem cells and regeneration relates to cell and gene therapy for liver disease. The liver is a major potential target for gene and cell therapy of metabolic liver disease, including liver diseases such as Crigler-Najjar syndrome and urea cycle disorders, as well as diseases that are not usually considered liver conditions, but are caused by a lack or defect of a gene that is expressed in the liver, such as hemophilia B, familial hypercholesterolemia, alpha-1-antitrypsin deficiency, acute intermittent porphyria, and primary hyperoxalosis. Studies in animal models suggest that these serious diseases could be ameliorated or even reversed by enabling moderate, but continuous expression of the normal gene in the liver. This is a major challenge, as shown in experiments using *ex vivo* and *in vivo* correction of gene defects in the liver carried out for many of these conditions, in which the replacement gene provided only transient correction of the metabolic error.

- *Research Goal:* To develop viable therapies using gene transfer and transplanted cells for inherited metabolic liver diseases (Matrix Cell C3; see also Chapters 4, 10, and 11, C3).

Because vectors carrying the gene typically deliver it directly to the liver, it is also the organ most likely to suffer complications of gene therapy, which can be severe and even fatal. Better data on the biodistribution, hepatic uptake, metabolism, and toxicity of gene vectors and therapeutic oligonucleotides (e.g., antisense and siRNA molecules) are important to accrue to help ensure the safety and efficacy of molecular and gene therapies.

- *Research Goal:* To improve the efficacy and safety of vectors for gene and cell therapy (Matrix Cell C1; see also Chapter 11, C3).

Several animal models have shown that transplanted hepatocytes and stem cells can be positively selected in host liver and reach therapeutically relevant levels in the tissue. Safe methods for conditioning the host liver to selectively support growth of the transplanted cells do not currently exist. Other approaches to enhancing or circumventing the need for homing of cells to the liver include use of cell-based platforms and extracorporeal hepatic devices.

- *Research Goal:* To develop methods and techniques to promote homing and engraftment of transplanted cells to the liver (Matrix Cell B1).

STEPS TO ACHIEVE RESEARCH GOALS

Investigator-initiated research into developmental biology and regeneration of the liver is a high priority and warrants full support and encouragement. Particularly important is the development of animal and cell culture models to elucidate the cellular and molecular steps involved in liver formation and regeneration. Interdisciplinary collaborations between cell biologists, developmental biologists, tissue engineers, and stem cell biologists would facilitate efforts to develop and study *in vitro* models of liver organogenesis.

While animal and *in vitro* models are useful in the fine definition of molecular and cellular pathways of regeneration, ultimately the findings warrant evaluation in humans. Studies with patients undergoing hepatic resection or suffering from chronic liver disease or acute liver failure can provide clinical samples that are critical to research elucidating regeneration in these conditions. Regeneration is a major

focus of the ongoing, NIDDK-funded Adult-to-Adult Living Donor Liver Transplantation Cohort Study (A2ALL). This study could benefit greatly from collaboration with basic research groups involved in the elucidation of the processes of regeneration. Another important clinical study that could enable valuable collaborations with experts on regeneration is the Acute Liver Failure Study Group (ALFSG), an NIDDK-funded collaborative group of medical centers that enroll patients with acute liver failure in a prospective database that includes collection of serum, peripheral blood, DNA, and tissue from well characterized patients. Clinical materials and investigator collaborations provided by these studies could prove inval-

able in developing biomarkers for assessing regeneration in transplant donors and recipients, as well as in critically ill individuals with acute liver failure, using state-of-the-art approaches such as gene expression arrays or proteomic methods.

An important part of the research goals outlined in this chapter is the translation of basic research findings to clinical practice. By identifying pathways involved in liver development and regeneration, specific molecular targets can be defined, some of which may be used in high-throughput screening for drugs or small molecules. Encouraging drug development in this area is a high priority.

Matrix of Research Goals in Developmental Biology and Regeneration

	Short Term (0-3 years)	Intermediate Term (4-6 years)	Long Term (7-10 years)
High Risk	A3. Define role of nonparenchymal cells in liver regeneration and liver development. Develop new animal model systems to study liver development.	B3. Delineate sequence of molecular and cellular events that lead embryonic stem cells to differentiate into mature hepatocytes.	C3. Develop practical gene or cell therapy for metabolic liver disease. Develop <i>in vitro</i> model of hepatic organogenesis.
Intermediate Risk	A2. Identify noninvasive biomarker or imaging method for assessing liver regeneration. Define role of inflammation, fibrosis, and cell injury in regeneration.	B2. Validate biomarkers of regeneration in living donor liver donation and acute liver failure. Identify pathways that stop proliferation of hepatocytes as liver returns to normal mass.	C2. Develop safe means of promoting normal liver regeneration for acute liver failure, liver resection, and transplantation. Delineate molecular and cellular events that lead from endodermal liver primordium to mature liver in fetal development.
Low Risk	A1. Identify and characterize hepatic stem cells in fetal and adult liver. Profile transcriptional network during endodermal specification, liver growth and regeneration.	B1. Develop methods to select transplanted donor cells and induce homing and engraftment of transplanted cells to the liver. Identify how deregulation of genes and pathways involved in normal regeneration contributes to carcinogenesis.	C1. Develop <i>ex vivo</i> and <i>in vivo</i> vectors for liver-directed gene therapy.