INVOLVEMENT OF SDF-1 IN STEM CELL-AIDED LIVER REGENERATION

By

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Stromal derived factor-1 (SDF-1) and its receptor CXCR4 have been shown to play a critical role in regulation of stem/progenitor cell activities, such as migration and proliferation. In this study, the role of SDF-1 and its receptor CXCR4 was investigated as a possible mechanism for oval cell activation in oval cell aided liver regeneration. In liver injury models where oval cell activation is involved, hepatocytes increase the expression of SDF-1. While in hepatocyte-driven liver regeneration model, SDF-1 is not upregulated. In addition, CXCR4 is expressed by oval cells. Lastly, *in vitro* chemotaxis assays demonstrated that oval cells migrate along a SDF-1 gradient, suggesting that the SDF-1/CXCR4 interaction is a mechanism by which the oval cell compartment could be activated and possibly recruit a second wave of bone marrow stem cells to the injured liver.

To further assess the role of SDF-1 in stem cell-aided liver regeneration, SDF-1 expression was knocked down in the liver of 2AAF/PHx treated rats using siRNA delivered by recombinant adenovirus, and oval cell activation in these animals was

examined. Results showed that the oval cell response was compromised in these animals, as evidenced by a decreased number of OV6 positive oval cells. In addition, knockdown of SDF-1 expression caused a dramatic decrease in AFP expression, implying impaired oval cell activation in these animals. These results indicate that SDF-1 is an essential molecule needed in oval cell activation.

Overexpression of SDF-1 in the liver of 2AAF/PHx treated rats fails to enhance the stem cell response to the injury. But in the long term, overexpression of SDF-1 during oval cell activation causes increased ECM deposition in the liver, which is similar to portal-portal liver fibrosis. The significance of SDF-1 in liver fibrogenesis is yet to be investigated.

In conclusion, these studies begin to shed light on some facets of the mechanisms of oval cell-aided liver regeneration and liver fibrosis, which may someday lead to development of novel strategies to enhance stem cell-aided liver regeneration or to resolve liver fibrosis.

CHAPTER 1
INTRODUCTION

The liver plays a critical role in maintaining metabolic homeostasis of the body. This includes the processing of dietary nutrients, and the synthesis of a large spectrum of serum proteins, enzymes and cofactors. The liver is the main detoxifying organ removing wastes and xenobiotics by metabolic conversion and biliary excretion. Functioning as a way station between splanchnic and systemic circulation, the liver is vulnerable to a wide variety of metabolic, toxic, microbial and circulatory insults. Thanks to evolutionary adaptation, the liver features an unusual regenerative capability and enormous functional reserve. Therefore, mild liver damage usually does not cause noticeable symptoms. But when progression of liver disease finally ablates a considerable portion of functional liver parenchymal cells, the consequence of hepatic failure could be life threatening.

For patients with terminal liver disease, a successful liver transplantation is their last resort nowadays. Besides the potential complications inherent to long-term immunosuppresion, liver transplantation faces another major hurdle, scarcity of donor organs. As in other fields of solid organ transplantation, the gap between the number of patients listed as candidates for liver transplantation and the number of transplants performed has grown over the last 10 years (through 2001). According to the report of United Network for Organ Sharing (www.unos.org/data), the waiting list increased more than 6-folds from 1993 to 2000 (from 2,902 patients to 18,047 patients). But the number of donor organs recovered only increased from 3800 to 5382. The prospect of hepatocyte transplantation also suffers from a shortage of cell sources.

Because of the vast need for liver transplants, many researchers sought alternative option(s). One option would be to exploit the potentiality of adult stem cells to repair damaged liver. Adult tissue stem cells are known to present in a large variety of tissues such as skin, intestine, bone marrow *et al* and represent a potential source of cells needed for normal tissue turnover and damage repairing. In the adult liver, a group of cells called oval cells have been shown to be able to differentiate to mature hepatocytes and bile duct cells after toxic injury or massive necrosis. These cells are facultative liver stem (progenitor) cells keeping a reservoir for liver regeneration.[1] A body of recent publications show that bone marrow stem cells also provide a reservoir for hepatocytes and oval cells.[2-4] It is of great interest that in adult animal a number of cell types, both hepatic and non-hepatic, retain the ability to differentiate into hepatocytes. Further understanding of these mechanisms governing this phenomenon could enhance the development of novel therapies for tissue regeneration, and not solely for hepatocytes.

This study aims to investigate the signal(s) that may control stem cell migration and activation during liver regeneration, and sets to exploit possible way to enhance recruitment of stem cells in the regenerative process.

Summary of Liver Biology

The liver is a large parenchymal organ consisting of complex array of cells and vasculature. It represents about 2% of the body weight in humans and 5% in the mice. The liver has two separate sources of afferent blood supply---the hepatic artery and the portal vein. The hepatic artery provides liver with oxygenated blood, and the portal vein brings in venous blood rich in nutrients (and digested toxins too) from the splanchic vascular bed. The efferent blood drains to the hepatic vein and further to the vena cava.

The bulk of the liver is primarily composed of the hepatocytes, stellate cells (Ito cells), Kupffer cells, sinusoidal epithelial cells, and biliary epithelial cells. The major organismal liver functions are carried out by hepatocytes, which represent about 90% of total liver weight and about 60% of the total cell number. Hepatocytes are large, cuboidal epithelial cells with a basal surface facing the sinusoids; and an apical surface (also called the canalicular surface) facing the bile canaliculi. Hepatocytes exchange metabolites with the blood on the basal surface and secrete bile at the canalicular surface. Although somewhat variable among species, a large proportion of adult hepatocytes are binucleated, with some nuclei being tetraploid. Thus, hepatocytes can have 2n, 4n, or 8n total DNA content. In a microscopic view, hepatocytes are arranged in a single-layer cell plate called the hepatic plate, which bifurcates and merges freely while extending from portal space to the central venule. The limiting plate is the first row of hepatocytes that separates liver parenchyma from the portal space. The continuous mass of hepatocytic plates is interpenetrated by hepatic sinusoids, a liver counterpart of capillary. Blood from the hepatic artery branch and portal vein branch enters the sinusoids, delivers oxygen and nutrients to the liver cells, picks up carbon dioxide and metabolic products from the liver cells, and drains into the central vein branch. Fenestrated endothelial cells form the wall between the sinusoids and hepatocytes, facilitating the exchange of materials between hepatocytes and blood. Bile canaliculi are lined by two adjacent plates of hepatocytes and drain bile into bile ductules, which are lined by biliary epithelial cells. The canaliculo-ductular junction connects the bile canaliculi and bile ductule, and is also known as canal of Hering. The portal triad harbors the branches of the hepatic artery, portal vein and bile duct. Hepatocytes vary in biochemical properties and pattern of gene expression

according to their proximodistal orientation to the portal triad. The term ‘metabolic zonation’ has been coined to indicate the different properties of zone-1, zone-2, and zone-3 hepatocytes. Zone-1 hepatocytes are close to the portal triad, zone-2 cells are in the middle, and zone-3 consists of cells directly adjacent to the central vein.

Kupffer cells represent about 5% of liver cells and reside in the sinusoids. These cells are macrophages of hematopoietic origin capable of replicating within the liver itself. Stelalte cells represent about 5-10% of the total number of hepatic cell and are located in space between endothelial cells and hepatocytes (Disse’s space). In addition to storing vitmin A, they are essential for the synthesis of extracellular matrix proteins and produce many growth factors that play an essential role in biology of liver regeneration.[5] Oval cells are the apparent progenitors of liver hepatocytes and epithelial cell, and are found in regenerating liver after partial hepatectomy or chemical damage.[1]

The liver involves in a wide variety of biochemical functions. The liver plays an important role in intermediary metabolism of carbohydrates, amino acids, and lipids, including gluconeogenesis, synthesis and storage of glycogen, production of glutamine, conversion of ammonia to urea, synthesis of very low density lipoproteins (VLDL) and so on. The liver is the center for biotransformation involved in detoxification and elimination of drugs as well as bilirubin. The liver is also the primary organ for synthesis and secretion of serum proteins such as albumin, transferrin, coagulation factors and complements. In addition, the liver produces bile, which is important for digestion and absorption of dietary lipid, and elimination of cholesterol and copper.

The liver develops from a diverculum of the floor of the foregut. [6] Under the induction of cardiac mesoderm, the founder cells (endodermal cells that give rise to the

epithelial elements of the liver) start to activate liver-specific genes and invade the mesenchyma of the septum transversum. Endodermal cells later generate hepatocytes and nonparenchymal epithelial cells, while the mesenchyma give rise to sinusoidal lining cells. The early hepatocytes coalesce around sinusoids in the mesenchma, forming the liver bud. Early cells in the liver primordium are positive for α-fetoprotein (AFP) and albumin. The more-differentiated parenchymal cells forming hepatic cords from day 10 to 17 in rat liver development are generally called hepatoblasts, and can differentiate into hepatocytes and bile duct cells and thus have been considered equivalent to fetal liver stem cells. [7, 8] In rats, mice, and most likely also in humans, AFP+albumin+ hepatoblasts located near large vascular space close to the hilus give rise to primitive intrahepatic bile ducts. [7] These structures contain cells that express AFP and albumin as well as cytokeratines (CK) 7, 8, 18 and 19. In the rat, CK-7 and CK-19 are expressed exclusively in the newly formed ducts, while the surrounding hepatoblastes contain only CK-8 and CK-18. [7] In humans, a significant proportion of hepatoblasts express CK-19 in early gestration. Expression of this marker increases in cells near vascular spaces forming ducts, but progressively decreases and is no longer detectable after 14 weeks of gestation in the hepatoblasts, which differentiate into hepatocytes in the rest of the parenchyma.[9] Cells of primitive intrahepatic bile duct can be considered transitional because they express markers of both hepatocyte (AFP, albumin, CK-8, and CK-18) and bile duct (CK-7, CK-19) lineages, a pattern of expression also found in the oval cell compartment of hepatocarcinogenesis. These data suggested that the primitive intrahepatic bile ducts formed from AFP+, albumin+ hepatoblasts are embryologic counterparts of the oval cell.[7]

Oval cells are the heterogeneous population of nonparenchymal epithelial cells emerging from the portal triads at the early stage of liver carcinogenesis induced by most chemical carcinogens. Ultrastructurally, oval cells closely resemble cells that form terminal bile ductules, and they form irregular duct-like structures that enclose lumens, which connect to adjacent, pre-existing bile ducts. The oval cell compartment contains transitional cells that express phenotypic features of hepatocytes and intrahepatic biliary epithelium. Lineage studies showed that these cells differentiate into hepatocytes and biliary epithelium.[10, 11]Given the proliferation pattern of the oval cell compartment in carcinogenesis and the differentiation of primitive intrahepatic bile duct in organogenesis, it is logical to expect putative stem cells in the adult liver should be localized in the canal of Hering---the smallest units of the biliary tree closest to the parenchyma.

Liver Regeneration

Basic Characteristics of Liver Regeneration

The liver has a remarkable capacity to regenerate after major tissue loss. This has been demonstrated by a number of experimental liver injury models as well as clinical observations. A widely used model of liver repair is the partial hepatectomy (PHx) model. This involves surgical removal of specific lobes amounting to approximately two-thirds of the liver mass. Liver regeneration is highly efficient. First described in 1931 by Higgins and Andersen, [12] rat liver grew back to its original mass approximately 10 days after the surgical resection. This process is fulfilled by enlargement of the remaining lobe rather than re-growth of the removed lobes. Thus, it is also called ‘compensatory hyperplasia of the liver’, although the term ‘liver regeneration’ is used more often. In addition to cell proliferation, which makes up for the lost liver mass, reformation of normal liver architecture also occurs to ensure full restoration of liver function. Similar

results are also observed in other animal models involving diffused liver damage, such as hepatocyte necrosis or apoptosis induced by hepatic toxin or virus.

The liver has an almost unlimited capacity to regenerate. In repeated PHx experiments, the rat liver was able to regenerate each time and achieved its previous mass, even after 12 sequential resections. [13] Serial transplantation and liver repopulation in experimental animals further demonstrated the almost unlimited replication capacity of adult hepatocytes. In the alb-uPA transgenic mice, which experience severe liver damage and postnatal death of most pups, repopulation of nearly the entire liver occurs through proliferation of a small portion (1%) of the hepatocytes that have undergone spontaneous transgene inactivation. [14] Subsequent studies by Rhim et al.[15,16] showed that transplantation of both syngeneic and xynogeneic (rat and human) adult hepatocytes could be used to reconstitute the damaged livers of the newborn alb-uPA mice. Using a gene knockout approach, Overturf et al.(1997) created a mouse model of liver repopulation based on the human disease hereditary tyrosinemia type I, which is due to a lack of the enzyme fumarylacetoacetate hydrolase (FAH), and renders a selective advantage to transplanted wild-type hepatocytes.[17] In hepatocyte transplantation-repopulation studies using the FAH-/- mouse model, they found that normal male adult hepatocytes, when transplanted to female FAH knockout recipients, could repopulate the recipient animals liver to >90% within 6 to 8 weeks. Rescue of FAH-deficient animals and restoration of liver function required as few as 1000 donor cells.[18] Furthermore, the genetically marked donor hepatocytes could be re-isolated from repopulated recipient liver and re-transplanted in limiting numbers. This process could be successfully repeated in a serial fashion at least eight times with no apparent decrease in proliferative potential

and with no evidence of abnormal liver function or hepatic architecture. This experiment demonstrated that the regenerative potential of hepatocytes exceeds 69 cell doublings, equivalent to a 7.3×10^{20}-fold expansion, and is similar to that of hematopoietic stem cells.

Liver regeneration is a tightly regulated process. After PHx, the normally quiescent hepatocytes rapidly re-enter cell cycle. In rat liver, the rate of DNA synthesis in hepatocytes begins to increase about 12 hours after PHx and peaks around 24 hours.[19] Other cells of the liver start DNA synthesis about 24 hours after the hepatocytes. The original liver mass is restores in 5 to 7 days. After that, DNA synthesis of hepatic cells abruptly stops. It is obvious the liver mass is precisely regulated. An example of this is when a large liver is transplanted into a relatively small recipient; the liver is gradually decreases in size and becomes proportional to the new body.[20] Many believe that regulation of liver mass is key to maintain the homeostasis of functional capacity of the liver. The set point for growth control is the ratio between liver mass and body mass rather than liver mass per se. In another words, the body tends to maintain a liver mass only enough to meet its metabolic demands.[21] How the body senses the liver/body mass ratio and control liver growth has been under intensive investigation for decades. Jirtle and Michalopoulos[22] showed that when isolated hepatocytes are transplanted into an extrahepatic site, those cells enter into replication after PHx. Molten and Bucher used parabiotic rats to show that hepatectomy of one member of pairs causes DNA synthesis within the hepatocytes of the intact liver of the other member, with the maximum effect seen when the liver of one animal is totally removed.[23] These early studies establish that factor(s) in circulation are responsible for triggering liver regeneration. Significant efforts have been exerted afterwards to identify these factors and dissect the signaling pathways

that control liver regeneration. Some growth factors and cytokines have been shown to be critical players during the regenerative process. (Discussed in section 'hepatocyte dependent liver regeneration')

Restoration of liver parenchyma after liver injury depends on a two-tier cell system composed of hepatocytes and progenitor (stem) cells, known as oval cells. What is unique to the liver is that the differentiated cells (hepatocytes) constitute the first line of response to injury or resection, while the progenitor cells (oval cells) function as a reserve compartment. This is distinctly different from other tissues, such as skeletal muscle, in which differentiated myocytes do not replicate, but regeneration after injury can occur through the proliferation of precursor cells (satellite cells). Mature hepatocytes are very effective in responding to replicative signals after PHx and have enormous proliferative capacity. As a general rule, replication of existing hepatocytes is the quickest way to repair damaged liver parenchyma. Oval cells replicate and differentiate into hepatocytes only when the replication of mature hepatocytes is delayed or entirely blocked, in situations such as 2-actylaminofluorene (2AAF) treatment followed by partial hepatectomy. Hepatocyte-dependent and oval cell-dependent liver regeneration will be discussed separately in following sections, using PHx and 2AAF/PHx as sample models respectively.

Hepatocyte-Dependent Liver Regeneration: PHx as a Sample

Liver regeneration after PHx is carried out by proliferation of all the existing mature cellular populations in remaining liver. These include hepatocytes; biliary epithelial cells; fenestrated endothelial cells; Kupffer cells; and Ito cells. All of these cells proliferate to rebuild the lost hepatic tissue. Hepatocytes are the first to proliferate. The kinetics of cell proliferation differs slightly from species to species. In rats, DNA

synthesis of hepatocytes typically starts at 10 to 12 hours post PHx. The first peak of DNA synthesis in hepatocytes occurs at about 24 hours, with a smaller peak between 36 and 48 hours. Because only two-thirds of the hepatic tissue is removed, restoration of the original number of hepatocytes theoretically requires 1.66 proliferative cycles per residual hepatocyte. Most of the hepatocytes (95% in young and 75% in very old rats) in the residual lobes participate in one or two proliferative events. Hepatocyte proliferation starts in the periporta areas and then precedes to the pericentral areas by 36 to 48 hours. The other cells of the liver enter into DNA synthesis about 24 hours after the hepatocytes, with a peak of DNA synthesis at 48 hours or later.[19] The kinetics of cell proliferation and the growth factors produced by proliferating hepatocytes suggest that hepatocytes provide the mitogenic stimuli leading to proliferation of the other cells. After 2 to 3 days of cell proliferation, new hepatocytes form clusters surrounding capillaries.

Typical hepatic histology is gradually restored with the help of Ito cells, which send processes that penetrate the hepatocyte clumps and start producing several types of laminin. Eventually, the small hepatocyte clumps become rearranged into the typical hepatocyte plates seen in the mature liver. The capillaries of the small hepatocyte clumps lose their basement membrane and change into true hepatic sinusoids, which are surrounded by very scant matrix and lined by fenestrated endothelial cells and Kupffer cells. By day 7, hepatic lobules are larger in size than those before regeneration. Hepatocytes are arranged in plates consisting of two cell layers as opposed to the one cell layer of the normal liver. It is not clear whether there is a net increase in the number of lobules or whether existing lobules merely become larger in size, though evidence suggests that both phenomena occur.

Hepatocyte regeneneration is accomplished by a sequence of distinct phases: an initiation phase, rendering hepatocytes in a state of replicative competence; a proliferation phase, where expansion of the cell population population occurs, and a termination phase, where cell growth is suppressed to terminate regeneration at a set point.

The iniatiation step of liver regeneration is characterized by priming quiescent (G0) hepatocytes to enter a cell division cycle. Priming involves the activation of a group of transcription factors, which are necessary, but not sufficient for the S phase completion; they comprise the nuclear factor for kappa chains in B cells (NFκB), signal transducer and activator of transcription-3 (STAT3), activator protein-1 (AP-1), CCAAT enhancer binding protein (C/EBPβ), and several immediate early genes, such as c-*fos*, c-*myc* and c-*jun*. The priming step is reversible until the cells have crossed the so-called G1 checkpoint, the cells thereupon being irreversibly committed for replication.

It is thought that loss of functional liver mass after PHx stimulates the release of a number of factors, acting in a cascade-like fashion. Gut-derived factors, such as lipopolysaccharide (LPS), are upregulated after liver injury or hepatectomy and reach the liver through the portal blood supply. They activate hepatic non-parenchymal cells (including Kupffer cells and stellate cells) and increase the production of tumour necrosis factor α (TNFα) and interleukin 6 (IL-6). Other factors are released from the pancreas (insulin), duodenum or salivary gland (epidermal growth factor; EGF), adrenal gland (norepinepherine), thyroid gland (triodothronine; T3) and stellate cells (hepatocyte growth factor; HGF). Cooperative signals from these factors push the resting hepatocytes

through cell-cycle checkpoint controls, i.e. to move from G0, through G1, to the S phase of the cell cycle. This leads to DNA synthesis and hepatocyte proliferation.[24]

Principal factors active in initiation phase comprise two cytokines: TNF-α and interleukin-6 (IL-6), which render hepatocytes in a state of replicative competence (priming). Kupffer cells produce most of the TNF-α and IL-6 in the liver after PHx. TNF-α acts through two different receptors, TNFR1 (CD 120a) and TNFR2 (CD 120 b) in affecting cell proliferation, differentiation, and cell death. Using TNF-receptor-1 (TNF-R1) knockout mice, it was shown that TNF-α signalling is required for a normal proliferative response after PHx.[25] This effect seems to be largely mediated by the ability of TNFα to induce IL-6, as treatment with IL-6 corrects the defect in DNA synthesis seen in TNF-R1 knockout mice after PHx. For unexplained reasons, however, the absence of TNF-α does not impair liver regeneration.[26] TNF-α induces the production of IL-6 by binding to its receptor on Kupffer cells and activating NF-κB, which in turn activates transcription of IL-6. The potentially noxious effects of TNF-α, such as the release of reactive oxygen species (ROS) from mitochondria, appears to be controlled by the prompt up-regulation of survival genes that regulate mitochondrial membrane permeability, and hepatocytes may even exploit a transient ROS surge to evoke a proliferative response.[27]

IL-6 is a key inducer of transcription factors involved in liver regeneration. Binding of IL-6 to its receptor IL-6R, which is associated with two subunits of gp130, stimulates the tyrosine-kinase activity of the associated Janus-kinase-family member — usually JAK1. Activated JAK then phosphorylates the associated gp130 and STAT3 on a Tyrosine residue, which results in the dimerization of STAT3. Dimerized STAT3

translocates to the nucleus and activates the transcription of target genes. Stimulation of gp130 also activates the MAPK signaling cascade, which is crucial for cellular proliferation, and recent evidence indicates that IL-6 signalling can also directly activate kinases that are involved in cell survival including phosphatidylinositol 3-kinase (PI3K) and AKT.

IL-6 is elevated in the first hours after PHx, i.e. much earlier than DNA synthesis in hepatocytes. This increase of IL-6 activates the target genes that control G0/G1 transition through two main pathways--- the STAT3 and MAPK signaling pathway. Liver regeneration after PHx is impaired in the livers of *IL-6*–/– mice, and is characterized by liver necrosis and liver failure, a reduced DNA-synthesis response in hepatocytes, and discrete G1-phase abnormalities, including the lack of STAT3 activation and selective abnormalities in gene expression.[28] The defect is limited to hepatocytes as the DNA-synthesis response seems normal in *IL-6*–/– non-parenchymal cells. Defective liver regeneration can be explained by the large number of immediate-early genes that are regulated, at least in part, by IL-6 pathway.[29] Treatment of *IL-6*–/– mice with IL-6 in the absence of partial hepatectomy induces a much smaller set of genes in the liver, which indicates that IL-6 cooperates with other factors that are induced by partial hepatectomy to activate the rest of the up-regulated genes. Stem-cell factor (SCF), which binds the receptor tyrosine kinase Kit, is one of the targets of IL-6 signaling. The mechanism by which IL-6 activates SCF is unknown. However, regenerative changes in *IL-6*–/– mice can be corrected by treatment with SCF. In addition, SCF can directly activate STAT3 independently of IL-6 signaling, and it is also known to activate the MAPK signal-transduction pathway.

Once the cells have been primed, the progression to G1 phase is dependent on the presence of continued stimulation by mitogens, such as EGF, TGF-α and most potent of all, hepatocyte growth factor (HGF).

HGF is synthesized by non-parenchymal cells, particularly stellate cells, and therefore affects hepatocytes in a paracrine manner. In the rat, plasma concentrations of HGF rise more than 20-fold within 1 hour after PHx.[30] HGF concentrations decline slowly during the first 24 hours but remain elevated for more than 72 hours, eventually returning to normal. Since HGF has been shown to be a potent mitogen for hepatocytes in culture,[31] it is reasonable to postulate that the rapid rise of HGF in the plasma 1 hour after PHx is responsible for leading hepatocytes to DNA synthesis 23 hours later. This scenario is compatible with the time kinetics of the appearance of blood-borne regenerative factors as well as the rapid changes in immediate early gene expression. HGF induces expression of some immediate early genes,[32] suggesting that HGF may be one of the stimuli leading to the rapid changes in gene expression after PHx.

The HGF precursor, pro-HGF, is rapidly activated by proteases—such as uPA (urokinase-type plasminogen activator) and its downstream effector plasminogen—after PHx or liver injury. Blocking uPA delays the appearance of HGF, and thereby delays liver regeneration, whereas blocking plasminogen-activator inhibitor (PAI) accelerates the release of HGF and thereby accelerates liver regeneration.

HGF regulates various processes in the liver, including direct stimulation of hepatocyte proliferation. As HGF and its receptor, Met, are important growth factors in various tissues, and knockout of their genes results in a lethal phenotype, A liver-specific conditional knockout of Met was developed to show that the HGF–Met pathway is

important for DNA synthesis after liver injury.[33] Also, pharmacologic tools, including antibodies and adenoviruses, have shown that HGF and a downstream regulator—TGFα—function in the mitogenic response of the liver.

Like many growth factors, HGF has pleiotropic effects on various mitogenic signaling pathways. HGF activates the receptor tyrosine kinase Met as well as various downstream pathways, which include those that involve PI3K, ERK, S6 kinase and AKT.[34] *In vitro* studies in isolated hepatocytes indicate that the mitogenic effect of HGF is mediated, in part, through upregulation of another growth factor, TGFα.

In contrast to HGF, which stimulates hepatocyte replication by a paracrine or endocrine mechanism, TGFα is an autocrine growth factor, that is, it is produced by hepatocytes and acts on these cells through binding the EGF receptor.[35] It can also function through a juxtacrine mechanism, which involves the binding of membrane anchored TGFα from one cell to the EGF receptor of another. In this way, both cell adhesion and activation of the receptor are accomplished. TGFα mRNA is induced in hepatocytes within 2 to 3 hours after PHx, rises to a peak between 12 and 24 hours, and remains elevated for at least 48 hours after PHx.[36] Enhanced expression of TGFα in hepatocytes under the influence of the albumin promoter leads to sustained high levels of hepatocyte DNA synthesis and eventually to tumor formation.[37,38]

Whether these findings apply to regeneration, however, is not entirely clear. In TGFα knockout mice, liver regeneration proceeds normally.[39] This, however, may be due to a compensatory increase in other members of the EGF receptor family of ligands.

Other growth factors involved in liver regeneration include epidermal growth factor (EGF), acidic fibroblast growth factor (aFGF) and vascular endothelial growth factor

(VEGF). Although EGF is not up-regulated early after PHx, it may play a mitogenic role in liver regeneration by abruptly becoming more available to hepatocytes after PHx. EGF is continually made available to the liver by the Brunner's glands of the duodenum, through portal circulation.[40] EGF is taken up by liver in one pass and, as with HGF; it deposits itself in the periportal matrix.[41] A decrease of hepatic mass to one-third by PHx increases the concentration of EGF (available through the portal circulation) per unit liver weight by 3-fold. In addition norepinephrine, a substance that also increases dramatically after PHx, stimulates secretion of EGF by the Brunner's glands,[42] which may further increase the amount of EGF entering the liver after PHx. Rapid tyrosine phosphorylation and down-regulation of the EGF receptor occur shortly after PHx,[43] suggesting that EGF may indeed play a mitogenic role early in the process. Production of aFGF and VEGF may be part of a programmed set of events that aim to restore normal histology, since both of them are well-established angiogenic signals.

Besides those growth factors that provide direct mitogenic signals to the progression of regeneration, some other factors function by enhancing the effect of mitogens and reducing the effect of inhibitory substance. These factors include hormones and neurotransmitters, such as insulin, glucagon, noradrenaline, adrenaline, thyroid and parathyroid hormones, as well as calcium and vitamin D.

HGF and TNFα–IL-6 signaling are necessary for liver regeneration, but, as mentioned above, other signals and transcription factors are involved in the liver-regeneration response that have not yet been linked to any one growth factor or cytokine. There are a few signal-transduction molecules (for example, ERK and JNK), transcription factors (e.g. AP1 and C/EBPβ) and other molecules (i. c. insulin-like-

growthfactor-binding protein (IGFBP1), which seems to be regulated by both growth factors and cytokines. This allows speculation about how the combination of cytokine and growth factor signals might lead to robust liver regeneration and repair after injury.

Less is understood about the mechanisms by which regenerative process is stopped after appropriate liver mass has been restored. The termination response should include arrest of cell proliferation and apoptosis of the redundant cells. The regenerative pathway has several checkpoints whereby the feedback inhibition of specific growth factor and cytokine-mediated pathways could regulate organ size.

Suppressors of cytokine signaling (SOCS), as the name suggests, are important negative regulators of cytokine signaling that prevent the tyrosine phosphorylation of STAT proteins. SOCS directly interact with phosphorylated JAK kinases and prevent the activation of STATs. It has been shown that IL-6 signaling in the liver causes the rapid upregulation of SOCS3, which correlates with the subsequent down-regulation of phosphorylated STAT3, thereby terminating the IL-6 signal.[44] This finding could explain why overexpression of IL-6 can, at times, inhibit cell growth and cause liver injury.[45] The notable upregulation of SOCS3 and other cytokine inhibitors by IL-6 could block its own signaling or the signaling of other cytokines. However, SOCS proteins are part of an important mechanism that prevents uncontrolled cytokine signaling. In addition, IL-6 itself could have a role in terminating the HGF signal by inducing PAI (plasminogen activator inhibitor), which blocks the processing of pro-HGF into active HGF.[46]

Perhaps the most well-known hepatocyte anti-proliferative factors within the liver are TGFβ and related TGFβ-family members such as activin.[47] TGFβ is produced mainly by hepatic stellate cells, and the up-regulated expression of TGFβ leads to liver fibrosis

and apoptosis. In the rat, TGFβ mRNA increases within 3 to 4 hours after PHx, reaching plateau amounts at 48 to 72 hours.[48] Because DNA synthesis in hepatocytes eventually stops at that time, it is reasonable to postulate that this may be mediated by a paracrine mito-inhibitory effect of TGFβ. Infusion of TGFβ after PHx suppresses the hepatocyte DNA synthesis peak at 24 hours, though DNA synthesis returns by 72 hours.[49] Hepatocytes isolated from regenerating liver 12 to 48 hours after PHx are resistant to TGFβ mitoinhibitory effects.[50] This might be a consequence of the downregulation of TGFβ receptors on hepatocytes during the same time frame.[51] Resistance to TGFβ by regenerating hepatocytes is an important phenomenon because it may allow hepatocytes to proliferate even though concentrations of TGF-β1 are increasing. Sensitivity to TGFβ returns by 96 hours; however, hepatocyte proliferation stops between 48 and 72 hours, a time when they are still resistant to TGFβ. Overall, the role that TGF-β1 plays during liver regeneration is not clear. Obviously hepatocytes proceed through regeneration despite the TGFβ increase. On the other hand, TGF-β1 is a mito-inhibitor and thus a logical candidate to cause the end of regeneration.

Similarly, activin is a pro-apoptotic member of the TGFβ family that blocks hepatocyte mitogenesis. Activin shows diminished signaling during liver regeneration when its cellular-receptor level is reduced, but the receptor level is restored once liver regeneration is terminated.[52]

Apoptosis holds a key position in the intricate balance between gain and loss of target cells, also within a regenerative response of the liver.[53] There is evidence that, in the liver, a physiological role of the Fas/Fas ligand system is operational in the removal of senescent hepatocytes. In liver regenration after PHx in the rat, Fas mRNA is

significantly decreased by 2 hours, this down-regulation continuing until 18 hours, which then slowly recovers after 6 days,[54] suggesting that physiological Fas expression is again achieved when liver mass has been reconstituted. Liver caspase-3-like protease, which is specifically activated in apoptosis, is increased at 18 through 48 hours after PHx in rat;[55] again suggesting that apoptosis is another key mechanism for termination phase.

Bile ducts and biliary ductules regenerate in concert with the arterial vessels accompanying them, and the cells making part of the periductal sheath may modulate the ductular growth behavior via factors secreted by hepatic stellate cells (Ito cells) and myofibroblasts situated here. Biliary epithelial cells (BEC) have a regenerative response different from hepatocytes. *In vivo*, BEC respond to PH with a delay of about 24 hours. In PHx animals, factors inducing a proliferative response of BEC include epidermal growth factor, hepatocyte growth factor, somatostatin, bile acids and IL-6.

Based on the close anatomical and functional relationship, the formation of arteries (arteriogenesis) during liver regeneration may go in parallel with the development of new bile ducts, but relatively few data are available in this respect. An altered oxygen supply may be a main driving force for this arteriogenetic response. As to the reconstitution of venous epithelium, it has been shown that angiopoietin, vascular endothelial growth factor (VEGF), and tubedown-1 (Tbdn-1 acetyltransferase) may play an essential role[56] in this process. VEGF is mainly expressed in the periportal (zone 1) hepatocytes. Following a regenerative signal in the liver, VEGF expression in this area is increased by 48-72 hours after initiation of the response, and results in an augmentation of sinusoidal endothelial cell proliferation.[57] This reaction is accompanied by a burst of hepatocyte proliferation within the same periportal zone, probably owing to sinusoid reconstruction

known to exert an influence on hepatocyte replication. Sinusoidal endothelial cells enter DNA synthesis cycle at 48 to 72 hours after PHx, and exhibit very early changes of their fenestration and porosity (open surface area) features. After an initial rise of porosity at 5 min post-PH, porosity gradually decreases by 72 hours, to augment again afterwards, remaining elevated up to 14 days. The porosity nadir at 72 hours goes in parallel with the initiation of DNA synthesis, compression of sinusoidal endothelial cells by proliferating hepatocytes, and enveloping by hepatic stellate cells.[58] Two types of endothelial progenitor cells take part in hepatic neoangiogenesis. Type A cells are vascular-derived endothelial precursors, being present in large numbers, migrating to the tip of the proliferating vessel, and exhibiting a short proliferative capacity. Type B cells derive from the circulating hemangioblast, are bone marrow-derived, migrate to sites of neoangiogenesis, occur in low numbers, and have a long-term proliferation potential.

After partial PHx, hepatocytes proliferate and form cell clusters of 10–14 cells.[59] These clusters do not resemble normal liver architecture and are devoid of extracellular matrix (ECM). Early changes in the composition of the hepatic extracellular matrix modulate the cellular regenerative response. The formation of hepatocyte clusters with desinusoidalisation and ECM loss may be related to the interaction between collagens and cell adhesion molecule expression. Hepatic ECM remodeling is thought to be a prerequisite process for hepatocyte proliferation in hepatic regeneration, involving matrix-degrading proteases. PHx is followed by a dramatic increase of membrane-type matrix metalloproteinase (MMP-14) and to a lesser extent of TIMP-1 (the MMP inhibitor) expression, whereas other MMP and TIMP are not induced.[60] This is thought to be related to pericellular fibrinolysis or fibrolysis required for a distinct function of

regenerating hepatocytes, and also to the desinusoidalization process. There is a relationship between induced hepatocyte proliferation and the expression of metalloproteinases involved in hepatic remodeling. After hepatectomy, inactive matrix metalloproteinases (pro-MMP-2 and pro-MMP-9) are elevated at 30 min already, and activated at 6 to 12 hours and at 3 to 6 hours, respectively.[61] TNFα induces MMP-9 expression, but not MMP-2, and the increased hepatocyte proliferation is suppressed by TIMP-1.

To restore normal tissue structure, stellate cells produce extracellular matrix about 4 days after partial hepatectomy, which re-establishes the connection between hepatocytes and the sinusoidal epithelium. In rat livers after PHx, mature stellate cells increase in number at day 7, i.e. much later than regenerating hepatocytes, but occur together with proliferating hepatocytes forming clusters, i.e. in the phase of effacement of the perisinusoidal space. In addition to stellate cells located to the perisinusoidal space of Disse, stellate cells are situated in portal tracts, i.e. in an extralittoral compartment. These cells are in close relationship to small bile ducts, and make part of the so-called periductal sheath. Together with biliary ductules, this cell system may be operational as a remodeling pacemaker.[62,63]

Stem Cell-aided Liver Regeneration

It is believed that stem cells participate in liver regeneration only when the ability of hepatocytes to divide and replace damaged tissue is compromised. Stem cells within adult liver are recognized as oval cells, which can be seen proliferating in many rodent experimental models (Table 1‑1) as well as human livers suffering from chronic injuries. Oval cells are small in size (relative to hepatocytes) with ovoid nucleus and high

nuclear to cytoplasmic ratio. Oval cells are observed to originate in the region of terminal bile duct within the periportal region, as liver damage progresses, they infiltrate into the parenchyma along the bile canaliculi between the hepatic cords. As they migrate through the parenchyma, oval cells proliferate rapidly, with labeling rates after pulse dose of ^{3}H-thymidine of 5-20% during peak proliferation.[64] As oval cells proliferate, individual and small group of small, highly basophilic hepatocytes typically appear among them, after which the oval cells disappear and the parenchyma is graduately reconstructed.

Oval cells express different combinations of phenotypic markers from both the hepatocyte and biliary lineage (Table 1–2), suggesting that the term 'oval cell' describes a heterogeneous cell compartment (or oval cell compartment) containing cells that may differ in their differentiation capacity and stage of differentiation. Some of these cells may function as hepatocyte progenitors, whereas others may commit to biliary epithelial lineage. Accordingly, oval cells are a multipotent cell population with the potential to differentiate into hepatocytes and biliary epithelia.[10,11]

During fetal liver development, hepatoblasts positive for AFP and albumin give rise to intrahepatic biliary epithelium, which is regarded as the embryologic counterparts of oval cell.[7] In adult animal, the existence of an oval cell precursor population was initially suggested when a population of oval like cells negative for the expression of classical oval cell markers was found in the liver of 2AAF and/or allyl alcohol (AA) treated mice.[65] These cells began to express AFP after 2 days, possibly because of a commitment to differentiate along the hepatocyte lineage and, accordingly, it was assumed that oval cells were derived from progenitor cells located endogenously in the liver. An examination of the 3-D relationship between CK19-expressing cells in massive

necrosis implies that the characteristic ductular reaction observed is caused by the proliferation of cells lining the canals of Hering, the junction between the hepatocyte canalicular system and terminal bile ducts.[66] Likewise, AAF-induced oval cells are generated by the proliferation of terminal bile ducts, and they form structures representing an extension of the canals of Hering.[67] Considering the first cells undergoing proliferation and differentiation into hepatocytes are found in this region, it is reasonable to assume that the canals of Hering are the source of hepatic progenitor cells and their direct progeny, the oval cell.

Another population of putative hepatic stem cells has been identified in developing mouse liver, which possess multilineage differentiation potential and self-renewing capability.[68] These cells are CD49+, CD29+, c-Met+, c-kit–, CD45–, Ter119–, and are designated 'hepatic colony-forming-unit in culture' (HCFU-C). However, unlike oval cells, they do not express hepatocyte or cholangiocyte specific markers and c-kit.[69] Cells derived from a single H-CFU-C expand *in vitro* and are capable of reconstituting hepatocytic, bile-ductal, pancreatic and intestinal structures *in vivo*.[68] Whether H-CFU-C's exist in the adult liver is not known, nor is their relationship between hepatoblasts or oval cells.

Some non-hepatic adult stem cells have also been postulated to participate in liver regeneration under certain circumstance. One of these stem cells is hematopoietic stem cells. Evidence of a cell lineage relationship between the hematopoietic system and the hepatic oval cells is initially suggested by the finding that classical hematopoietic markers, including Thy-1, c-kit and CD34, are expressed on the surface of oval cells.[70-73] Additionally, bone marrow transplantation of purified c-kit +, Lin– and Sca-1+

hematopoietic stem cells rescued fumarylacetoacetate (FAH)-deficient mice, an animal model of fatal hereditary tyrosinemia type 1.[4] Cross-gender, or cross-strain, bone marrow and whole liver transplants in mice have identified cells in the bone marrow that are capable of repopulating the liver.[2,3] These results are supported by human studies using archival liver biopsy specimens from recipients of cross-gender therapeutic bone marrow transplants who later developed chronic liver damage due to recurrent disease. Analysis for the presence of the Y chromosome in cells of liver biopsy specimens showed that bone marrow-derived cells give rise to hepatocytes alone, or both hepatocytes and cholangiocytes.[74,75] However, there is disagreement on the extent of engraftment. Alison *et al*. reported a relatively low frequency of Y-chromosome positive hepatocytes (0.5–2%); whereas Theise *et al*. reported that, even in mild conditions, significant engraftment (5–21% for hepatocytes and 4–18% for cholangiocytes) occurred; while in cases of severe injury, up to 64% of periportal hepatocytes and 38% of cholangiocytes were donor derived. [74, 75] Hence, hematopoietic cells are capable of migrating to the liver and differentiating into hepatocytes in rodents and humans; however, dispute still exists about whether the bone marrow-derived hepatocytes are the products of cell fusion or trans-differentiation.

Other bone marrow cells have been shown to differentiate into hepatocytes in vitro, although their role in liver regeneration remains unknown. Multipotent adult progenitor cells (MAPC) are a unique population of adult stem cells that can be isolate from the marrow of multiple mammalian species, including human, rat, and mouse. MAPCs are generated in culture by plating nonhematopoietic adherent cells and serial passaging. They are telomerase positive and grow stably in culture for many passages if kept at low

density. These cells have properties similar to embryonic stem cells in that they can be differentiated toward many different lineages in vitro under the appropriate conditions.[76,77] These cell types include mesenchymal lineages such as muscle, cartilage, and bone; neuroectodermal lineages such as different classes of neurons; and, endodermal lineages such as hepatocytes. Human and murine MAPCs, grown in matrigel in the presents of FGF-4 and HGF, changed their phenotype and expressed multiple hepatocyte functions, including urea synthesis, albumin secretion, Phenobarbital-inducible cytochrome p450 induction, and others. In addition, the cells morphologically appeared hepatocytic and were frequently binucleated.[78]

Stem cell –aided liver regeneration is a highly regulated process involving interactions of multiple cell lineages and signaling molecules. In response to hepatocellular damage, the liver initiates an immune response. A complex array of cytokines and chemokines were secreted most importantly by Kupffer cells and hepatic stellate cells. Those cytokines may act in concert to direct the migration of oval cell or bone marrow stem cell to the damaged area, to control the proliferation and differentiation of stem cells and most likely regulate the remodeling of liver parenchyma. An incomplete list of these cytokines may include HGF, TGF-α, acidic fibroblast growth factor (aFGF), TNF, leukemia inhibitory factor (LIF), stem cell factor (SCF), stromal cell-derived factor 1 (SDF-1), and IL-6 et al.

TNF is an important cytokine associated with oval cell-aided liver regeneration. Inhibition of TNF by dexamethasone administration impairs the proliferation of hepatic cell populations following 2-AAF/PHx, completely suppressing activation of the oval cell compartment.[79] Impaired oval cell proliferation has also been observed in TNF receptor 1

(TNFR1) knockout mice, suggesting that TNFR1 downstream signaling events are required for maximal oval cell proliferation.[80] In addition, *In vitro* studies have shown that TNF stimulates proliferation of the LE/6 murine oval cell line. These studies have outlined the importance of the TNF family of ligands and receptors in the activation/proliferation of oval cells. Although some TNF production can be attributed to infiltrating inflammatory cells, the majority of hepatic TNF produced during liver regeneration is Kupffer-cell derived. Depletion of Kupffer cells by treatment with gadolinium chloride prior to bile duct ligation completely ablates oval cell induction, but not ductular proliferation, suggesting that multiple cytokines produced by Kupffer cells are crucial to the process.[81]

Similarly, oval cell number decreased in the liver of 2-AAF/PHx rat when IL-6 production was inhibited by dexamethasone. This suggests its involvement in the activation of the oval cell compartment.[79] However, comparison of the oval cell response to cocaine induced periportal injury in normal and IL-6 –/– mice demonstrated increased proliferation of periportal oval cells in IL-6 –/– mice, probably to compensate for the decrease in restorative proliferation of hepatocytes, biliary epithelia and sinusoidal cells in those animals.[82] Ten days after injury, the liver was completely repaired in all mice, indicating that IL-6 is not essential for oval cell proliferation. It is feasible that other members of the IL-6 family, including leukemia inhibitory factor (LIF) and/or oncostatin M (OSM), may compensate for the absence of IL-6 in these mice.[83] Indeed, LIF is increased and remains elevated during oval cell induction by 2 AAF/PH, suggesting that it may have a role in the expansion and differentiation of the oval cell compartment.[84] Additionally, *in situ* hybridization has demonstrated LIF, LIF receptor (LIFR) and

glycoprotein (gp)130 mRNA expression in oval cells, with weak expression in parenchymal cells. Oncostatin M has recently been implicated in the maturation of fetal hepatocytes *in vitro* and *in vivo*,[85] and it may have a similar role in the hepatic differentiation of oval cells.

γ-Interferon (IFN- γ) is another inflammatory cytokine considered to play an integral role in controlling stem cell-aided liver regeneration.[86] Suppression subtractive hybridization following 2-AAF/PH identified genes associated with the proliferation of oval cells including IFN- γ, IFN- γ receptor α subunit (IFN- γRα), IFN-γR β primary response genes (gp91phox), IFN-γR β secondary response genes such as ICE, CD54/ICAM-1 and uPAR, cytokines that induce expression of IFN- γ, which include IL1-b and IL-18, and cell adhesion molecules that regulate the interactions between lymphocytes and epithelial cells; lymphocyte function associated molecule 1-a (LFA-1a/CD11 and CD54/ ICAM-1). These proteins are all part of the complex cellular response associated with the IFN-g signaling cascade. γ-Interferon alone is not a hepatic mitogen, however, it can act synergistically with other growth factors such as EGF. Therefore, it has been suggested that interferon functions to prime certain cell populations to respond to mitogenic stimuli.

Evarts et al have shown that hepatic stellate cell proliferation is closely associated with oval cell proliferation. [87] Hepatic stellate cells express hepatocyte growth factor (HGF), acidic fibroblast growth factor (aFGF), transforming growth factor-a (TGFα), and transforming growth factor-b (TGF- β). Interestingly, these cytokines have all been identified in regenerating liver following PH. Although oval cells induced by 2-AAF/PH do not express HGF, they express mRNA for the HGF receptor, c-met.[88,89] High levels of

HGF mRNA are expressed by hepatic stellate cells proliferating in close proximity to oval cells, suggesting that hepatic stellate cell-derived HGF may cause oval cell proliferation via the paracrine activation of c-met.[88] Furthermore, infusion of human recombinant HGF into rats following 2-AAF treatment results in the expansion of several liver cell populations, including the hepatic stellate cells and oval cells.[90] Evidently, this situation also occurs in humans, as elevated levels of serum HGF are present in individuals with chronic hepatitis and cirrhosis,[91-93] both conditions in which oval cell proliferation is well documented.[94,95]

A marked increase of aFGF levels in the liver has been reported at the peak of oval cell proliferation in the 2-AAF/PHx model, and levels greatly exceeded those observed after PHx alone, suggesting a prominent role for aFGF in oval cell-aided liver regeneration.[89] High levels of TGF-α expression are observed not only in hepatic stellate cells, but also in oval cells in the 2-AAF/PHx model.[87,96] TGFα expression is detected in oval cells and foci of oval cell-derived hepatocytes.[97] In contrast, TGF- β is implicated as a negative regulator of oval cell activation. Transforming growth factor- β1 expression on smooth muscle actin (SMA)-positive hepatic stellate cells coincides with oval cell proliferation in the 2-AAF/PHx model and correlates with maximal oval cell apoptosis.[98] As TGF- β1 is proposed to be a negative growth signal that controls liver size by the induction of apoptosis during compensatory hyperplasia, it is possible that TGF- β1 may assist in the remodeling of liver parenchyma during oval cell-mediated liver regeneration by terminating oval cell activation.[98]

The plasminogen activator and plasmin proteolytic cascades also have an important function in stem cell-aided liver regeneration, which is always associated with changes in

the extracellular matrix. The plasminogen activator/plasmin system involves many proteins including urokinase type plasminogen activator (uPA), tissue type plasminogen activator (tPA), the uPA receptor (uPAR), and plasminogen activator inhibitor 1 (PAI-1).[99] The upregulation of uPA mRNA has been observed during oval cell proliferation, and infusion of uPA enhanced the mitogenic response of cells located near bile ducts after the administration of 2-AAF. Expression of uPA, uPAR and PAI-1 is upregulated in the 2-AAF/PHx model of oval cell induction, and localized to the ductal structures formed by the oval cells.[100] uPA expression was also detected in non-parenchymal cells along the hepatic sinusoids. All these data suggest a significant role of plasminogen activator/plasmin system in remodeling of liver parenchyma and migration of oval cells.

Stem cell factor (SCF) and its receptor, c-kit, play a fundamental role in survival, proliferation, differentiation and migration of a variety of stem cells and may similarly affect oval cells. Stem cell factor is induced early in the activation of oval cells by 2-AAF/PHx, but this is not observed following PHx alone.[101] Oval cell precursors express both SCF and c-kit, suggesting that an autocrine mechanism may be involved, although the precise role of this receptor–ligand system in liver regeneration is unclear. Oval cell induction is significantly suppressed in Ws/Ws (white spotting on the skin) rats, in which the c-kit receptor tyrosine kinase (KIT) activity is severely impaired,[70] implying an important role of SCF/c-kit interaction in oval cell activation.

Stromal cell-derived factor 1 (SDF-1) has also been thought to play a role in stem cell-aided liver regeneration.[102-104] SDF-1 is up regulated in the liver of oval cell induction animal models but not in non-oval cell induction models. During oval cell induction in the rat, SDF-1 is expressed in the hepatocytes, while the receptor CXCR4 is

seen on the surface of oval cells. *In vitro* migration assay showed that oval cells responded to SDF-1 gradient and migrated to a high concentration of SDF-1.[102] These data implied that SDF-1 may play a role in oval cell activation. A series of extensive studies have been conducted to investigate the actual role of SDF-1/CXCR4 interaction in stem cell-aided liver regeneration; and will be elaborated in the following chapters of this manuscript.

Stromal Cell-Derived Factor 1

SDF-1 was initially cloned by signal sequence trap strategy to detect polypeptides secreted from bone marrow stromal cells. [105] It is produced by multiple bone marrow stromal cell types, such as osteoblasts, endothelium and adipocytes,[106-108] and by epithelial cells in many organs, such as brain, thymus, heart, lung, liver, kidney, spleen, stomach and intestine.[105-106] It is also expressed during embryogenesis, for example in brain, liver, heart and bone marrow spindle-shaped stromal cells.[109]

SDF-1 belongs to CXC chemokine family. Its only known receptor CXCR4 is a seven transmembrane G-protein coupled receptor that widely expressed by a variety of cell types in developing embryo and adult organs such as brain, lung, heart, thymus, lymph node, spleen mouse, bone marrow, stomach, small intestine, colon, liver and kidney.[110]

SDF-1 was originally identified as a significant growth factor for pre-B cells.[106] Studies with knockout models revealed that murine embryos which lack SDF-1 or CXCR4 have multiple defects which cause high degree of embryonic and perinatal lethality. These mutant mice exhibit a lack of circulating B lymphocytes that is associated with a severe reduction of their precursors in the liver and bone marrow, a defect in myelopoiesis limited to the bone marrow compartment (although normal in the

extramedullary hematopoietic sites), while the developments of T cells and thymus appear normal.[109,111] transplantation of CXCR4 –/– fetal liver cells to irradiated wild-type mice perform poorly in reconstitution of adult bone marrow B cell and myeloid precursors.[112,113] The decreased numbers of B cell and myeloid precursors in CXCR4–/– fetal liver and reconstituted bone marrow are accompanied by the aberrant appearance of the precursors in the bloodstream, indicating that CXCR4 is necessary for retention of these progenitor cells within the fetal liver and bone marrow.[112]

These results demonstrate an essential role for SDF-1/CXCR4 axis in B cell lymphopoiesis and myelopoiesis during embryonic development. In addition, SDF-1/CXCR4 interaction has been implicated in homing of more primitive hematopoietic progenitor cells (HPCs) to the bone marrow. Human CD34+ (multipotential) HPCs express CXCR4 and migrate in response to SDF-1 *in vitro*,[114,115] and pretreatment with blocking antibodies against CXCR4 inhibited engraftment of human CD34+ HPCs in NOD/SCID mice,[116] consisting with a role in HPC recruitment to the bone marrow. CD34+ cells migrating toward SDF-1 include cells with a primitive phenotype (CD34+CD38+ and CD34+HLA-DR-) as well as CFU-mix (=CFU-GEMM), BFU-E, and CFU-GM. HPC recruitment to the bone marrow is critical for engraftment in clinical bone marrow transplantation and may also be important during embryogenesis when hematopoiesis moves from the fetal liver to the bone marrow. SDF-1 expression can be detected in murine fetal liver as early as embryonic day E10.5–E12.5, the period of fetal liver colonization by HPCs; SDF-1 falls off sharply as HPCs begin to emigrate from the liver to the bone marrow (E14.5).[117] On the other hand, mobilization of HPCs from bone marrow to peripheral circulation is also partially controlled through SDF-1/CXCR4

interaction. Elevation of SDF-1 level in the serum or disruption of the CXCR4 function by its antagonist induce mobilization of HPCs.[118,119] These findings indicate that SDF-1/CXCR4 axis not only influence the efficiency of stem cell engraftment into the bone marrow but also modulate the mobilization of the stem cells from bone marrow to peripheral blood.

Beisdes the defect in lymphopoiesis, the SDF-1/CXCR4-deleted mice also display defects in the development of heart, large vessel, gonad and cerebellum.[109,111,120] These facts suggest that SDF-1/CXCR4 axis may play more general role during organogenesis. Indeed, a whole body of evidence has accumulated that functional

CXCR4 is also expressed on the surface of several tissue committed stem/progenitor cells such as primordial germ cells,[120] skeletal muscle satellite progenitor cells,[121] neural stem cells,[122] retinal pigment epithelium progenitors.[123] Finally, we recently found that functional CXCR4 is also expressed on rat hepatic oval cells.[102] Furthermore, CXCR4 ligand, SDF-1 is expressed/secreted by several tissue/organs not only during organogenesis but also in responding to tissue damage. Based on these observations, it is possible that SDF-1/CXCR4 axis also regulate tissue regeneration by tissue-committed stem cells. Although the role of SDF-1/CXCR4 interaction in these processes is not fully elucidated, compelling evidences suggest that the mechanisms for SDF-1 function may include enhancing the survival, proliferation, motility, adhesion, chemotaxis, and secretion of certain factors in the target cells bearing the receptor CXCR4. All of these effects result from the activation of several crucial signaling pathway elicited by SDF-1/CXCR4 interaction on the target cells.

Binding of SDF-1 to CXCR4 triggers the association of the latter with Gαi protein and subsequent signaling cascade. The most important pathways include activation of calcium flux, focal adhesion components, such as proline-rich kinase-2 (Pyk-2), p130Cas, focal adhesion kinase, paxilin, Crk and Crk-L, protein kinase C, phospholipase C-γ (PKC-γ) as well as MAPK p42/44-ELK-1 and PI-3K-AKT-NF-κB axes.[124-128] Strong phosphorylation of focal adhesion components and MAPK p42/44 and serine–threonine kinase AKT is observed within seconds after CXCR4 activation.[129] CXCR4 signalling also involves several src-related kinases and T-cell activating molecule ZAP-70.[130] JAK2 and JAK3[124] and Tyk-2[131] may also associate in some cell types with CXCR4 and are activated, probably by transphosphorylation, in a Gαi-independent manner. As a consequence of this several members of the STAT family of transcription factors may become recruited and phosphorylated. However, involvement of STAT proteins in signaling from activated CXCR4 may depend on the cell type. For example, while STAT 2 and 4 but not STAT 1, 3, 5 and 6 become activated in the haematopoietic CTS progenitor cell line,[132] STAT 1, 2, 3 and 5 but not STAT 4 or 6 become activated in MOLT4 cells.[131]

SDF-1 induced cell mobility and chemotaxis are associated with PI-3K-AKT axis, which regulated rearrangement of F-actin in the leading edge of the migrating target cells. The migration of normal hematopoietic cells is inhibited by employing LY290042, a specific inhibitor of PI3-kinase.[133] The role of the PI-3K-AKT pathway in cell motility is further supported by the observation that murine cells with a disruption of a single gene encoding PTEN (phosphatase and tensin homologue), a negative regulator of AKT phosphorylation/activation, show enhanced phosphorylation of AKT, and as a result

enhanced chemotaxis to SDF-1.[134] SDF-1- mediated cell motility and chemotaxis, however, is affected by several other signaling events probably involving MAPK p42/44 and phosphatases.[135]

The activation of PI-3K- AKT pathway by SDF-1/CXCR4 interaction also leads to the activation of NFκB which in turn induces cell secretion of MMPs (e.g., MMP-2 and MMP-9), nitric oxide and some angiopoietic factors such as VEGF.[136,137] These factors secreted by target cells may play an important role in remodeling of extracellular matrix and facilitating angiogenesis indispensable for tissue repairing and even tumor metastasis.

It is known that SDF-1 modulates adhesion of cells to fibrinogen, fibronectin, stroma and endothelial cells.[116,129,136,138] This pro-adhesive effect of SDF-1 is mediated by the activation of various adhesion molecules (e.g., integrins) on the surface of target cells rather than by increasing their *de novo* expression on the cell surface. [116,136] The integrins activated by SDF-1 include LFA-1 (lymphocyte function associated antigen-1), VLA-4 (very late activation antigen-4) and VLA-5 (very late activation antigen-5).[116] SDF-1 induced firm adhesion and transendothelial migration in human CD34+ haematopoietic cells through LFA-1/ICAM-1 (intracellular adhesion molecule-1) and VLA-4/VCAM-1 (VLA-4/vascular adhesion molecule-1) interactions. These interactions were inhibited by pertussis toxin and cytochalasin D, indicating the involvement of Gαi protein downstream signalling and the requirement of an intact cytoskeleton.[116,136]

The effect of SDF-1 on cell proliferation and survival remains controversial. It has been shown in some experimental conditions that SDF-1 can stimulate the proliferation and survival of haematopoietic cells, and the pro-survival effect of SDF-1 on purified

CD34+ CD38+ bone marrow mononuclear cells was PI-3K-AKT axis dependent.[139-141] In their studies, Zhou and Bonavia found that SDF-1 can also stimulate the proliferation and survival of astrocytes and some tumour cell lines.[142,143] The proliferation effect of SDF-1 on astrocytes was MAPK p42/44 dependent because it is sensitive to inhibition by PD98059, an inhibitor of MEK kinase –the activator of MAPK p42/44.[143] SDF-1 was found to be a survival factor for glioma and glioblastoma cells. This effect correlated with a prolonged activation of the pro-survival kinases AKT and MAPK p42/44.[142,143] However, several other tumour cell lines did not respond by proliferation or increased survival to stimulation by SDF-1. However, according to Majka and Kijowski, SDF-1 did not affect the proliferation/survival of certain cells of hematopoietic lineage such as human CD34+ stem/progenitor cells, erythroblasts, megakaryoblasts, myeloid cells and T-, B-lymphoid cell lines, although it induced the phosphorylation of MAPK p42/44 and AKT.[136,145] These data suggest that in addition to MAPK p42/44 and AKT, other complementary pathways also had to be activated simultaneously in the target cells in order to increase their proliferation/survival.

The activity of SDF-1/CXCR4 axis is modulated by several external factors. The N-terminus of CXCR4 and the first extracellular loop are crucial for SDF-1. Thus enzymatic cleavage of CXCR4 N-terminus by the leucocyte-derived proteases inhibits CXCR4 signaling. The proteases released from activated leucocytesmay also cleave SDF-1 and negatively regulate SDF-1 activity. In addition, SDF-1 may also be N-terminally truncated by cell surface expressed CD26/dipeptidylpeptidase IV, and as a result, the truncated SDF-1 does not possess chemotactic activity and may act even as an antagonist of CXCR4. Furthermore, it has been reported that CXCR4 signaling may also

be desensitized in B- and T-lymphocytes by MIP-1α or RANTES which activate another G-protein-coupled chemokine receptor – CCR5.[146,147] The molecular mechanism of this cross-desensitization between chemokine receptors is unclear, but it is likely that it involves regulators of G-protein signalling (RGS) proteins.

Recently several molecules had been identified that may increase the sensitivity/responsiveness of CXCR4+ cells to SDF-1. These molecules include inflammation factors such as anaphylatoxin C3a, uPAR, thrombin, hylauronic acid, platelet-derived microvesicles and ECM components such as fibronectin, fibrinogen and VCAM-1. They were found to significantly increase the chemotaxis of haematopoietic cells to low/threshold doses of SDF-1.[148-151]

In conclusion, the SDF-1–CXCR4 axis is an important player during organogenesis and tissue regeneration. One of the major effects of this important signal is to direct the migration of stem/progenitor cells including hepatic oval cells. The present study is focused on the involvement of SDF-1/CXCR4 axis in stem cell-aided liver regeneration.

Table 1-1. Rodent models of oval cell induction

Treatment	Reference
Rat	
Azodyes (AZD)	53
2-actylaminofluorene and partial hepatectomy (2-AAF/PH)	54
Galactosamine	55
Choline deficient, ethionine supplemented (CDE) diet	56
Diethylnitrrosamine (DEN)	57
Retrosine and partial hepatoectomy	58
Mouse	
Dipin	59
3,5, -Diethoxycarbonyyl-1,4-dihydrocollidine (DDC)	60
Phenobarbital, cocaine and partial hepatectomy	61

Table 1-2. Liver cell lineage markers

Marker	oval cells	hepatocytes	biliary duct epithelium
Albumin	+	+	+
α-fetoprotein (AFP)	+	fetal only	-
π-GST	+	fetal only	+
cytokeratin 8 (CK8)	+	+	+
cytokeratin 14 (CK14)	+ (human)	-	-
cytokeratin 18 (CK18)	+	+	+
cytokeratin 19 (CK19)	+ (rat, human)	-	+
OV6	+ (rat, human)	-	+
OC.2	+ (rat)	-	+ (rat)
A6	+ (mouse)	-	+ (mouse)

CHAPTER 2
EXPRESSION OF SDF-1 DURING OVAL CELL ACTIVATION

Introduction

Hepatic oval "stem" cells are recognized as playing an important role in the etiology of liver growth and development, as well as in hepatic carcinogenesis.[10,152-154] Initiation of hepatic oval cells is necessary when a severe liver necro-inflammatory insult cannot be corrected through replication of mature hepatocytes. The signal(s) triggering oval cell participation in the regenerative process is still unknown. A key assumption to oval cell research to date has been that the oval cells are native to the liver. This approach may be only partly representative of a wide range of regenerative responses responsible for reconstituting the hepatic mass. For instance, we now know that hepatocytes and bile duct ep-ithelium can also be extra-hepatic derived from bone marrow.[2-4,74,75] What is unknown, however, is how bone marrow–derived cells, presumably "stem" cells, migrate and trans-differentiate into hepatocytes and bile duct epithelial cells. For this to happen, two events must occur: (i) the mobilization of precursor cells residing in the bone marrow, and (ii) the homing and engrafting of precursor cells into the regenerating liver. Cytokines or other humoral agents, released into the blood circulation by the injured liver, could very well promote the first event and determine its magnitude. The same or similar agents, along with the extent of liver lobular architectural disruption created by the injury, could affect the efficiency of the second event.

We set out to identify a possible mechanism for oval cell activation in the injured liver. During embryogenesis the liver develops from a diverticulum of the floor of the

foregut,[6,155,156] where the founder cells invade the mesenchyme of the septum transversum. Endodermal cells eventually give rise to hepatocytes and the bile duct epithelial cells, while the mesenchyme gives rise to cells that make up the sinusoidal lining. During fetal development, the liver functions as the hematopoietic organ.[157,158] The hematopoietic cells found in the developing liver are of extra-hepatic origin, being derived from stem cells of the yolk sac[159,160] and the aorta-gonad-mesonephros (AGM).[161] Recruitment of the extra-hepatic cells to the embryonic liver is required for proper development, but the signals required by which HSCs respond and the mechanism of their movement within the fetus is not totally understood. It has been suggested that this movement could be controlled through the SDF-1/CXCR4 homing interaction between the hematopoietic and stromal cells.[162,163] The importance of SDF-1*a* and CXCR4 in hematopoiesis is supported by observations of embryonic lethality in knockout mice with targeted disruption of the genes for either SDF-1[109] or its receptor CXCR4.[111,113,164] The expression of CXCR4 on a majority of the CD-34 positive and negative cells and a demonstrated role of SDF-1*a*–inducing chemotaxis in these cells strongly suggests that the most primitive hematopoietic population including stem cells is responsive to a SDF-1 chemotactic gradient.[165-167] To date, several reports have shown hepatic oval cells and HSCs share a similar immunohistochemical profile, being positive for Thy-1, CD-34 and c-Kit.[71,72,101,168] In addition, the findings of Lagasse et al. (2000) demonstrated that it was indeed the $Sca^{+}/Thy^{lo}/kit^{+}/lin^{-}$ sub-population of the bone marrow cells that was capable of becoming hepatic tissue.[4]

Using various types of rat liver regeneration models, normal (non-oval cell–aided [i.e. allyl alcohol, AA and CCl_4]) and oval cell–aided models (i.e., 2–acetylaminofluorene

(2–AAF)/CCl_4, 2–AAF/PHx and 2–AAF/AA),[72,168] we tested whether or not SDF-1 protein regulation was affected in a positive or negative manner. Western immunoblotting was used to determine if the type of liver regeneration model affected SDF-1 protein regulation. In addition, immunohistochemistry for SDF-1 was performed on rat liver sections to determine what cell type, if any expresses the protein. Also, immunohistochemistry for CXCR4 expression was performed on sorted cells to determine if oval cells expressed the only known receptor for SDF-1. The results described herein show that, when oval cells are involved in the regenerative process, SDF-1 is up-regulated during the oval cell proliferation process. However, under normal, non-oval cells–aided regeneration, SDF-1 protein expression was not detected. In the former condition, it was found that the oval cells express the SDF-1 receptor, CXCR4, while SDF-1 was expressed by the liver parenchyma. These data indicate a possible mechanism by which oval cells are activated in the injured liver.

Materials and Methods

Materials and Subjects

Carbon tetrachloride (CCl_4), 99% pure HPLC grade and 2-acetylaminofluorene (2-AAF) were purchased from Aldrich Chemical Co. (St. Louis, MO). 2-AAF crystals were incorporated into time released pellets (70 mg/pellet over 28–day release, 25. mg/day) by Innovative Research Inc. (Sarasota, FL). Male Fischer 344 rats (150–170 g) were obtained from Fredericks Laboratories (Frederick, MD). Microscope Superfrost Plus slides and buffered Formalin-Fresh were obtained from Fisher Scientific (Pittsburgh, PA). Iscoves Media was obtained from Invitrogen Corporation (Carlsbad, CA). Unless otherwise stated, all other chemicals were purchased from Sigma (St. Louis, MO). All animal protocols have been approved by the University of Florida Animal Care and

Usage Committee and were conducted within those guidelines. SDF-1*a* antibody and protein was purchased from R&D Systems Inc. (Minneapolis, MN) and CXCR4 antibody was bought from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Thy-1 antibody was obtained from BD Pharmigen (San Diego, CA). Secondary antibodies (Vector Blue and DAB Peroxidase) were purchased from Vector Laboratories (Burlingame, CA).

Liver Regeneration Models

The basic design of the liver injury alone and the 2–AAF/Injury models have been described previously.[169, 170] Briefly, a 2-AAF pellet (70 mg/28 day release, 2.5 mg/day) was inserted subcutaneously 7 days prior to induction of hepatic injury by protocols similar to those described by Novikoff et al. (1996) and Hixson et al. (1990).[171, 172] Normal rat liver was used as a zero time control. For the chemically induced liver injury, a single LD50 dose of AA (37 mg/kg body weight) was administered by intra-peritoneal (i.p.) injection of a 1:50 dilution (vol/vol) in 0.9% saline solution, or a single LD50 dose of CCl_4 (1,500 mg/kg) was administered i.p as a 1:1 (vol/vol) dilution in corn oil. Partial hepatectomies (PHx; 70%) were performed as described by Higgins and Anderson (1931).[12]

Protein Preparation and Western Blot Analysis

The protocol for protein preparation is fully described by Kim et al. (1997).[173] Briefly, total liver lysates were prepared by homogenizing livers of rats subjected to either normal (non oval cell) or oval cell aided regeneration protocols with 10 mM Tris-HCl (pH 7.6) containing 1% sodium dodecyl sulfate (SDS) in the presence of 100 mg/mL bovine serum albumin, 5 mM Na2–ethylenediamineatatraacetic acid (EDTA), 0.1 mM sodium o-vanadate, 1 mM phenylmethylsufonyl fluoride, fluoride, 50 mM sodium fluoride, 30 mM sodium pyrophosphate decahydrate (PP1), 10 mg/mL pepstain, 5 mg/mL

trans-Epoxysuccinyl-L-leucylamido–(4-guanidino) butane (E64), 10 mg/mL aprotinin, and 50 mM 1,10–phenanthroline. Insoluble material was removed by centrifugation at 14,000*g* before assaying for protein concentration.

For the crude soluble and insoluble fractions, the liver tissue from rats subjected to the above mentioned liver regeneration protocols were isolated at the indicated time points (n=3–6) and then homogenized with 10 mM Tris-HCl (pH 7.6), 5 mg/mL E64 1 4 mM diisopropylphosphate, 10 mg/mL each of proteinase inhibitors pepstain and leupepin, 1 mM $Na3VO_4$, and 1 mM EDTA. To prepare the insoluble- and soluble-enriched fractions, the homogenates were centrifuged at 15,000*g* for 3 h at 4°C. The crude soluble and insoluble fractions or whole lysate (5 mg protein) were mixed equal volumes of SDS sample load-ing buffer and separated by 8–18% gradient SDS–polyacrylamide gel electrophoresis gels in the presence of 2–mercaptoethanol as described by Laemmli et al. (1970).[174] Proteins were electrotransferred onto Immun-Blot PVDF membrane (BioRad, Hercules, CA), membranes were then blocked with 2% bovine serum albumin (Boehringer Mannheim) for 1–2 h at room temperature (RT) or overnight at 4°C, followed by incubation with primary antibody, Biotinylated Anti-SDF-1*a*/PBSF, in 1 3 TBST for 2–3 h at RT. The membrane was washed with 1 3 TBST for 30 min at RT with a minimum of three wash changes, then incubated with Strepavidin-Peroxidase Polymer Labeled (Sigma, S9420) for 1 h at RT. The membranes were then washed with 1 3 TBST for approximately 10 min with three changes of wash, the first being a quick rinse of the membrane and the others lasting from 3 to 7 min at RT. After the rinse, the membrane was incubated with ECL1Plus solution (Amersham,

Arlington Heights, IL) according to the protocol provided by the company. Chemiluminescence was detected using x-ray film (LPS, Rochester, NY).

Immunohistochemistry

A basic immunohistochemical protocol previously described by Michalopoulos et al. (1999) was used with slight modification to conform to each particular antibody.[175] SDF-1 was used at a dilution of 1:10. While immunohistochemistry for CXCR4 was carried out using a 1:25 dilution. Secondary anti- bodies were used at 1:100 dilution. Liver tissue was divided and fixed in either 10% buffered formalin or placed in OTC compound and frozen in cold 2–Methylbutane (Fisher Scientific, Pittsburgh), then stored at 280°C until needed. All staining procedures for light microscopy were carried out on 6–mm-thick frozen sections or 4-*m*m-thick paraffin sections. Routine histological examinations were made for all liver tissue samples on sections (paraffin and frozen) stained with hematoxylin and eosin. Single cells suspensions were collected on glass slides by cytocentrifugation and air-dried. Cytocentrifugation was done using a Cytospin 3 Cytocentrifuge (Shandon Inc, Pittsburgh) 6 min at 600 rpms. Immunohistochemistry on cytospin preparations (100,000 cells/slide) was performed using the techniques described above. For each antibody negative controls were performed by either blocking with appropriate non-immune serum or by omitting the primary antibody from the protocol.

Migration Studies

Primary Thy-1^+ hepatic oval cells were isolated as previously described by Petersen et al. (1998a).[72] Motogentic assays were performed on polycarbonate membrane 5-mm pore size tissue culture-treated Transwell inserts (Costar, Cambridge, MA). Cells were plated at high density (100,000/mL) for 2 h in serum free Iscoves media supplemented with gentamycin (50 mg/mL) and insulin (1×10^{-7} mol/L). After 2 h, cells were

stimulated with SDF-1 protein (100 ng/mL), either adding it to the bottom chamber or to the top chamber or in both chambers. Time points of 4 and 6 h post SDF-1 exposure were examined for cell migrations through the membrane (n=3/time point). As a control, cells were treated in the same manner with the exception that no SDF-1 was introduced to the medium. Cells on inserts were fixed and stained as previously described. The cells that attached to the top of the membrane were completely removed by rubbing with a cotton-tipped applicator after the staining. Cell debris was washed away using 1% PBS. Cells that had migrated through to the bottom of the membrane were counted and compared to wells that did not receive SDF-1 exposure. Data obtained was subjected to Student's *t* test to generate a *p* value to show significance.

Results

SDF-1 Protein Expression in Various Liver Regeneration Models

We have previously shown that oval cells do not play an important role in normal liver regeneration brought on by CCl_4 poisoning[170] or PHx[156]. In addition, we have also shown that, to fully activate the hepatic oval cells to proliferate, an inhibitory factor, such as 2–AAF, must be involved.[169],[170] Protein lysate from liver were obtained from rats exposed to acute dose of CCl_4 (alone) and 2–AAF/CCl_4 at various time points following hepatic injury. In order to study liver regeneration in these two models, different sets of time points were used. Compensatory regeneration (i.e., PHx, CCl_4) is completed within 120 h post-injury; therefore, it is necessary to use early time points during the regenerative process. Subsequently, hours were used instead of days, whereas in oval cell–aided regeneration, it is necessary to use time points in days based upon the fact that the peak of oval cell proliferation is reported to be around day 9 post-hepatic injury.[170] If SDF-1 were to play a role in the normal liver regeneration process, one would assume

that the SDF-1 protein would be up regulated at some point during the process. As seen in Figure 2‐1A, no protein was detected at any time during the normal repair process. However, when the hepatocytes have been exposed to 2–AAF followed by CCl_4 injury, a band for SDF-1 can be observed as early as day 1 and continues until day 13 post-hepatic injury (Fig. 2‐1B). The day-13 time point has been reported to show the differentiation of oval cells to hepatocytes.[170]

To further characterize these results, we separated liver lysates into crude soluble and insoluble fractions. SDF-1 has been reported to be associated with membrane bound proteins, including integrins to direct stem cells to specific sites within the body,[176] we performed Western immunoblotting on the membrane fraction of these two different hepatic injury models (Fig. 2‐2). Again, in the acute CCl_4 injury model (Fig. 2‐2A), no signal can be seen, indicating that SDF-1 is not involved in liver regeneration under these circumstances. As seen in the oval cell model of 2AAF/CCl_4 (Fig. 2‐2B), the protein is highly up-regulated, confirming our results seen in Figure 2‐1. Because SDF-1 is associated with the membrane and the total lysate was fractionated, this may explain why there is such a difference between Figures 2‐1 and 2‐2. The inflammatory response brought about by CCl_4 poisoning could possibly account for the more active oval cell proliferation observed in the 2–AAF/CCl_4 model than that seen the 2–AAF/ PHx model.

Liver injury induced by PHx has been shown to up-regulate similar chemokines as CCl_4[177] but does not elicit an inflammatory response. We have previously shown that in the 2AAF/PHx model there appears to be a less pronounced activation of oval cell proliferation, at least by histological technique.[170] To confirm the 2AAF/CCl_4 results, we

examined SDF-1 protein expression in another oval cell model, 2AAF/PHx. As seen in Figure 2-3C, the 2AAF/PHx models exhibit SDF-1 expression. Although expression appears to be not as intense as the 2AAF/CCl_4 model it too appears to exhibit the same pattern of expression.

In the 2AAF/PHx model at about day 13 post-hepatic injury, it appears that the protein is down-regulated. This is the time that oval cells have been reported to begin their differentiation into hepatocytes.[87, 170] Figure 2-3C confirms the data seen in Figures2-1B and 2-2B, showing that SDF-1 is up-regulated when oval cells are present in the regenerating liver. If SDF-1 were involved in the inflammatory response, the protein would have been observed in the acute CCl_4–or AA-alone models, but this was not the case. In addition, because 2AAF/PHx model does not induce an inflammatory response, but does induce an oval cell response as well as expression of the SDF-1 protein, this provides a second model to link the SDF-1 protein to oval cell activation pathway.

Immunohistochemistry for SDF-1α and CXCR4

Figure 2-4 represents immunohistochemistry of SDF-1 on liver tissue sections from both oval cell aided regeneration models (2AAF/CCl_4 and 2AAF/PHx). As shown in Figure 2-4A, there appears to be a slight staining pattern of SDF-1 in the normal liver. As seen in low power magnification fields, the 2AAF/CCl_4 and 2AAF/PHx (Fig. 2-4C,D), respectively, the staining for SDF-1 was on hepatocytes that appeared to be closest to the proliferating oval cells. At higher magnification the individual hepatocytes can be seen staining positive for SDF-1 (Fig. 2-4E,F). It may also appear that the level of expression may depend upon the type of damage (inflammatory, C and E; or non-

inflammatory, D and F). The immunohistochemistry does support our Western analysis. The fact that SDF-1 is up regulated further goes to supporting the concept that oval cells could be activated or recruited to an injured liver in response to a chemotactic gradient. It should be noted that the staining patterns we show appears to be consistent in staining described by Pablos et al. (1999).[178]

In order to complete the pathway for SDF-1, its receptor must also be involved. Figure 2-5 represents immunohistochemistry of CXCR4 on tissue sections from both models of oval cell–aided regeneration (2-AAF/CCl4 and 2-AAF/PHx). Little to no staining can be seen in normal rat liver (Fig. 2-5A). As seen in the photomicrographs only the oval cells appear to be expressing the receptor (Fig. 2-5C, D).

We have previously shown that oval cells express the hematopoietic stem cell marker Thy-1 and through cell sorting techniques we can obtain a highly purified population of Thy-1+ oval cells. In addition, we have previously reported this isolated population of cells to be lineage negative as well, meaning they do not express markers that would define them to be committed toward a specific lineage.[72] Thy-1–FITC labeled sorted cells were cytocentrifugated (Fig. 2-5G green) on to microscope slides and stained for CXCR4–Texas Red the receptor for SDF-1 (Fig. 2-5E, red). As seen in Figure 2-5F, the merged image (yellow) reveals that the oval cells do indeed express the receptor indicating a possible protein-receptor interaction pathway for activation/recruitment of oval cells in the injured liver.

Previously we have reported that the 2AAF/AA model produces a small oval cell response.[170] In order to determine whether or not the results in Figure 2-3A, B were

credible, we performed immunohistochemistry on liver section obtained from 2AAF/AA exposed rats.

As seen in Figure 2-6A, there was little to no staining observed on the hepatocytes. This would appear to confirm our protein results. However, there were a few clusters of smaller cells in close proximity to the proliferating oval cells that were positive for SDF-1. These smaller cells could be stellate cells or fibroblast cell found in the liver. In contrast to the 2-AAF/PHx and 2AAF/CCl_4 models, where the oval cells stained positive for CXCR4, the 2AAF/AA oval cells were negative for CXCR4 (Fig. 2-6B,C). This may suggest there may be a significant difference between the different types of oval cell proliferation models. Immunohistochemistry was also performed on liver section obtained from acute AA exposed rat. Figure 2-6D reveals that, even in areas where high levels of damage are seen with an inflammatory response, there was little evidence of staining observed. Again, this may suggest that it takes more that just injury to induce the SDF-1/CXCR4 pathway.

To test if oval cells respond to the chemotractant ability of SDF-1 has been reported to exert on stem cells, experiments were designed in such a way to show the specificity of the oval cells to migrate to a gradient of higher concentration of SDF-1. Figure 2-7 represents the ability of oval cells to migrate to the area where there is a higher concentration of SDF-1. When SDF-1 was placed in the bottom chamber of the culture system, there was an approximately 11–fold increase in the number of cells that migrated through the membrane as compared to controls. However, to illustrate that the homing effect was due to motility and not from chemokinesis, SDF-1 was placed in the top chamber or in both chambers (top and bottom) to determine if oval cells would still

migrate through the membrane. As shown in Figure 2-7, there was some movement of oval cells as compared to controls, but the fold increase was negligible in both conditions. When the data was subjected to a Student's *t* test, a *p* value equaling 0.00964 was obtained for the 4-h time point with a similar *p* value for the 6-h time point.

Disscusion

In recent years there has been an increasing body of evidence supporting the concept that adult stem cells have a far greater degree of plasticity than once thought. Bone marrow associated stem cells have been able to produce endothelial, mesenchymal and epithelial cell types found in the body.[2,179,180] Neural stem cells have been shown to be capable of differentiating into blood cells and then back to brain cells.[181] In addition, brain stem cells have been shown to produce an entire mammalian system (mouse).[182] These studies were conducted in rodent models, and only suggest that adult stem cells may be exploited to treat dysfunction found in the body. Theise et al. (2000) and Alison et al. (2000) both reported[74,75] that human adult bone marrow stem cells could differentiate into mature hepatocytes, thereby providing a link from animal studies to human studies, and proof of concept, which may lead to clinical applications in the near future. The next critical step is to utilize the rodent liver to dissect the mechanisms regulating oval cell activation and/or the bone marrow to liver migration process.

HSCs and progenitor cells can be found throughout the entire body, mainly within the bone marrow of an adult, but they are also present in a number of different organs. For instance a lethally irradiated animal can be rescued and have its bone marrow reconstituted through liver transplant.[183] Stem cells and progenitors traffic to different organs during embryonic and fetal development and can move from organ to organ

during adult life, especially during times of stress- and injury-induced states such as during infection.[166,167] There are over 50 different chemokines, most of which have suppressive functions in HSC proliferation.[166] Such redundancy is a phenomenon found throughout nature and in many biological systems, but the chemokine system appears more prone to have back-ups to back-ups. Perhaps this redundancy is the means by which HSC proliferation is kept in balance in organs in which proliferation is not needed or warranted during steady-state functions such as normal liver regeneration.

The movement or homing of cells to organs also needs integrin-mediated adhesion to the ECM components like fibronectin. This adhesion can be activated by certain cytokines such as stem cell factor (SCF).[166] It is also reported that SDF-1 is constitutively expressed in most major solid organs found in the body (i.e. brain, liver, pancreas, lung, heart, kidney, and spleen) as well as the bone marrow and the surrounding cells of the germinal center.[166] Also, there is only one known receptor for SDF-1*a*, which is CXCR4. It also was found that CD-34- cells had a higher expression of CXCR4 (77%) than CD-34+ population, which were only (61%),[184] possibly indicating that CD-34- cells are more primitive than CD-34+ cells. With the results from LaGasse et al. (2000), indicating that a population of bone marrow stem cells with a Sca-1+/Thy-1lo/kit+/Lin-/CD-34- phenotype are the cell type within the bone marrow capable of differentiating into hepatocytes.[4] In addition, there are recent reports showing that mutlipoten adult progenitor cell (MAPC) of mesenchymal lineage could trans-differentiate into hepatocytes both *in vivo* and *in vitro*.[76,78]

To speculate and place into context a number of different reported events that occur in the liver during the oval cell–aided regeneration process, we suggest the following

scenario. SDF-1 is produced in the liver, which then activates oval cells to proliferate. As the progenitor cells enter the liver lobule they come in contact with adhesion molecules like fibronectin. It has been reported that there is an increase in soluble fibronectin, an ECM molecule that would facilitate their engraftment.[185] The expansion in the number of stellate cells that occurs in the periportal regions of the liver[186] would next result in an increased production of the growth factors such as hepatocyte growth factor (HGF) and transforming growth factor–alpha (TGF*a*).[187,188] At the same time HGF and TGF*a* are being up regulated, transforming growth factor–beta (TGF-*b*1) is also being up-regulated by the expanding Kupffer and stellate cell population.[189,190] In order for stem cells to differentiate down the hepatic lineage, the differentiation of HSC down the hematopoietic lineage would have to be blocked. TGF-*b*1 and tumor necrosis factor–alpha (TNF*a*) have been shown to suppress the differentiation of progenitor HSCs into megakaryocytes and down the myeloid lineage.[191,192] In addition; HGF would act as strong promoter of differentiation toward the hepatic lineage.[175] Both oval cells and HSCs express the HGF receptor c-Met.[193] In addition, stores of HGF and epidermal growth factor (EGF) in the periportal region of the liver[194] can be released from the ECM by an up regulation of uPA.[100] Thus, the necessary contributors to the processes of homing, engrafting and differentiation would be in the right place at the right time to signal to the oval cell of its final fate in the liver. These factors could be the signals, plus, as of yet undetermined signals, which oval cells need to begin to differentiate down the hepatic lineage. In a recent study, Muller et al. (2001) showed that the CXCR4 is highly expressed in human breast cancer cells and metastases. In addition, they reported that SDF-1*a* exhibited peak levels of expression in organs representing the first destination of breast cancer

metastasis.[195] Their study showed that chemokines and their receptors have a critical role in the movement of breast cancer metastasis.

The SDF1/CXCR4 interaction is fairly unique, meaning that CXCR4 is the only known receptor for SDF-1 and because of its specificity and its location throughout the body it makes this protein- receptor interaction a good candidate for homing bone marrow derived cells to various sites of injury. This interaction could also be viewed as a signal to initiate the oval cell compartment in certain forms of liver regeneration. Our data begins to shed light on a possible mechanism for oval cell activation and a possible signal, which could aid in the recruitment of bone marrow derived stem cells to the liver as a second wave of cells enter the injured organ. This may someday lead to a better understanding of the hepatic and hematopoietic interaction in oval cell activation and proliferation. This may in turn lead to better clinical relevance in treating patients through stem cell therapies, but further experiments will need to be performed.

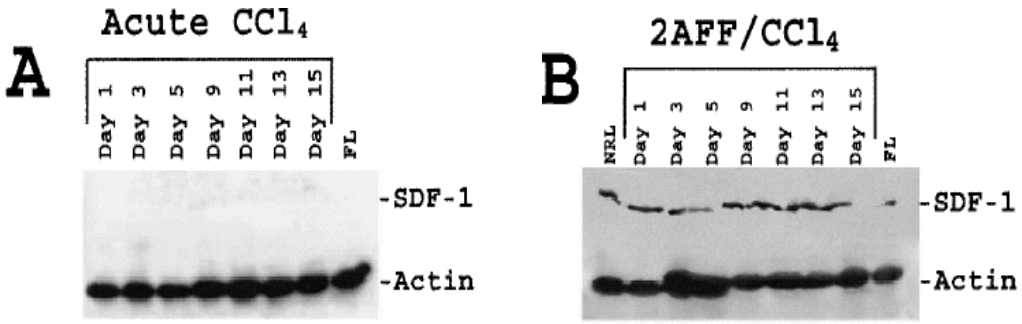

Figure 2-1. Western blot analysis on whole cell lysate obtained from male Fisher 344 rats treated with CCl4 A) and 2AAF/CCl4 B), with 5mg of protein loaded in each well. Note there is expression of SDF-1 in the 2–AAF/CCl4 model in B, whereas there is no expression in the acute model. Actin bands are shown to provide a reference to loading consistency. Protein was pooled from n= 3–6 samples per time point.

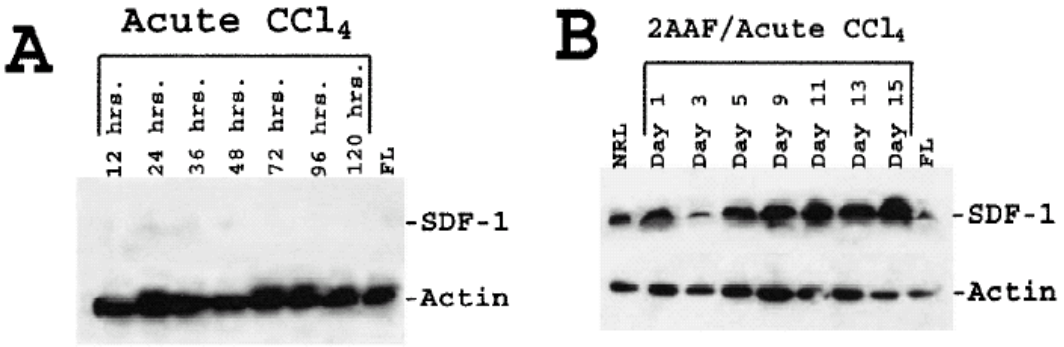

Figure 2-2. Western blot analysis for SDF-1a on crude insoluble extracts from both CCl4 A) and oval cell B) aided model. 5 mg of protein loaded in each well. Notice that, even in the enriched fraction of membrane, there appears to be no expression of SDF-1 in the CCl4 model. As seen in the whole cell lysate fraction B), the expression of SDF-1 is highly expressed in the oval cell aided model of regeneration. Actin bands are shown to show loading consistency. Protein was pooled from n=3–6 samples per time point.

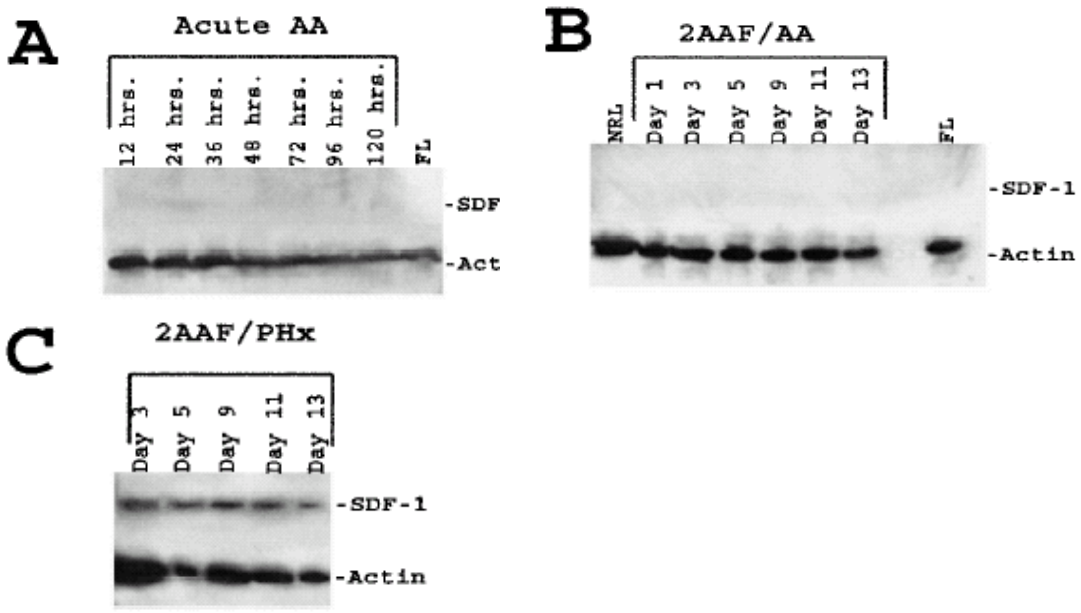

Figure 2-3.. Western blot analysis for SDF-1 in the crude insoluble extracts from acute AA A), 2-AAF/AA B), and 2AAF/PHx C) treated male Fisher 344 rats. 10 mg of protein was loaded in each well for A and B; only 5 mg of protein was loaded for C. In both the acute AA model and the 2AAF/AA model, there appears to be no expression of SDF-1, even though samples were overloaded as seen by actin expression. To confirm the results seen in the 2AAF/CCl4 model, we tested the insoluble fraction from rat livers exposed to the 2AAF/PHx protocol. And again we observed an up-regulation of SDF-1. Protein was pooled from n =3–6 samples per time point.

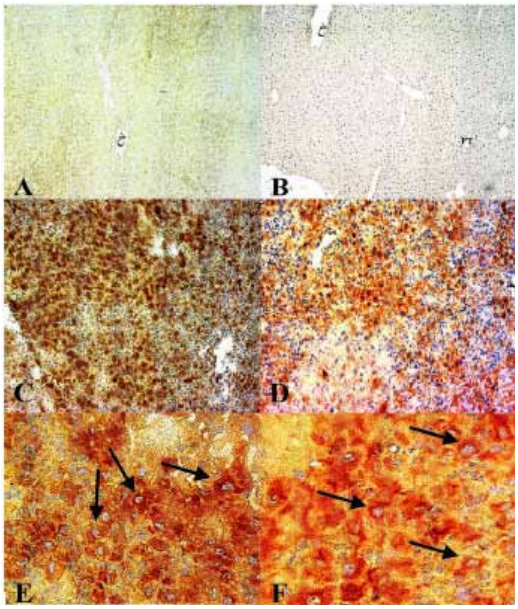

Figure 2-4. Immunohistochemistry for SDF-1 on frozen rat liver sections obtained from male Fisher 344 rat. A) Normal (control) liver for representation of normal levels of SDF-1 expression. B) From normal rat liver without antibody to serve as the negative control. C), D) Oval cell–aided regeneration model of 2AAF/CCl_4, day 9, and 2AAF/PHx, day 9, respectively. E) 2AAF/CCl4 model day 9 post-injury. F) 2AAF/PHx day 9 post-injury. CV, central vein; arrows point to a few representative individual hepatocytes.

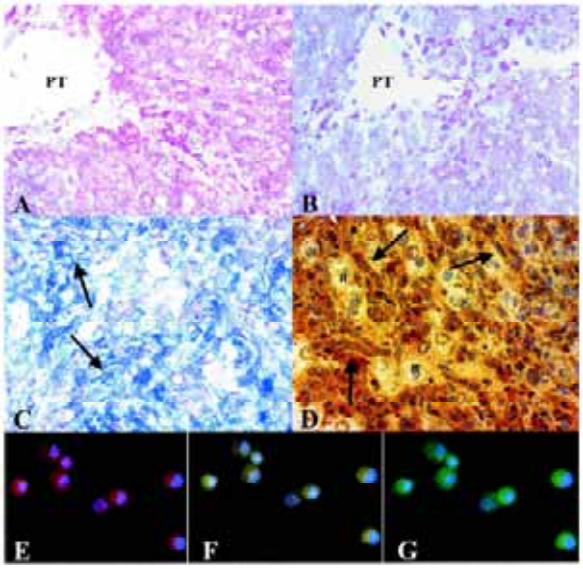

Figure 2-5. Immunohistochemistry for CXCR4 on frozen rat liver sections obtained from male Fisher 344 rat. A) Normal liver showing no CXCR4 expression. B) From normal rat liver without antibody to serve as the negative control. C, D) Oval cell–aided regeneration models of 2AAF/CCl4, day 11, and 2–AAF/PHx, day 13, respectively. E,G) Cytospin of Thy-11–FITC-labeled cells (stained green), sorted from animals on the 2–AAF/CCl4 protocol, stained for CXCR4–Texas Red (E-red stain). A–C were stained using vector blue detection and counter-stained with nuclear fast-red. D was visualized using DAB-peroxidase (brown) and counter-stained using Mayors hematoxylin. The nuclei for E–G were counter-stained using DAPI (blue staining). PT, portal triad; H, representative hepatocytes; arrows point to representative oval cells that are positive cells.

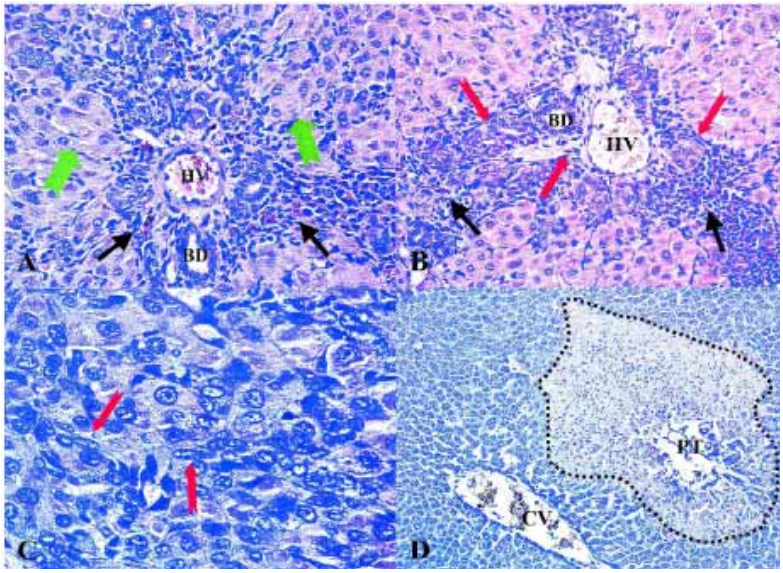

Figure 2-6. Immunohistochemistry for SDF-1 and CXCR4 on liver sections obtained from rats exposed to 2AAF/AA day 9 post-injury. A) There is very little to no staining for SDF-1. The smaller positive cells could be stellate cells, which are known to be in close proximity to proliferating oval cells. B,C) No staining for CXCR4 on the oval cells. D) Staining for CXCR4 on tissue obtained from rat liver exposed to AA 24-h time point. The area inside the dashed line represents the damage brought on from AA exposure, and even though there is a considerable amount of inflammation, no staining is observed. All photomicrographs were taken using an Olympus BX51 microscope. A and B have an original magnification of 40X. C was taken under 100X oil immersion, and D magnification was 20X. Green arrows in A indicate hepatocytes, while black arrows in A and B point to small clusters of positive cells, SDF-1, and CXCR4, respectively. Red arrows in B and C point to representative individual oval cells. HV, hepatic vein; BD, bile duct; CV, central vein; PT, portal triad.

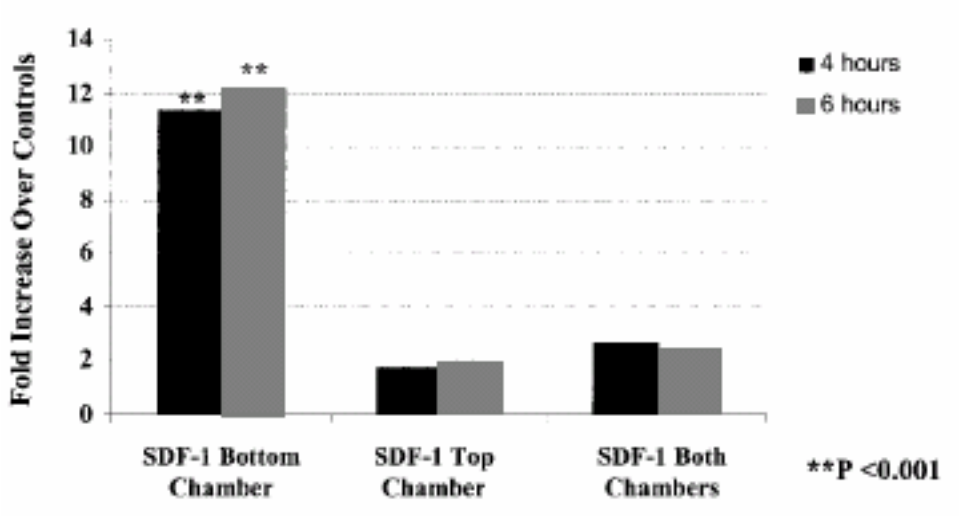

Figure 2-7. Comparison of the motogenetic effects of SDF-1 on primary cultures of liver oval cells plated at high density. SDF-1 was added to the cultures 2 h post-plating, either in the bottom chamber, the top chamber, or in both chambers. Time points of 4 and 6 h were examined for cell migration through the trans-membrane. When SDF-1 was only added to the bottom chamber, there was an approximately 11-fold increase of cells moving to the side with the higher concentration of the protein as compared to control cultures. When SDF-1 was added to either the top chamber or to both chambers, the fold increase of cells migrating was minimally increased and did not reach the levels seen when the protein was only in the bottom chamber. Values are given as mean of $n = 3$.

CHAPTER 3
KNOCKDOWN OF SDF-1 COMPROMISES OVAL CELL ACTIVATION

Introduction

In general the liver relies on two types of responses to regenerate after major tissue loss: (1) proliferation of existing hepatocytes and (2) to a lesser extent, the activation of stem/progenitor cell compartment. Mature hepatocytes have a remarkable replication capability and are very efficient in restoring hepatic parenchyma after liver injury caused by a variety of methods (i.e. partial hepatoectomy (PH), hepatic toxins, hepatotrophic virus infection and so on); and thus are enlisted as the first line of regeneration. However, in some situations where hepatocyte replication is suppressed, such as following treatment with 2-acetylaminofluorene (2-AAF), oval cells will proliferate and differentiate to replenish the hepatic mass. Oval cells in this case have been regarded as facultative liver stem cells capable of differentiating into both hepatocytes and bile duct epithelial cells.[10, 11]

The question of the origin of oval cells remains open. It has been suggested that oval cells or their precursors reside within or adjacent to the canal of Hering, and expand into the liver parenchyma after activation.[67] Other evidence demonstrates that bone marrow stem cells might be an alternative source of the liver progenitor cells,[2-4]migrating to and engrafting in the liver, giving rise to oval cells then hepatocytes. Regardless of the origin, oval cells or their precursors must depend on proper signal(s) to mediate activation, migration and differentiation. The molecular signaling microenvironment at the site of liver injury consists of a complex array of growth factors, cytokines,

chemokines, extracellular matrix (ECM) as well as cell-cell contacts. Factors that have been associated with the oval cell response include, but are not limited to, hepatocyte growth factor (HGF); [91-93,188] transforming growth factor-α (TGF-α);[89,96] acidic fibroblast growth factor (aFGF); [89,196] tumor necrosis factor (TNF); [79,80] leukemia inhibitory factor (LIF); [84] stem cell factor (SCF); [70,101] γ-interferon (INF-γ) [197] and plasminogen activator/plasmin system [100], but the precise roles of these proteins are unclear.

SDF-1 is a member of CXC chemokines first identified from bone marrow stromal cells, and later found in most major solid organs in the body including liver. One of the functions of SDF-1 is to direct cell migration along a SDF-1 gradient, from low concentration to high concentration. This is triggered by binding of SDF-1 to the G-protein coupled receptor CXCR4 on the surface of responding cells. The SDF-1/CXCR4 axis plays an essential role in hematopoiesis presumably through directing hematopoietic stem cells (HSCs) to their final niches.[198,199] Besides, SDF-1/CXCR4 interaction may have a more general role during embryogenesis and postnatal tissue regeneration involving various tissue-committed stem cells. For example, some neural precursors,[200] endothelial progenitors[201] and primordial germ cells[202] also express functional CXCR4 on their surfaces and the importance of SDF-1/CXCR4 interaction on these cells have been illustrated by the defects of brain,[111] large vessel[164] and germ cells[202] found in the embryos of CXCR4 -/- mice. Previous findings from this laboratory have reported that SDF-1 was up-regulated during oval cell activation but not during normal liver regeneration. In the 2-acetylaminofluorene/partial hepatectomy (2AAF/PHx) oval cell activation model, SDF-1 was expressed by hepatocytes,[102] while its receptor CXCR4 was expressed on the oval cell surface. [102,103] *In vitro* migration assays demonstrated that oval

cells migrate to a gradient of higher SDF-1 concentration.[102] These observations bring up the possibility that the SDF-1/CXCR4 axis may play a role in oval cell activation, although the significance of this interaction on the oval cell response is yet to be determined.

In the present study, RNA interference was employed to knock down the SDF-1 signal in the livers of 2AAF/PHx treated rats, providing a more clear view of the role of the SDF-1/CXCR4 axis during oval cell activation. The oval cell response was assessed by histology, as well as Northern and Western analyses. These results indicate that the oval cell response was compromised when SDF-1 expression was suppressed within the regenerating liver, suggesting an important role of SDF-1 in oval cell activation.

Materials and Methods

Animal Experiments

Two-month old Female F-344 rats were used for all experiments. A 2AAF pallet (70mg, released over a period of 28 days) was implanted into the peritoneal cavity. 70% partial hepatoecomy was performed 7 days after 2AAF implantation, and 6×10^{10}pfu of recombinant adenovirus was infused through tail vein immediately following PHx. Animals were sacrificed at day 9 after PHx and liver tissues collected for further studies. All animal studies were conducted according to the NIH guidelines for animal use and institutionally approved protocols.

Recombinant Adenovirus

SiRNA Expression Cassette. Invert repeat DNA fragments based on rat SDF-1 coding sequence was inserted at the +1 position of mouse U6 promoter. The transcribed RNA is therefore predicted to form a small hairpin, which will be further processed into

siRNA within the target cell. Control vector was constructed in a similar way except that a scrambled sequence was used.

Mouse U6 promoter (-315 to +1) was amplified from mouse genomic DNA by PCR using primer pairs: 5'-ACT AGT GAT CCG ACG CCG CCA TCT CTA GGC-3' and 5'-GGG CCC AAA CAA GGC TTT TCT CCA AGG GAT ATT TA-3'. Oligonucleotide pairs: 5'-TGT GCA TTG ACC CGA AAT TTC AAG AGA ATT TCG GGT CAA TGC ACA CTT TTT GGT AC-3' and 5'-CAA AAA GTG TGC ATT GAC CCG AAA TTC TCT TGA AAT TTC GGG TCA AGT CAC A-3' were annealed to form invert repeat DNA template for siRNA against SDF-1 (siSDF). Oligonucleotide pairs for scrambled siRNA (si-scramble) templates are: 5'-GCA TAT GTG CGT ACC TAG CAT TCA AGA GAT GCT AGG TAC GCA CAT ATG CCT TTT TTG GTA C-3' and 5'-CAA AAA AGG CAT ATG TGC GTA CCT AGC ATC TCT TGA ATG CTA GGT ACG CAC ATA TGC-3'.

Generation of Recombinant Adenovirus. Adeno-X expression system (BD biosciences clontech, CA) was used to make adenoviral vectors containing the siSDF expression cassette and control adenoviral vector containing the si-scramble cassette. U6 promoter and invert repeat DNA were inserted into pShuttle vector and the expression cassettes were further transferred to BD Adeno-X vector. The adenoviral vectors were then used to transfect AD-293 cells (Stratagene, TX) to produce adenovirus. Recombinant adenoviruses were enriched and purified using ViraBind Adenovitus purification kit (Cell Biolabs Inc. CA). All procedures were performed following the manufacturer's instructions. The adenovirus containing the siSDF cassette was designated as Ad-siSDF, and the control virus Ad-scramble.

Immunohistology

Immunofluorescent Staining for SDF-1. Formalin-fixed liver tissues were embedded in paraffin and cut into 5μm sections. After deparaffinization and hydration, sections were microwaved for 7 minutes in 0.01 M citrate buffer (PH6.0). The sections were then washed with Tris-buffered saline (TBS) and incubated with goat polyclonal anti-SDF1 (sc-6193, diluted 1:50, Santa Cruz Biotechnology) for 1 hour at room temperature. Fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG (FI-5000, diluted 1:200, Vector Laboratories) was used as the secondary antibody.

Immunostaining for OV6. Cryostat sections (5μm) were fixed in acetone (-20oC) for 10 minutes. After serum blocking and avidin/biotin blocking, sections were incubated with mouse anti-OV6 antibody (diluted 1:150, gift from Dr. Stewart Sell) for 1 hour at room temperature and later with biotinylated anti-mouse IgG (Vector Laboratories) for 30 minutes at room temperature. The staining reaction was developed using Vectastain elite ABC kit (PK-6200, Vector Laboratories) and diaminobenzidine tetrahydrochloride (DAB) substrate (SK-4100, Vector Laboratories).

Immunostaining for Ki67. 5μm paraffin sections were microwaved for 7 minutes in 0.01 M citrate buffer (PH6.0) after deparaffinization and hydration. The sections were then washed with TBS. After normal serum blocking and avidin/biotin blocking, sections were incubated with mouse anti-Ki67 antibody (diluted 1:100, PharMingen 556003) for 1 hour at room temperature and later with biotinylated anti-mouse IgG (Vector Laboratories) for 30 minutes at room temperature. The staining reaction was developed using Vectastain elite ABC kit (PK-6200, Vector Laboratories) and diaminobenzidine tetrahydrochloride (DAB) substrate (SK-4100, Vector Laboratories).

Northern Blotting

Total RNA was isolated from liver tissues using RNA-Bee^{TM} reagent (CS-501B, Tel-Test, Inc.) 10μg of total RNA were loaded onto a denaturing 1% formaldehyde agarose gel, electrophoresed in the presence of formaldehyde and transferred to Genescreen nylon membrane (NEN Life Science). Hybridization and stripping were performed according to manufacturer's instructions. DNA probes for AFP, SDF-1 and Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) were labeled with (α-^{32}P) dCTP using Megaprime DNA labeling system (Amersham Bioscienses) and following manufacturer's instructions.

Western Blotting

Liver lysates were separated by SDS-PAGE and transferred to Immun-Blot PVDF membrane (Bio-Rad) using standard technique. For α-fetoprotein (AFP) detection, goat polyclonal anti-AFP IgG (sc-8108, Santa Cruz Biotechnology) and horseredish peroxidase (HRP) conjugated anti-goat IgG (sc-2352, Santa Cruz Biotechnology) was used as primary and secondary antibody, respectively. ECL plus western blotting detection kit (RPN 2132, Amersham Biosciences) was used for development of the membrane. Membranes were stripped and blocked before detection of different proteins. For SDF-1 detection, goat polyclonal anti-SDF1 (sc-6193, Santa Cruz Biotechnology) was used as primary antibody and HRP-conjugated anti-goat IgG (sc-2352, Santa Cruz Biotechnology) as secondary antibody. For β-actin detection, mouse anti-actin IgG (ab6276, Abcam) was used as primary antibody and HRP-conjugated anti-mouse IgG (Amersham Life Sciencenses) as secondary.

TUNEL Analysis

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL) assay was performed on paraffin-embedded liver sections to detect apoptotic cells using the ApoAlert™ DNA fragmentation assay kit (Cat. 630107, BD Biosciences Clontech), and following manufacturer's instruction.

Results

SDF-1 Expression in Rat Liver after 2AAF/PHx

SDF-1 expression was not detected by immunostaining on quiescent liver cells before oval cell induction (Figure 3‐1A). An increasing number of hepatocytes were decorated with SDF-1antibody over time as the oval cell reaction progressed. By day 7 and day 9 after 2AAF/PHx, most of the hepatocytes located within pericentral region were positive for SDF-1, while most of the cells located within portal regions were SDF-1 negative. (Figure 3‐1B and C)

Suppression of SDF-1 Expression by SiSDF in the Livers of 2AAF/PHx Rats

Recombinant adenovirus has been used for hepatic gene transfer to express therapeutic gene products in mice, rats, rabbits, and dogs. From these studies, intravenously administered adenovirus has proven to be highly efficient in transducing non-dividing hepatocytes.[203] In the present study, adenovirus was used to deliver the siRNA expression cassette into rat liver after 2AAF/PHx. 9 days after virus infusion, SDF-1 expressions in Ad-siSDF transduced livers were remarkably knocked down in more than 90% of the hepatocytes (Figure 3‐2E), while the Ad-scramble treatment did not inhibit up-regulation of SDF-1. (Figure 3‐2C) Northern blot and Western blot analyses of Ad-siSDF treated livers further confirmed decreased levels of SDF-1 mRNA

and protein. (Figure 3‐4, lane 3) It is unlikely that suppression of SDF-1 production was due to non-specific inhibition of protein synthesis by foreign RNA or adenovirus infection because similar levels of SDF-1 mRNA and protein were seen between Ad-scramble treated 2AAF/PHx liver and 2AAF/PHx liver (Figure 3‐4, lane1 and lane 2).

Inhibition of Oval Cell Response by Knocking Down SDF-1

Day 9 after 2AAF/PHx and Ad-scramble infusion, numerous oval cells appeared at the portal regions (Figure 3‐2D). Oval cells featured an ovoid-shaped nucleus and high nucleus/cytoplasm ratio when stained with hematoxylin and eosin. A remarkable decrease in oval cell proliferation was seen in the liver of 2AAF/PHx rats treated with Ad-siSDF (Figure 3‐2F). Consistent with the morphologic findings, the number of $OV6^+$ oval cells was dramatically decreased in Ad-siSDF treated 2AAF/PHx rats as compared to that of Ad-scramble treated rats (Figure 3‐3C, 3‐3D versus Figure 3‐3A, 3‐3B).

Northern blot and Western blot analyses on α-fetoprotein (AFP) showed that this oval cell marker protein was dramatically reduced at both the RNA and protein levels in the livers of Ad-siSDF treated 2AAF/PHx rat (Figure 3‐4), which further confirmed that the oval cell activation was compromised in these animals.

Decrease of Oval Cell Number Was Not Related to Oval Cell Apoptosis

Since SDF-1 has been shown, in some studies, to be capable of protecting $CD34^+$ progenitor cells from apoptosis, [141] it was considered that the decreased number of oval cells seen in Ad-siSDF treated animal was the result of apoptosis of those cells. To address this issue, TUNEL staining of the liver sections was used to assess apoptosis in the livers of all rats used in these studies. Apoptotic cells were rarely detected in all three

groups of rats (Figure 3‐5). Hence, suppression of SDF-1 did not increase oval cell apoptosis evidently. The decrease in oval cell numbers seen in Ad-siSDF treated animal results likely from suppressed proliferation of oval cells.

Consistent with this notion, Ki67 staining revealed that the number of cells entering active cell cycle was far lower in Ad-siSDF treated rat than in control rat in day 13 after the treatment. (Figure 3‐6) The cells proliferating around day 13 included oval cells and their progenies---new hepatocytes and bile duct cells. This result indicates that knockdown of SDF-1 expression in the damaged liver inhibits the proliferation of hepatic cells, and thus hinders oval cell-aided liver regeneration.

Discussion

A role for the SDF-1/CXCR4 axis in stem cell-aided liver regeneration has been suggested by several studies, all of which demonstrated a correlation between SDF-1 expression and stem cell accumulation in the liver. [102-104] In the present study, the well-characterized 2AAF/PHx model was used to determine the role of SDF-1 in oval cell activation. In 2AAF/PHx model, oval cell number increases markedly by day 5 following PHx and peaks at approximately day 9.[170] Consistent with the previous findings, SDF-1 was expressed mostly in the pericentral region of the liver during oval cell activation (Figure 1). These findings are in contradiction with those of Mavier *et al* (2004), who demonstrated in a similar model that SDF-1 was strongly expressed by oval cells while only rarely detected in hepatocytes.[103] The discrepancy might stem from the use of different antibodies. According to our present findings, production of SDF-1 was polarized across the liver lobule with high levels of expression near the central vein and virtually no expression around the portal triad. SDF-1 is known to bind heparin sulfate

associated proteoglycan on cell membrane and extracellular matrix (ECM).[204] SDF-1 is critical for cell trafficking in a number of important biological events including hematopoietic stem cell homing, [198, 199] cancer metastasis[205] and primordial germ cell migration.[202, 206] During oval cell reaction in 2AAF/PHx rat model, oval cells proliferate and radiate from the periportal region (possible from canal of Hering). Since oval cells express CXCR4 on their surface, it is possible that oval cells arising from periportal region respond to the SDF-1 gradient across the liver lobule and migrate into the parenchyma, where the microenvironment is favorable for their differentiation into hepatocytes.

The interaction of SDF-1 with CXCR4 also increases secretion of matrix metalloproteinase (MMPs) by target cells. These proteolytic enzymes play an important role in stem cell migration through vascular basement membranes.[137]At early stages of oval cell activation; oval cell ductules are surrounded by basement membrane, which disappears from the oval cell foci when they begin to differentiate into small hepatocytes.[207] An *In vitro* study showed that hepatic stem cells express an hepatocyte phenotype only when losing touch with basement membrane matrix, [208] suggesting that degradation of basement membrane may be critical for oval cells to differentiate down hepatocytic direction. A SDF-1 signal elicited in oval cell activation might stimulate MMPs secretion from oval cells or other nonparenchymal cells (Kupffer cells, stellate cells et al) proximal to the oval cell foci. The degradation of the surrounding basement membranes by these MMPs may facilitate oval cell migration into liver lobules and allow them to differentiate into small hepatocytes. AFP is a widely used oval cell marker indicative of hepatocytic commitment. In this study, AFP expression was lost in the liver

when SDF-1 signal was knocked down during oval cell activation; supporting our hypothesis that SDF-1 may function to direct oval cell movement into the hepatic parenchyma and differentiation into hepatocyte in 2AAF/PHx rat model.

The effect of SDF-1 on cell survival/anti-apoptosis remains controversial. Recent studies found that activation of the SDF-1/CXCR4 axis prevents certain hematopoietic stem/progenitor cells or cell lines from apoptosis *in vitro*, [139-141] while others did not.[136, 145] Apoptosis has been reported in later stages of oval cell-aided liver regeneration. Yano et al (2004) observed apoptosis of hepatocytes surrounding oval cells at day 9 post 2AAF/PHx.[209] In another study, oval cell apoptosis was seen to peak at day 10 after 2AAF/PHx, concurrent with the secretion of TGFβ by hepatic stellate cells. It is believed that apoptosis of these cells represents a mechanism for modulating hepatic cell number and remodeling of liver parenchyma in the final stages of oval cell-aided liver regeneration.[189] In our oval cell activation model, apoptosis was not evident at day 9 after 2AAF/PHx under either SDF-1 suppression or up-regulation. However, since survival of oval cells was not compromised by knocking down of SDF-1, the decrease in the number of oval cells observed in treated animals most likely reflect a decrease in proliferation of oval cells, suggesting a possible role of SDF-1 in oval cell proliferation.

SDF-1 was found to promote the proliferation of astrocytes[210] and some tumor cell lines such as glioblastoma cells[211] and ovarian cancer cells[212]. This effect has been shown to correlate with activation of ERK1/2 and PI3K-AKT pathways in the target cells.[210, 212] However, other *in vitro* studies have showed that SDF-1 induced activation of these pathways did not affect proliferation/survival of several hematopoietic cell lines.[136] This suggests that activation of other signaling pathways is required in the target cells to

enhance their proliferation or survival. Moreover, there is evidence showing that SDF-1 acts synergistically with other cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF) and thrombopoietin (TPO) enhancing survival of CD34+ progenitor cells. [213] SDF-1/CXCR4 interaction also induces epidermal growth factor (EGF) receptor phosphorylation to enhance proliferation of ovarian cancer cells.[212] All these data support the notion that crosstalk between SDF-1/CXCR4 axis and other cytokine signaling pathways play an important role in regulating stem cell proliferation or survival. As shown by the present study, down-regulation of SDF-1 expression in the liver causes impaired oval cell responce, implying an important role of SDF-1 in promoting oval cell proliferation.

Oval cell-aided liver regeneration is regulated by an array of cytokines and chemokines such as HGF, TGFα, aFGF, TNFα, LIF, SCF and INF-γ et al.[70,79,80,84,89,91-93,96,101,196,197] SDF-1/CXCR4 interaction may represent an essential component of this complex controlling network. Besides its direct action on oval cells, it is possible that SDF-1 also has an effect on other hepatic cells. Further studies aimed to elucidate the function of SDF-1 on various hepatic cells and to dissect the connections between SDF-1 and other cytokine signaling pathways in the context of oval cell-aided liver regeneration will be critical for a better understanding of pathophysiology of liver regeneration.

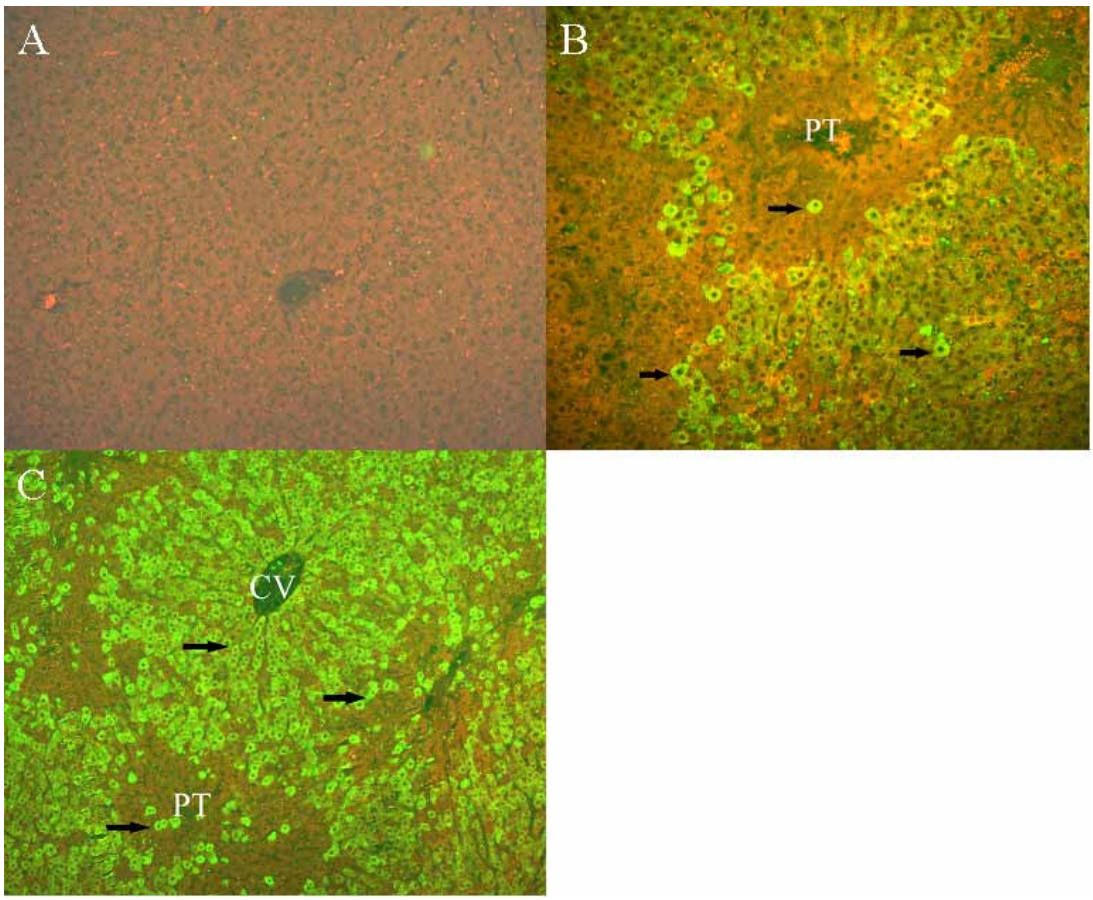

Figure 3-1. Immunofluorescent staining of SDF-1 in normal rat liver and 2AAF/PHx rat liver. A) Normal liver; B) 2AAF/PHx rat, day 7; C) 2AAF/PHx rat, day 9. Arrows point to SDF-1 positive hepatocytes. SDF-1 (green) expression can be seen in most of the hepatocytes within the pericentral region on the liver of 2AAF/PHx rat. In this model, most cells within portal triads are SDF-1 negative (B and C). SDF-1 expression is not detected in normal liver (A). (PT: portal triad; CV: central vein. Original magnification: 20X).

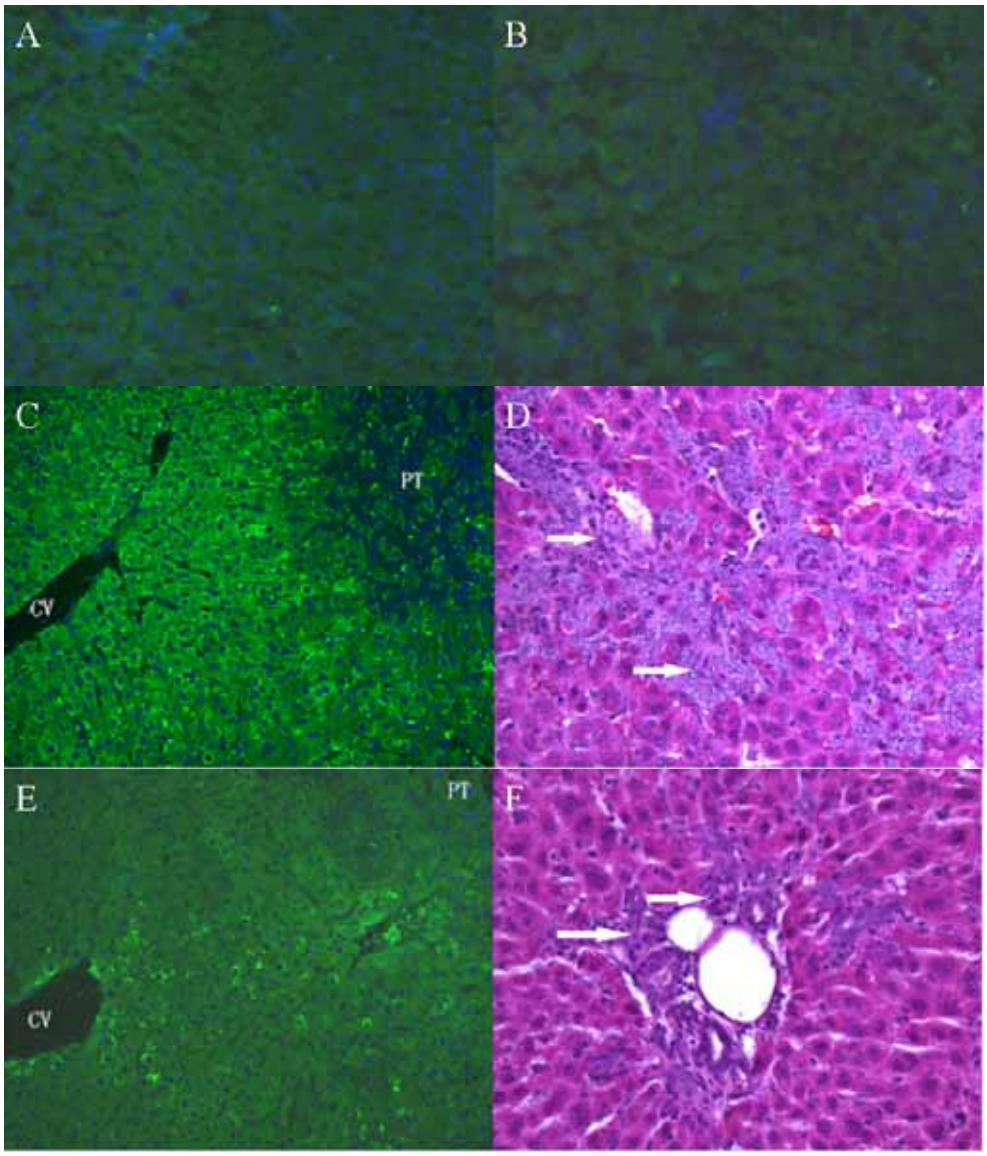

Figure 3-2. Ad-siSDF knocks down SDF-1 expression in 2AAF/PHx rat livers and inhibits the oval cell reaction in these animals. A)Normal rat liver; B) 2AAF/PHx, day 9, primary antibody omitted (negative control); C-D) liver sections from 2AAF/PHx rat treated with Ad-scramble, day 9; E-F) Liver sections from 2AAF/PHx rat treated with Ad-siSDF, day 9. A-C, E) immunofluorescence of SDF-1 (green) counter-stained with DAPI (blue). SDF-1 was knocked down in Ad-siSDF treated animal liver (compare E to C). D) and F) are H&E staining of liver sections showing suppressed oval cell response in Ad-siSDF treated animals F) compared to control animal D). Arrows point to oval cells. (Original magnification: 20X)

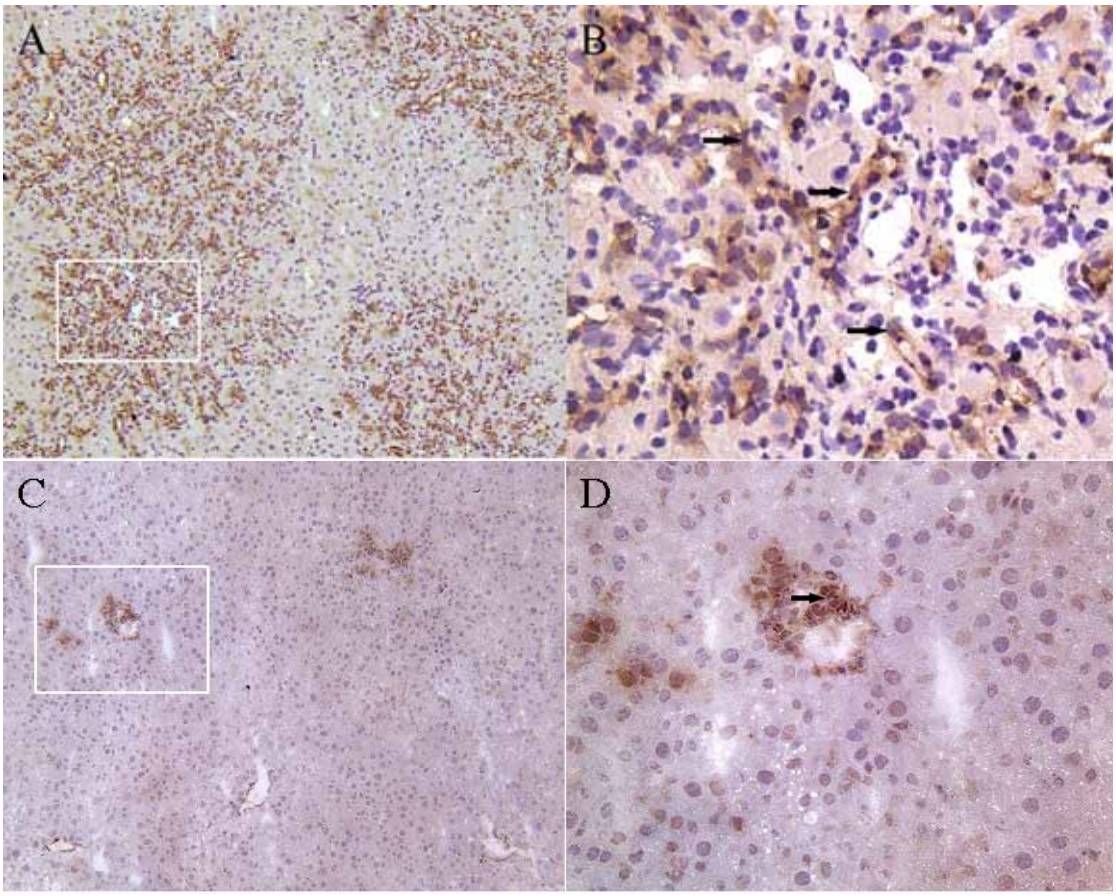

Figure 3-3. OV6 immunostaining of rat liver sections. A) 2AAF/PHx rat treated with Ad-scramble, day 9; B) High magnification of the bracketed area in A); C) 2AAF/PHx rat treated with Ad-siSDF, day 9; D) High magnification of bracketed area in C). Arrows point to oval cells. Less OV6+ (brown) oval cells are seen in Ad-siSDF treated animals C-D) than those seen in control animals A-B). (Original magnification: 10X for A and C; 40X for B and D)

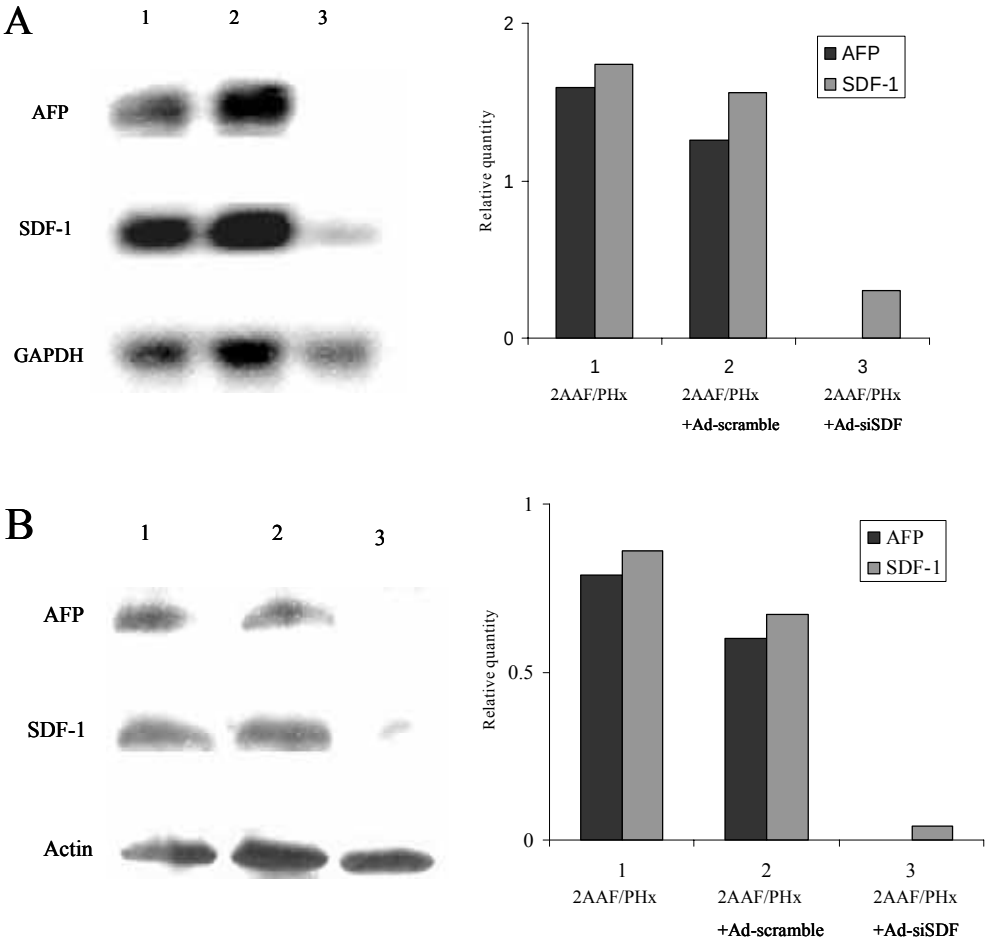

Figure 3-4. Knockdown of SDF-1 and the expression of AFP. A) Northern blot analysis of AFP and SDF-1 mRNA in rat livers; B) Western blot analysis of AFP SDF-1 protein expression in rat livers. In both A) and B), samples in lane 1 are from livers of 2AAF/PHx rats; lane 2 2AAF/PHx/Ad-scramble treated rats and lane 3 2AAF/PHx/Ad-siSDF treated rats. 10μg of pooled total RNA samples or 5μg of pooled protein samples from same group of animals (n=3 in each group) was loaded to each lane. Relative quantity of each band is determined by normalizing its total density to that of loading control in the same lane, and is plotted in accompanying graph. Ad-siSDF-induced RNA interferences result in considerable decrease in SDF-1 expression in both mRNA and protein level, accompanied by an almost absent of AFP expression from these rat livers (lane 3). Ad-scramble infusions do not cause significant decrease in SDF-1 and AFP expression in control animals (lane 2 compared with lane 1).

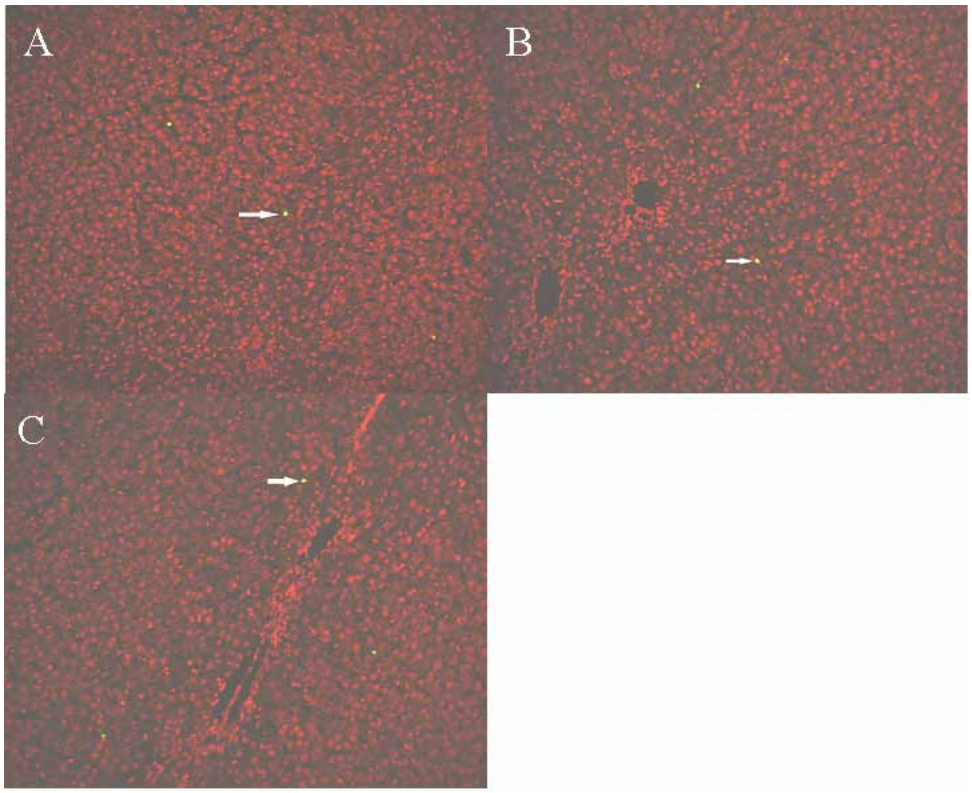

Figure 3-5. No significant apoptosis detected by TUNEL staining in 2AAF/PHx rat livers. A) 2AAF/PHx rat, day 9; B) 2AAF/PHx/Ad-scramble treated rat, day 9; C) 2AAF/PHx/Ad-siSDF treated rat, day 9. Arrows point to apoptotic cells (green), which are sporadic on the liver sections from all animals. The extents of apoptosis in the liver are similar between all three groups at this time point. (Original magnification: 10X)

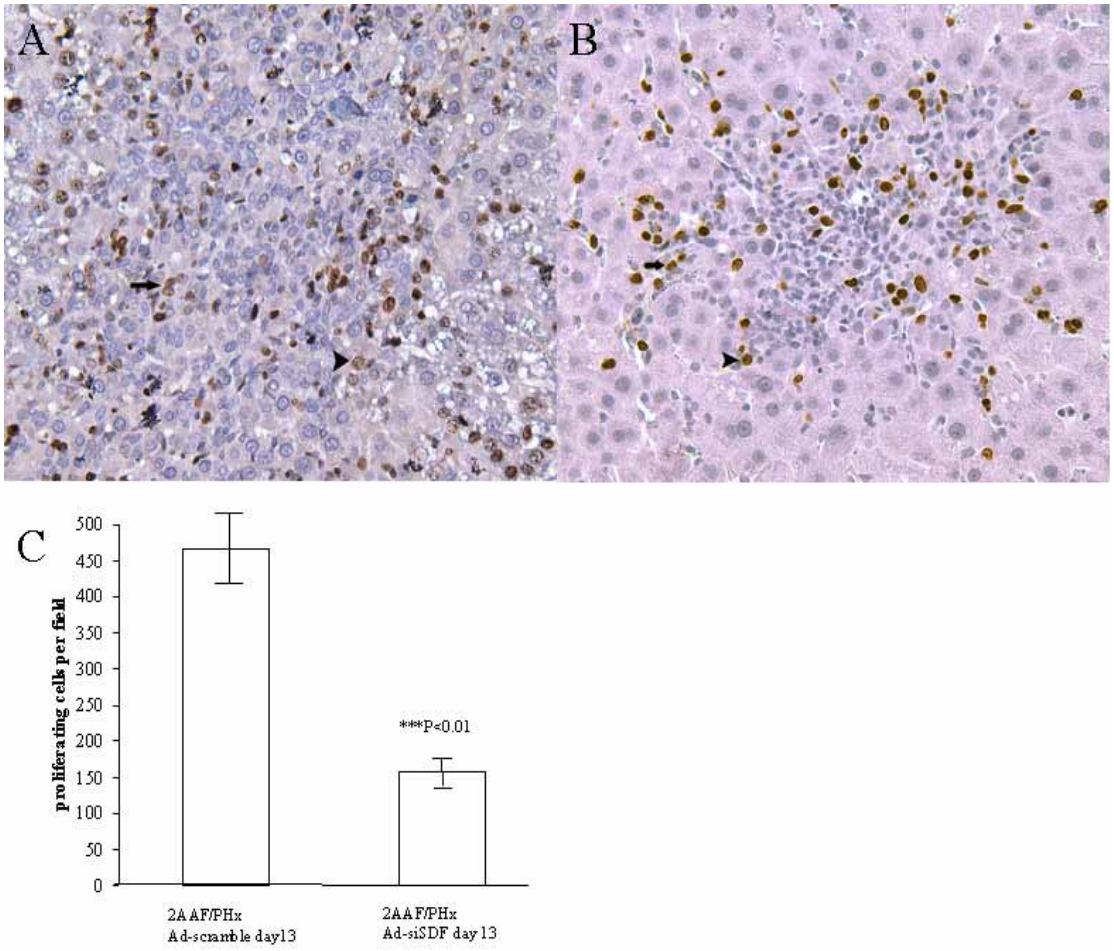

Figure 3-6. Knockdown of SDF-1 hindered hepatic cell proliferation. A) 2AAF/PHx/Ad-scramble treated rat, day 13; B) 2AAF/PHx/Ad-siSDF treated rat, day 13. Arrows point to proliferating oval cells and arrowheads point to proliferating hepatocytes. (Original magnification: 20X) C) Comparison of the number of proliferating cells in different group of animals. Ki67 positive cells (brown nucleus) were counted as proliferating cells from 25 randomly selected fields in sections of each group. The cells residing within sinusoids were intentionally ignored since they were not related to oval cells. The number of proliferating cells per field (under 20X magnification) was 465.8±53.7 in control rat and 157.5±20.2 in Ad-siSDF treated rat. (Mean±2SD, students' t test, $P<0.01$)

CHAPTER 4
EFFECTS OF SDF-1 OVEREXPRESSION DURING OVAL CELL-AIDED LIVER REGENERATION

Introduction

It has been documented that hepatocytes can be generated from hematopoietic stem cells (HSCs).[2,3,4] This has raised the hopes that HSCs could in the future be used for regeneration and reconstitution of damaged liver, or serve as carriers of therapeutic gene for gene therapy.

In order for HSCs residing in bone marrow to become hepatocytes in the liver, these cells must first move out to the circulation; when they reach the liver, they must engraft into the liver and eventually differentiate into hepatocytes. The mechanisms governing each steps of this process are not fully understood.

The studies on bone marrow derived hepatocytes have revealed that generation of hepatocytes from HSCs occurs in a fairly low frequency. The contribution of HSCs to the hepatocytes in the regenerating liver vary from 0.8%~8%, depending on the species, the injury model, the time after liver damage, and the techniques used to identify bone marrow derived hepatocytes.[3,214,215] To significantly increase the bone marrow contribution to the liver parenchyma, the signals have to be elucidated that control the mobilization of HSCs from bone marrow and homing of these cells to injured liver; then the ways to modify those signals need to be found to improve the efficiency of these processes.

Studies on murine and human subjects have shown that the SDF-1/CXCR4 interaction is involved in regulating mobilization of HSCs and has been implicated in migration of stem cells during organogenesis and adult tissue repairing, including homing of HSCs to bone marrow.[111,198-202] Our research elaborated in the previous chapters also suggested that the SDF-1/CXCR4 axis was a critical player in stem cell-aided liver regeneration. It is of great interest to see whether augmentation of SDF-1 in the liver will lead to enhancement of oval cell activation, or increase of contribution of bone marrow stem cell to the hepatocytes. To this end, bone marrow transplantations were performed from DPPVI+ male rats to lethally irradiated DPPIV- female rats. One month after bone marrow transplantation, the DPPIV- rats were put into 2AAF/PHx treatment and received one dose of injection of recombine adenovirus carrying SDF-1 expression cassette immediately after PHx. Animal tissue were collected and examined at day 9 and day 28 after PHx and virus infusion.

Materials and Methods

Recombinant Adenovirus

SDF-1 Overexpression Cassette. SDF-1α cDNA coding region was PCR-amplified from mouse liver cDNA and inserted downstream of HBV promoter (a kind gift from Dr. chen liu, University of Florida). The primer set used is 5'-GTC CAC CTC GGT GTC CTC TT-3' and 5'-CCA CGG ATG TCA GCC TTC CT-3'.

Generation of Adenovirus. Adeno-X expression system (BD biosciences clontech, CA) is used to make adenoviral vectors containing SDF-1α expression cassette. The adenoviral vectors are then used to transfect AD-293 cells (Stratagene, TX) to produce adenovirus. All procedures are performed following manufacturer's manual. The

adenovirus containing SDF-1 overexpression cassette is designated Ad-SDF-GFP and the control virus Ad-GFP.

Bone Marrow Transplantation

Dipeptidyl peptidase IV (DPPIV) negative female F-344 rats were used as recipients. These rats were subjected to a lethal dose of irradiation of 900 rads (450 radsX2, with 3 hours interval) before receiving bone marrow cell infusion. Normal male F-344 rats were used as bone marrow donors. Bone marrow cells were isolated from the femurs and the tibias of the donor rats. Briefly, donor rats were killed by Nembutal overdose (100mg/kg) and disinfected by immersion in 70% ethanol; after the bones were removed and cleaned of soft tissue, bone marrow was exposed by cutting the end of the bone and bone marrow cells were expelled by inserting a needle and forcing Iscov medium through the bone shaft. Bone marrow cells were then passed through a nylon mesh to remove any bone pieces. Approximately $60X10^6$ male bone marrow cells were infused to each recipient via tail vein. Chimera was detected by PCR screening for Y-chromosome 30 days after transplantation. Established chimeras were used for oval cell induction.

DPPIV Staining

5μm frozen sections were fixed in -20^{o}C Ethanol:glacial acetic acid (99:1) for 5 minutes, then transferred to 4^{o}C 95% ethanol for 5 minutes. The slides were left at room temperature to air dry. After that, substrate solution was applied on the slides and the slides were incubated at 37^{o}C for 20 minutes. The slides were washed with TMS buffer twice and incubated with 0.1 M $CuSO_4$ for 2 minutes at room temperature. The slides were then washed and conterstained with hematoxylin for 1 minute.

Substrate solution: GPMN (Gly-Pro-4-Methoxy-Beta-Naphlylamide): 2.5 mg in 150 µL 0f Dimethylformamide. Fast blue BB salt: 5mg in 5 ml of TMS buffer. Mix and filter immediately before use. TMS buffer: 0.1 M Tris maleate, 0.1 M NaCl, PH6.5.

Laminin Staining

Formalin-fixed paraffin-embedded liver tissues were cut into 5µm sections. After deparaffinization and re-hydration, the sections were covered with proteinase K (20µg/ml, in TE buffer, PH8.0) and incubate for 15 minutes at 37^{o}C in a humidified chamber. The sections were allowed to cool to room temperature before washing with TBS. After normal serum blocking and avidin/biotin blocking, sections were incubated with polyclonal anti-laminin IgG (diluted 1:100, Dakocytomation Z0097) for 1 hour a room temperature. After wash and blocking of endogenous peroxidase activity, the sections were incubated with biotinylated anti-rabbit IgG (Vector Laboratories) for 30 minutes at room temperature. The staining reaction was developed using Vectastain elite ABC kit (PK-6200, Vector Laboratories) and diaminobenzidine tetrahydrochloride (DAB) substrate (SK-4100, Vector Laboratories).

Immunofluorescence of Desmin/Laminin

Cryostat sections (5µm) were fixed in acetone (-20^{o}C) for 10 minutes. After washing and normal serum blocking, sections were incubated with mouse anti-desmin antibody (diluted 1:50, Dako 0904) and rabbit anti-laminin IgG (diluted 1:100, Dakocytomation Z0097) for 1 hour at room temperature. Texas red-conjugated anti-mouse IgG and Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (diluted 1:200, Vector Laboratories) was used as secondary antibody.

Immunofluorescence of OV6/Laminin

Cryostat sections (5μm) were fixed in acetone (-20^{o}C) for 3 minutes. After washing and normal serum blocking, sections were incubated with mouse anti-OV6 antibody (diluted 1:150, gift from Dr. Stewart Sell) and rabbit anti-laminin IgG (diluted 1:100, Dakocytomation Z0097) for 1 hour at room temperature. Texas red-conjugated anti-mouse IgG and Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (diluted 1:200, Vector Laboratories) was used as secondary antibody.

Immunofluorescence of OV6/Desmin

Cryostat sections (5μm) were fixed in acetone (-20^{o}C) for 3 minutes. After washing and normal serum blocking, sections were incubated with mouse anti-OV6 antibody (diluted 1:150, gift from Dr. Stewart Sell). After washing, the sections were incubated with secondary antibody Texas red-conjugated anti-mouse IgG (diluted 1:200, Vector Laboratories) for 1 hour at room temperature. The sections were then microwaved in 0.01 M citrate buffer (PH 6.0) for 10 minutes to deactivate unbounded primary antibody. After wash and normal serum blocking, the sections were incubated with mouse anti-desmin antibody (diluted 1:50, Dako 0904) for 1 hour at room temperature, followed by secondary antibody Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG.

ELISA for SDF-1

Quantikine mouse SDF-1/CXCL12 immunoassay kit (R&D system, MCX120) was used to determine SDF-1 level in rat serum following the manufacturer's instruction. 10μl of serum from each animal was diluted in 90 μl of calibrator diluent RD6Q (1:10 dilution) and used in the assay.

Results

Overexpression of SDF in the Liver Does Not Enhance Oval Cell Activation at Day 9

The serum SDF-1 level was elevated in the rats after 2AAF/PHx, consistent with the findings of Western blot and immunohistochemistry (Figures 2-2 and 3-1, respectively). In the rats receives the infusion of Ad-SDF-GFP, serum SDF-1 level were higher than that of control rats infused with Ad-GFP (Figure 4-1A), as a result of transgene expression. The GFP expression by the hepatocytes further confirmed the expression of transgene within the liver (Figure 4-1B).

Day 9 after PHx and virus infusion, the oval cell activation in both Ad-SDF-GFP treated rats and control rats were comparable to each other, as seen in the H&E stained liver section. (Figure 4-2) DPPIV staining was also performed in the liver sections from these animals to detect bone marrow derived hepatocytes. However DPPIV positive hepatocytes were not seen at this time point.

Overexpression of SDF in the Liver Promote ECM Deposition in the Liver at Day 28 after 2AAF/PHx

Pathological symptoms were noticed in the rats that received 2AAF/PHx/ Ad-SDF-GFP. Animals were lethargic since day 20 after PHx and Ad-SDF-GFP infusion, and suffered from progressive dehydration and body waste with minimal response to supplement of diet vitamin and infusion of isotonic fluid. No similar symptom was noted in control animals treated with 2AAF/PHx/Ad-GFP. Animals were sacrificed at Day 28. Gross exam revealed numerous small nodules in the livers of Ad-SDF-GFP infused rats. Other findings include enlarged spleen; extended colon pouch stuffed with grainy undigested food, thin colon wall in the color of dark pink; no blockage were found distal

to the extended pouch. H&E staining on the liver sections from these animals showed cell-rich, fibrotic-like septa between portal regions. (Figure 4-3)

Most of the cells within the septa can be classified into three categories by their morphology: cells with spindle-shape nucleus, possibly (myo)fibroblasts; cells with ovoid-shape nucleus, possible oval cells; and newly generating hepatocytes.

DPPIV staining displayed a minimal number of bone marrow-derived cells incorporated in the livers of both 2AAF/PHx/Ad-SDF-GFP treated rats and control rats, with similar frequency (Figure 4-4). Those bone marrow-derived cells are not likely hepatocytes because they fail to show the typical DPPIV staining pattern of canaliculi membrane of hepatocytes. Further studies are required to identify these cells.

Laminin staining was performed to delineate the fibrotic-like septa. The liver of 2AAF/PHx/Ad-SDF-GFP treated rat exhibited heavier laminin deposit than control liver (Figure 4-5A-D). However, collagen deposit typically seen in liver fibrosis is not evident in either rat, as shown by trichrom staining (Figure 4-5E-F).

Double staining of OV6 and Laminin demonstrated that majority of the cells within the septa were OV6 positive and were surrounded by laminin (Figure 4-6A). The desmin signal did not co-localized with OV6 signal, and desmin expressing cells were much lower in number than $OV6^+$ cells (Figure 4-6B). Furthermore, although desmin positive cells were in close proximity to the laminin, they were not circled in laminin, but rather resided adjacent to hepatocytes (Figure 4-6C).

Discussions

In our previous studies, SDF-1 has been shown to upregulated in rat liver; and when SDF-1 expression is suppressed, oval cell activation is inhibited. These findings

suggest that SDF-1 is an important molecule in stem cell-aided liver regeneration. Day 9 after infusion of Ad-SDF-GFP into the 2AAF/PHx rats, SDF-1 was overexpressed in the liver, adding more to the already upregulated endogenous level. However, oval cell activation in these animals was not enhanced accordingly, neither was the engraftment of bone marrow stem cells at this time point. The reason is probably that the endogenous upregulation of SDF-1 after 2AAF/PHx itself is significant enough to reach its maximal potency in stimulating oval cell activation. As a consequence, the overexpression of exogenous SDF-1 would not show significant add-on effect.

The overexpression of SDF-1, however, might have a broader set of effects on the functions of multiple cell types during liver regeneration, as suggested by the finding that the SDF-1 overexpressing livers developed heavy laminin deposition between portal triads 28 days after 2AAF/PHx. The mechanism for the dysregulation of extracellualr matrix is unclear in these animals. Based on the cellular composition within the fibrotic-like septa, a working model integrating the function of multiple cell types must be considered.

Chronic liver damages tend to induce the accumulation of extracellular matrix (ECM) proteins in the liver, a process called liver fibrosis. The excessive ECM proteins are produced by the activated myofibroblasts, which could be of multiple origins within the liver. In portal-portal fibrosis, like the ones we see in this study, the myofibroblasts that contribute most to the ECM are activated portal fibroblasts.[216,217] hepatic stellate cells were later be recruited to the interface between the fibrous septa and the parenchyma and be activated to myofibroblasts.[217,218]

Whether SDF-1 plays a role in activating fibroblast or hepatic stellate cells is unknown. If yes, overexpression of SDF-1 could directly promote liver fibrosis in the hosts. It has been known that the activation of fibroblasts and hepatic stellate cells is controlled by a complex array of cytokines secreted by inflammatory cells such as Kupffer cells, nature killer cells and lymphocytes, in responding to liver injury.[219] The overexpression of SDF-1 might increase the recruitment of inflammatory cells to the site of liver damage. As a consequence, the production of pro-fibrosis factors might increase, leading to excessive ECM deposition by activated fibroblasts and hepatic stellate cells.

The ECM proteins found to excessively deposit in the fibrotic liver include three large families of proteins—glycoproteins such as fibronectin, laminin, hyaluronic acid *et al*; proteoglycans and collagens.[220] But the most characterized feature of liver fibrosis is increased deposition of type I and type III collagen.[220] However, in our model of SDF-1 overexpressing liver, laminin was the dominant ECM deposit, instead of collagens, as evidenced by comparing the laminin staining to trichrome staining results. (Figure 4‐5) The meaning of this discrepancy and how it relates to overexpression of SDF-1 is unclear. Another interesting finding was that a large number of OV6+ cells were seen within the laminin-rich septa. The identity of these OV6+ cells is ambiguous based on the limited data on hand. They could be proliferating bile duct epithelial cells, which are also activated and contribute to ECM deposition during portal tract fibrogenesis.[221] Considering that the primary goal of the injury (2AAF/PHx) was to induce oval cell activation in these animals, it is a nature view that the OV6+ cells thriving in the portal-portal septa are oval cells or their progenies. In 2AAF/PHx rat model, oval cell activation usually peak around day 9-13. After that most of the oval cells start to differentiate.[170] If

this is the case, the oval cells residing within the septa till day 28 must result from the arresting of differentiation of these cells into hepatocytes. The factor that prevent oval cell from differentiating could be the excessive laminin, which is a major component of basement membrane. At early stage of oval cell activation; oval cell ductules are surrounded by basement membranes, which disappear from the oval cell foci when they start to differentiate into small hepatocytes.[207] *In vitro* study also showed that hepatic stem cells expressed hepatocytic phenotype only when losing touch with basement membrane matrix.[208] It is possible that oval cell-laminin contact is an inhibitory signal for oval cells to differentiate down hepatocytic direction. In fact, a number of hepatocytes were seen residing within the septa, which were presumably newly derived from oval cells. These hepatocytes were not embedded in laminin and were closely proximal to desmin positive cells, presumably hepatic stellate cells. In addition to being a major source of ECM production, activated hepatic stellate cells also show increases in expression of MMPs, which serve as modulators of ECM accumulation. It is possible that hepatic stellate cells also secret MMPs to degrade laminin and allow the oval cells to differentiate into hepatocytes. In our SDF-1 overexpression rat model, the SDF-1 is mainly expressed by hepatocytes within the liver lobule, thus the SDF-1 level in portal region is relatively low (but still higher than that of non-overexpression animals). If SDF-1 is the chemotactic factor for stellate cells, one may expect to see fewer hepatic stellate cells recruited to portal-portal septa, because a steeper gradient of SDF-1 exist in the SDF-1 overexpresing liver compared to non-overexpressing animals, with high concentration end in liver lobule and low end in portal region. This could partially explain the heavier laminin deposition and oval cell detention in the overexpression rats.

Our present overexpression study brings up the possibility that SDF-1 signaling may be involved in portal-portal fibrosis. To ask better defined questions and to explore the answers, more comprehensive study must be conducted to gather the information about the course of the whole process of fibrosis after oval cell activation, including the identities of the cell types involved, the transition, migration and secretion of these cells.

And future studies regarding the putative effects of SDF-1 on portal fibroblasts, hepatic stellate cells and oval cells are pivotal to better understand of the mechanism of liver fibrosis after oval cell activation.

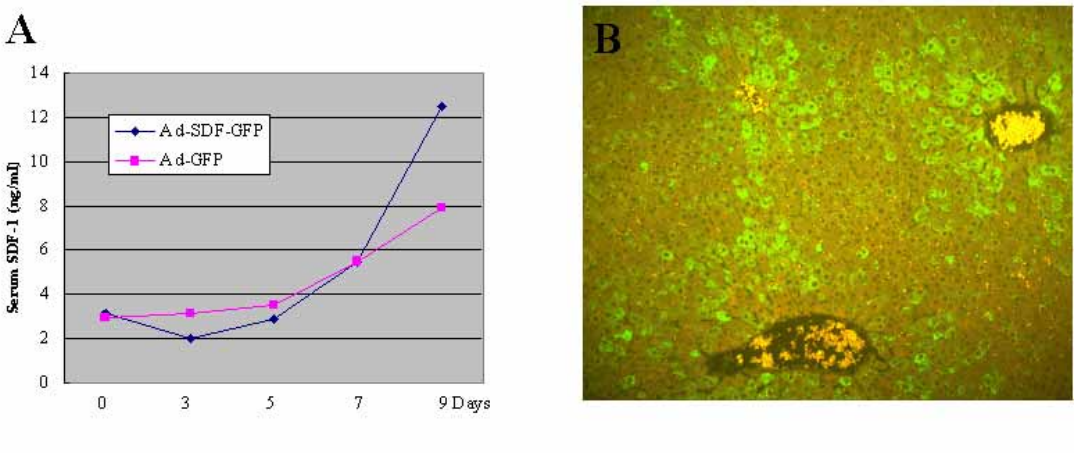

Figure 4-1. Overexpression of SDF-1. A) Elevation of serum SDF-1 level after infusion of Ad-SDF-GFP to 2AAF/PHx treated rat. B) GFP expression (green) within the liver of 2AAF/PHx/Ad-SDF-GFP treated rat. (Original magnification 20X)

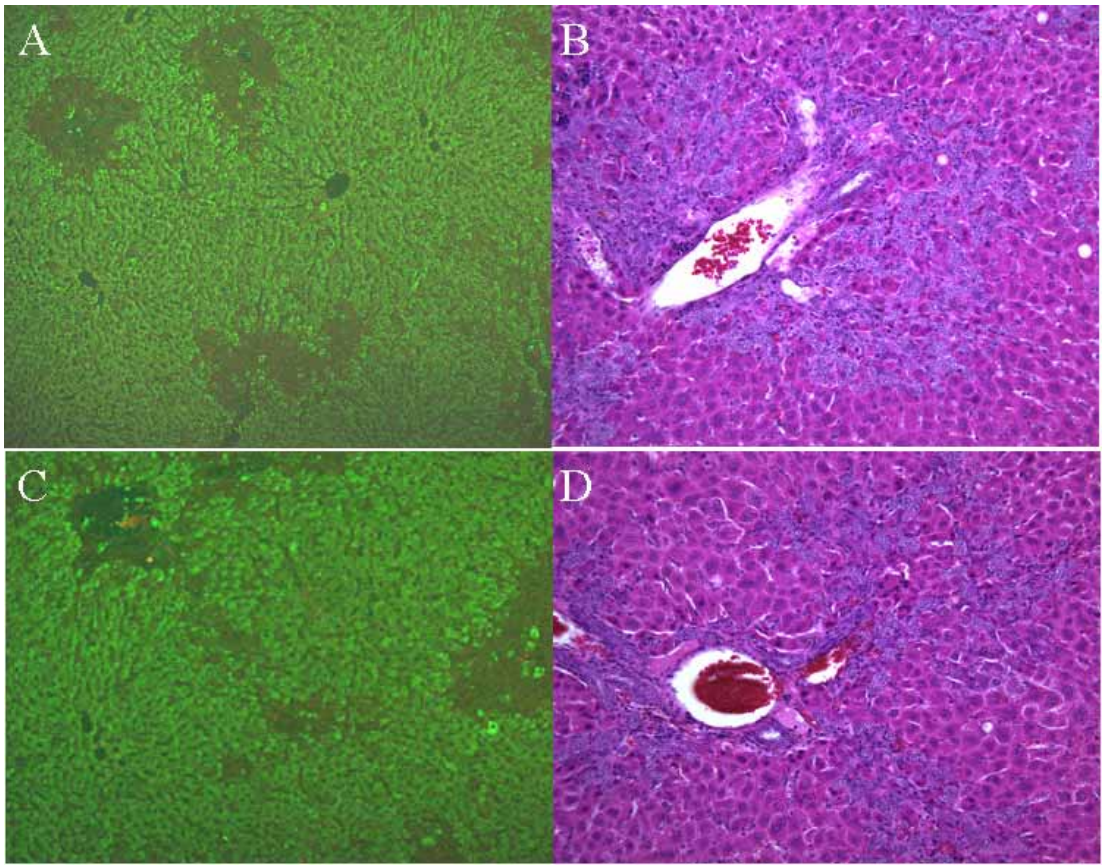

Figure 4-2. Oval cell activation at day 9 after 2AAF/PHx with or without SDF-1 overexpression. A) SDF-1 expression (green) within the liver of 2AAF/PHx/Ad-SDF-GFP treated rat. B) H&E staining of the liver of 2AAF/PHx/Ad-SDF-GFP treated rat. C) SDF-1 expression (green) within the liver of 2AAF/PHx/Ad-GFP treated rat. D) H&E staining of the liver of 2AAF/PHx/Ad-GFP treated rat. (Original magnifications 20X)

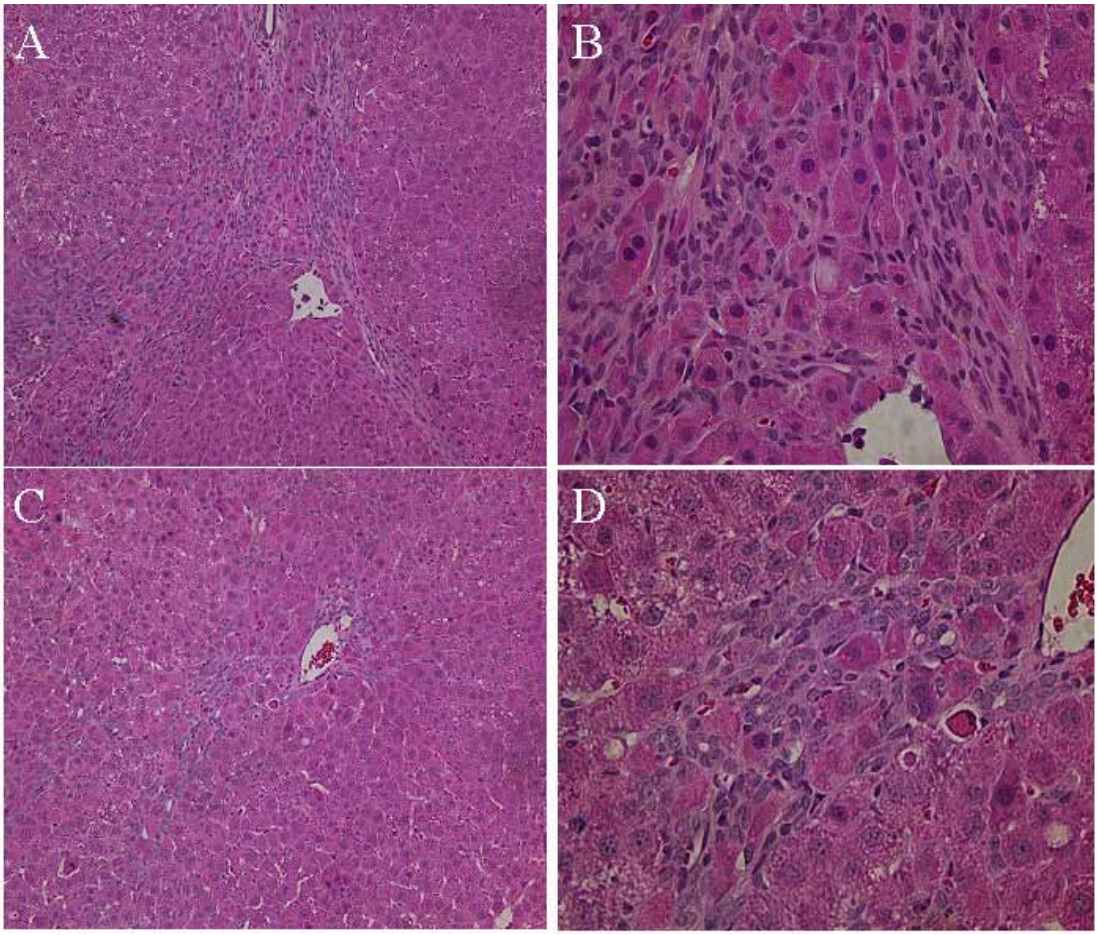

Figure 4-3. H&E staining of the liver of 2AAF/PHx treated rat with or without SDF-1 overexpression (day 28). A) and B) 2AAF/PHx/Ad-SDF-GFP. C) and D) 2AAF/PHx/Ad-GFP. (Original magnification: A and C, 4X; B and D, 40X)

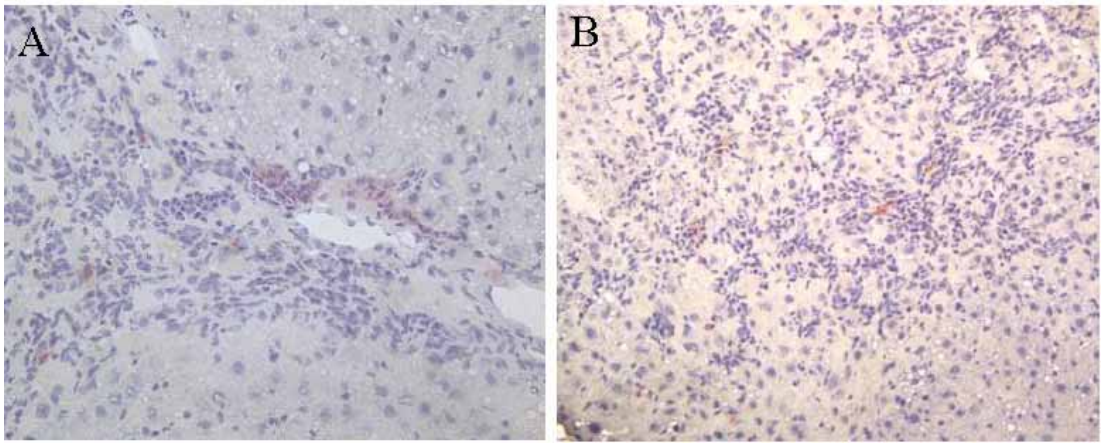

Figure 4-4. DPPIV staining of the livers of 2AAF/PHx treated rat with or without SDF-1 overexpression (day 28). A) 2AAF/PHx/Ad-SDF-GFP. B) 2AAF/PHx/Ad-GFP. (Original magnifications 4X)

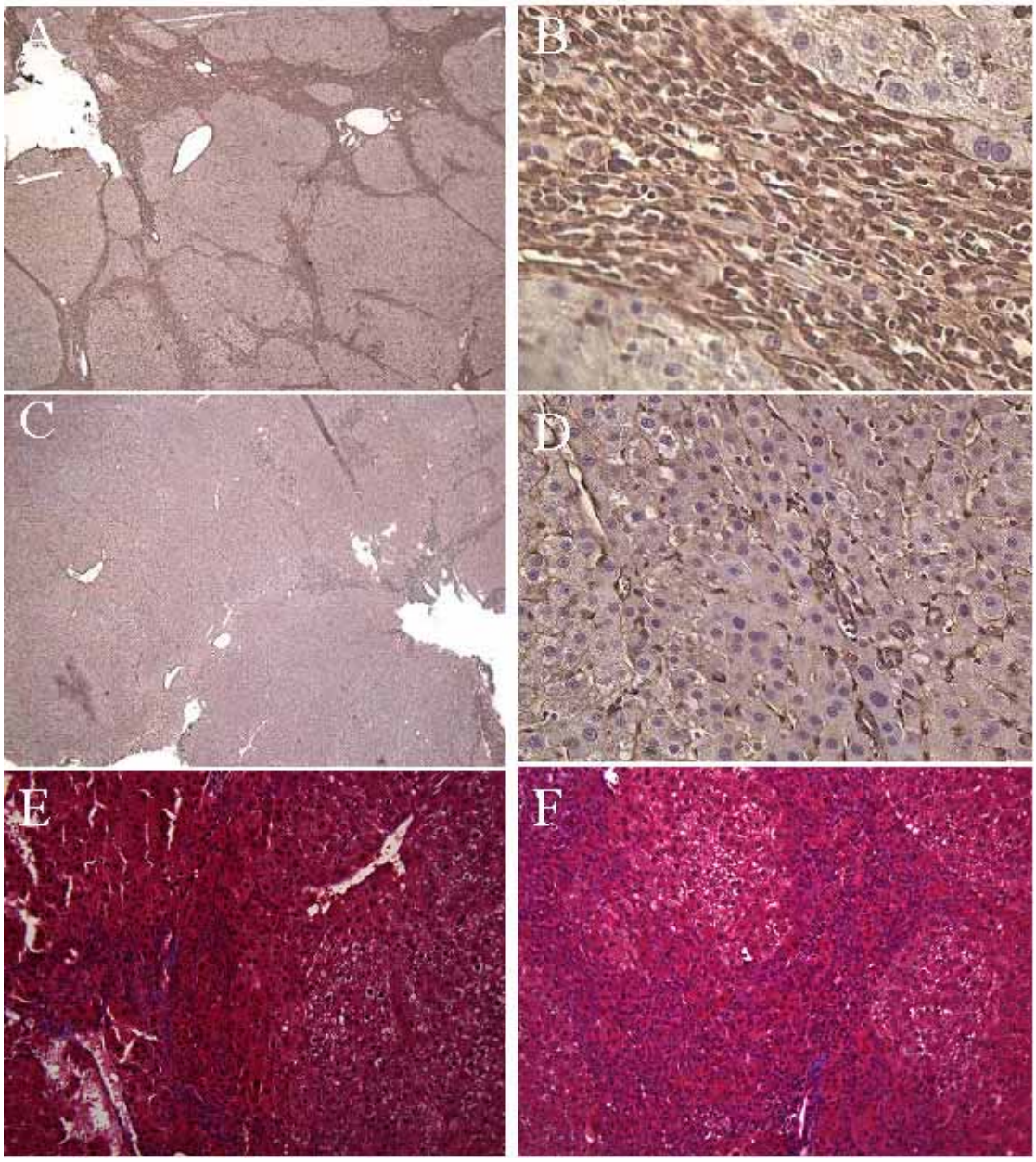

Figure 4-5. Extracellular matrix deposition with the liver of 2AAF/PHx treated rat with or without SDF-1 overexpression (day 28). A) and B) 2AAF/PHx/Ad-SDF-GFP. Stained for Laminin (brown). C) and D) 2AAF/PHx/Ad-GFP. Stained for Laminin (brown). E) and F) Trichrome staining for collagens (blue) within the liver of 2AAF/PHx/Ad-SDF-GFP treated rat and control (2AAF/PHx/Ad-GFP treated) rat, respectively. (Original magnification: A and C, 4X; B and D 40X; E and F, 20X)

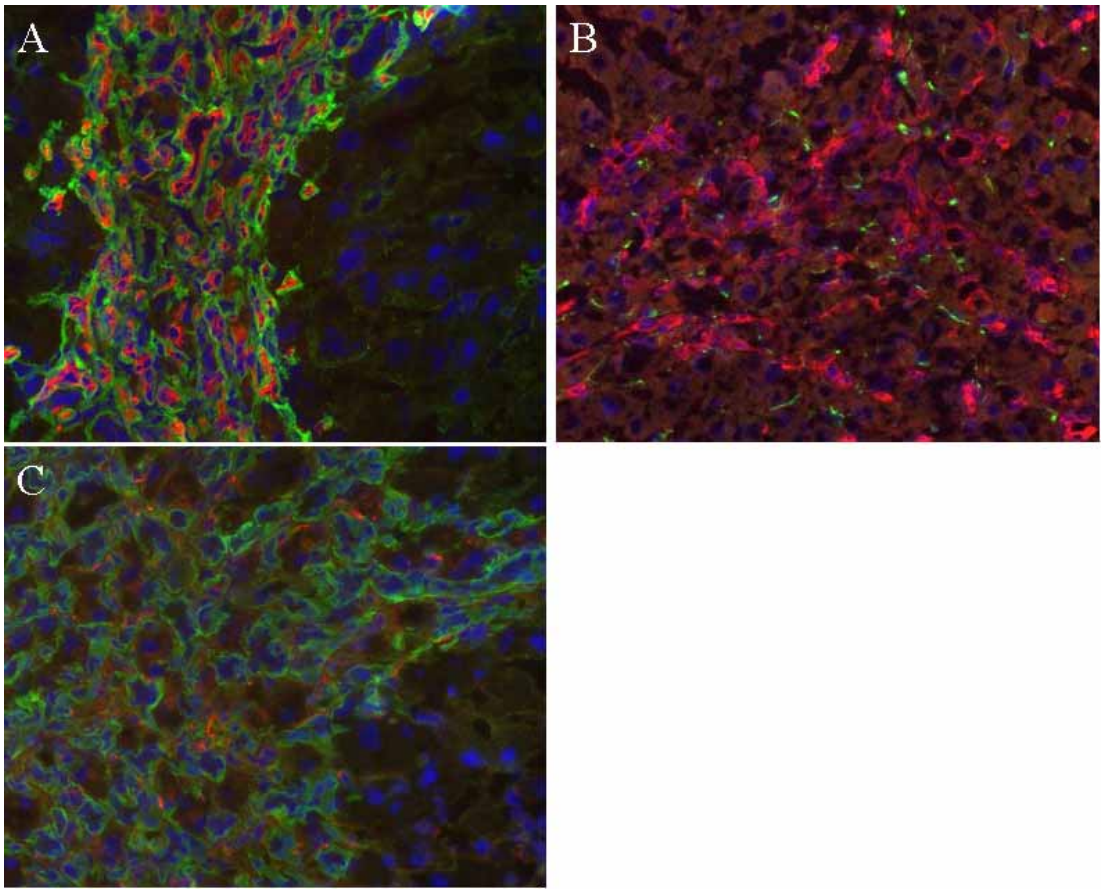

Figure 4-6. Double staining of cell markers and laminin in the liver of 2AAF/PHx/Ad-SDF-GFP treated rats (day 28). A) OV6 (red) and laminin (green). B) OV6 (red) and desmin (green). C) Desmin (red) and laminin (green). (original manification: 40X)

CHAPTER 5
GENERAL CONCLUSIONS

The primary goal of this project is to assess the role of SDF-1 during stem cell-aided liver regeneration. To this end, the expression patterns of SDF-1 and its receptor CXCR4 were examined in rat models of oval cell activation. The data reveals that SDF-1 up-regulation is correlated with oval cell activation---SDF-1 up-regulation only occur in oval cell induction models but not in other regeneration models. During oval cell activation, SDF-1 is expressed by hepatocytes within the liver lobules while its receptor CXCR4 is expressed by oval cells. Furthermore, isolated oval cells responded to SDF-1 gradient by migrating to high concentration of SDF-1 in vitro. These data suggest that SDF-1 play an important role in oval cell activation.

To further characterize the role of SDF-1in this regeneration pathway, RNA interference technique was employed to knockdown SDF-1 expression in the oval cell induction model 2AAF/PHx. As a result, oval cell number was decreased in these animals and the expression of AFP almost lost; the proliferation of oval cells and hepatocytes inhibited, suggesting compromised oval cell response and hindered liver regeneration in these animals. This line of data further confirmed that SDF-1 play an essential role in stem cell-aided liver regeneration, probably by guiding stem cells to migrate to favorable microenvironment that allow them to proliferate/differentiate.

Over-expression of SDF-1 in the liver of 2AAF/PHx rat was used as another strategy to further assess the role of SDF-1 in stem cell-aided liver regeneration, as well as to explore the possible way to enhance the recruitment of stem cells to the liver during

regenerative process. The results showed that oval cell activation was not enhanced by SDF-1 over-expression in day 9 after the treatment, neither was the recruitment of bone marrow stem cells. However, the over-expression of SDF-1 leaded to excessive deposition of ECM and robust OV6+ cell accumulation in the liver 28 days after PHx, suggesting SDF-1 may play a role in liver fibrogenesis related to oval cell activation. Future studies to elucidate the actions of SDF-1 and crosstalk between cytokines in the contexts of oval cell activation and liver fibrogenesis will be critical for the development of novel therapeutic strategy for liver fibrosis based on the modulation of SDF-1 signal.

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