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Liver regeneration: mechanisms and models to clinical application

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Abbreviations: APOLT, auxiliary partial orthotopic liver transplantation; HPCs, Hepatic progenitor cells; LSEC, liver sinusoidal endothelial cells; MSCs, mesenchymal stem cells; OLT, Orthotopic liver transplantation; VEGF, vascular endothelial growth factor;

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Key points

- **Liver regeneration is well described and occurs efficiently in the normal liver to restore normal architecture, size and function**
- **Chronic injury severely impairs liver regeneration through excess inflammation, scarring and epithelial abnormalities, this is less well studied but clinically important.**
- **Zebrafish are an excellent new tool to study liver regeneration and enable large scale chemical screening assays.**
- **There is a gap between the generally utilised animal models of liver regeneration and clinically important scenarios of severe liver injury and impaired liver regeneration**
- **Understanding and promoting regeneration and repair of the failing liver is a key challenge of major clinical significance**
- **Modern imaging techniques will allow non-invasive real-time assessment of liver structure and function.**
- **Cell therapies that have been successful in animal models are now being trialled in the more challenging clinical arena.**

Introduction

Why the clinical need to understand measure and promote liver regeneration?

Although the normal liver has a fantastic regenerative capacity following acute injury or resection this regenerative ability becomes overwhelmed in two important scenarios: (1) in the setting of severe acute liver injury or (2) when there is severe chronic liver injury with aberrant liver architecture and marked liver fibrosis. These are clinically relevant scenarios that often result in serious morbidity and mortality. Whilst there have been decades of excellent and clinically informative research into understanding the signals that control regeneration of the normal liver[1] the mechanisms at play when the abnormal liver attempts regeneration are less well described[2]. Understanding how regeneration fails or is impaired in the severely damaged liver is an important goal. Lessons learned from relevant animal models may have a have importance in the clinical setting and aid the development of new therapies to either promote regeneration or prevent complications that arise during the period of liver regeneration. A clinical scenario where an improved understanding of regeneration of the compromised liver includes liver transplantation, where the increasingly common use of partial livers such as split livers and living donor transplants relies upon regeneration of the donor graft to reach the correct liver mass. Failure of regeneration in these settings results in poor or delayed graft function, prolonged intensive care stays, occasionally a requirement for re-transplantation or ultimately even death of the recipient. By understanding the pathological mechanisms

driving these adverse conditions it is hoped that the period of regeneration can be more predictable and the associated clinical complications ultimately preventable. The ability to predict or improve liver regeneration when the liver is compromised, for example in the setting of cirrhosis when surgical resection of hepatocellular cancer (HCC) is commonly performed, or following the resection of colorectal hepatic metastasis, when the liver has received prior chemotherapy, would allow clinicians to optimise cancer resection approaches. Furthermore by understanding the mechanisms underlying normal liver regeneration and aberrant liver regeneration in chronic liver injury it is hoped that we will be able to promote "healthy regeneration" or remodelling in chronic liver disease. Such a scenario would be liver cirrhosis where the initial insult may now be directly treated such as viral hepatitis or autoimmune hepatitis, but the liver tissue is left severely damaged and still susceptible to the clinical consequences of liver failure, portal hypertension and an increased risk of HCC. This review cannot be comprehensive but seeks to describe briefly the mechanisms underpinning liver regeneration, the models used to study this and then discuss areas where failed or compromised liver regeneration is clinically relevant – with a view to highlighting areas for future research.

1. Mechanisms and models of Liver regeneration

In the following section we will review some of the animal models that have been used to understand liver regeneration. These have traditionally been in rodents but new models are emerging such as the zebrafish. A general theme is that there has been much work important work understanding how the normal liver regenerates in these models, but there is less information about how the damaged or compromised liver regenerates.

The Rat model of liver regeneration (see figure 1)

The normal liver will attempt to retain an appropriate size relative to the rest of the body. Following injury or resection the remaining liver undergoes a rapid series of co-ordinated changes to regain its original volume and structure[1]. Interestingly, this need to retain the previous size to body weight ratio appears after liver hypertrophy has been induced by growth factors such as Tri-iodothyronine, when the liver shrinks back to its original size[3].

The rat partial hepatectomy model is the classic model of liver regeneration and has been studied for decades. In a landmark paper from 1931, Higgins and Anderson reported that removal of the two anterior lobes of the rat liver (the median and left lateral lobes) equated to a 70% reduction in liver size.[4] This standardised procedure is well tolerated and produces a reliable result. Whilst the normal adult liver is mitotically quiescent with only minor hepatocyte proliferation detectable, following 70% or "two thirds partial hepatectomy" the remaining liver remnant undergoes a series of rapid vascular endothelial, inflammatory and epithelial changes (**See Figure 2A**). The peak of liver regeneration, as measured by the number of hepatocytes in DNA synthetic phase, termed "S phase", occurs about 24 hours following

resection. By 7 to 10 days following hepatectomy the rat has largely regrown a normal sized liver (93%) by hyperplasia of the remnant lobes, and by 20 days following hepatectomy the liver has fully regained its starting volume. This simple and repeatable experimental procedure has enabled many important new insights into regeneration of the normal liver[1]. Following such "normal regeneration" the non-parenchymal cells in the liver, namely the stellate cells, liver sinusoidal endothelial cells (LSECS) and macrophages act in a coordinated fashion and help to control the epithelial regenerative response[5]. In a classic parabiosis experiment by Moolten and Bucher, carotid-to-jugular cross circulation was established between a rat that has been subjected to partial hepatectomy and a normal rats. This induced "liver regeneration" in the non-hepatectomized rats with normal livers. This suggested factors were circulating from the hepatectomized rat to the normal rat to induce the regenerative response and thereby pointed to there being circulating blood derived factors that help to stimulate and co-ordinate liver regeneration following partial hepatectomy[6]. Interleukin-6, tumor necrosis factor-α (TNF- α), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and thyroid hormone have been discovered as humoral factors that control liver regeneration[7, 8]. Whilst the multiple important mechanisms controlling normal liver regeneration that have been identified in the rat, these have been well reviewed elsewhere[1] and we will therefore not write further on this.

The volume of liver resected in the rat can be increased to 90%, effectively modelling the clinical syndrome termed "small for size". In both the 90% hepatectomy model and the clinical situation survival is compromised with death from liver failure a significant risk[9]. In the 90% hepatectomy model and the clinical situation, if the volume of resection increases beyond a threshold then the regenerative capacity of the remaining hepatocytes actually begin to fall, thereby contributing to a rapidly developing scenario of liver failure. Understanding why there is a failure of appropriate regeneration by the remaining liver is a clinically important goal. The contributory mechanisms of this failure of regeneration are likely to be multiple but involve vascular shear stress in the livers sinusoids caused by the portal blood passing through a small parenchymal volume which can cause periportal sinusoidal endothelial damage and parenchymal inflammation[10].

Various strategies have been deployed in this model of 90% hepatectomy to increase liver regeneration and /or improve the survival following this operation. Ren et al. showed that 90% but not 70% hepatectomy increased portal and systemic endotoxin levels. Following this observations they used selective bowel decontamination with Gentamycin and showed that this reduced lipopolysaccharide levels, enhance liver regeneration and increased the survival following 90% hepatectomy from 24% to 56%[11]. Given that sepsis due to gut related organisms is a major cause of death following major hepatic resection this is a potentially important finding[12]. Another potentially clinically relevant treatments for this syndrome is octreotide which has been shown in the rat 90% hepatectomy model to reduce mortality from 63% to 33%[13]. Interestingly, although octreotide had this beneficial effect on mortality it actually reduced early hepatocyte proliferation,

however it did reduce liver injury and necrosis and modified the hepatic methionine cycle reaction, causing an increase in methionine and 5′-methylthioadenosine, which was thought to be important for the beneficial effect. An important study by Ninomiya and colleagues challenged the assumption that the promotion of regeneration would be beneficial in the rat 90% hepatectomy model. They hypothesized that the rapid regenerative response of small remnant liver is actually responsible for the poor outcome seen. They administered dHGF which promoted the rate of liver regeneration in the 70% hepatectomy model. However, in the 90% hepatectomy this had no benefit upon the survival rates. Instead they sought to delay the regenerative response through the administration of either NS-398 (an ERK1/2 inhibitor) or PD98059 (a selective MEK inhibitor). Deceleration of the regenerative response by NS-398 or PD98059 treatment resulted in a significant and exciting improvement in day 7 survival (approximately 70%) compared to the vehicle treated group (10%) [14]. Interestingly the lobular spatial integrity was better preserved in animals that had their regenerative response lowered. Presumably this enables the portal blood flow and resulting physiological function to be maintained during the regenerative phase.

The rat has further been widely used to study liver regeneration when the regenerative capacity of mature hepatocytes is compromised. Here hepatocyte proliferation is inhibited by the chemical 2-Acetaminofluorene (AAF). This can be combined with either partial hepatectomy or the hepatotoxin carbon tetrachloride to prompt liver regeneration[15, 16]. In a classic paper from the Thorgeirsson laboratory [3H]thymidine was administered to the AAF treated rats at 6 days following partial hepatectomy. The [3H]thymidine labelled the only epithelial cells that were proliferating in the liver at this time, the oval/HPCs. When the rats were subsequently sacrificed from 9 to 13 days the [3H]thymidine was then identified in hepatocytes. Whilst not definitive proof that the oval cells/HPCs were the source of hepatocytes in the rat, this was suggestive of a product-precursor relationship[17] Later work in the rat AAF/partial hepatectomy model confirmed that cell proliferation was limited to the oval cells/HPCs and that hepatocytes were senescent (i.e. unable to proliferate) and p21 positive, making them an unlikely source of regeneration[18]. However, a recent report has challenged the concept that oval cells/HPCs contribute to liver parenchyma regeneration in the AAF/PH model in the rat and following careful observations suggested that he replication of mature hepatocytes mainly contributes regenerate the liver, even in these circumstances where there is a challenge to hepatocyte regeneration[19]; presumably these hepatocytes, if able to regenerate hepatocytes, would have initially escaped the effects of AAF. Such results emphasize the need for reliable lineage tracing systems to make the claims of regenerative potential of various cell populations secure and the technology to achieve this is not well developed in the rat compared to mouse[20].

The mouse as a model of liver regeneration.

More recently the mouse has been used as a model of liver regeneration, this has facilitated the use of the many mouse transgenic strains that enable the understanding of the role of various genes that control or

modulate liver regeneration[21]. The transgenic mice may have a permanent over or under-expression of a normal or mutated gene, a refinement of this is that the gene may be "conditionally" deleted from a particular cell type in the liver at a set time- for example before a partial hepatectomy. The physiology of regeneration in the mouse following partial hepatectomy is similar to the rat although following partial hepatectomy the peak of regeneration, as measured by BrdU incorporation into hepatocytes in DNS synthesis (S phase), occurs slightly later, at 24-48 hours[22].

The mouse has been used to model liver regeneration in the setting of chronic liver damage where "normal liver regeneration" is impaired. The most frequently used model of iterative liver damage is the chronic carbon tetrachloride (CCl_4) model of liver injury (which has also been used in the rat). Following CCl_4 administration there is predictable parenchymal necrosis most prominently surrounding the central veins peaking at 24 hours which is then followed by liver regeneration. After repeated dosing of CCl_4 liver fibrosis begins to develop, with the activation of stellate cells into scar forming myofibroblasts, the deposition of excess liver scar tissue, and ultimately the development of nodular liver cirrhosis. The collagen scar becomes increasingly crosslinked over time, making degradation of the scar more difficult, and further inhibiting regeneration[23]. Upon cessation of CCl_4 administration, there is regeneration of the liver parenchyma which is combined with scar degradation and resolution of the inflammation. In this setting the importance of macrophages in collagen scar regression[24, 25],has been shown to be critical for effective liver regeneration[26] .

There are several dietary models of liver injury in mouse commonly used to model liver disease including the 1,4-dihydro-2,4,6-trimethyl-pyridine-3,5-dicarboxylate (DDC) diet[27], which induces biliary injury and regeneration. Following the diet there is a proliferation of primitive ductules with poorly defined lumens that spread from the portal tract into the hepatic lobule. This "ductular reaction" is associated with significant fibrosis[28, 29] and thus is a model of biliary injury and fibrosis. Mice subject to the DDC diet respond poorly to partial hepatectomy[27]. Another diet model which is commonly used in mice is the modified CDE diet which was developed by the Yeoh Group[30] which induces hepatocellular injury with a degree of steatosis and a secondary "ductular response" where oval/ductular cells spread from the portal tract[28, 31, 32]. These ductular reactions are important for biliary regeneration following injury and if their proliferative response is impaired following biliary injury then then there is an increase in hepatic necrosis[33]. Whether the ductular reactions contain bipotential hepatic progenitor cells capable of regenerating hepatocytes as well as biliary cells is a controversial area. In mouse, in the absence of significant hepatocyte senescence, then hepatocyte self-replication seems to provide practically all hepatocyte regeneration with little or no contribution from hepatic progenitor cells[34, 35]. However, in the context of severe liver injury ad hepatocyte replication failure then hepatic progenitor cells may have hepatocyte regenerating capacity[36]- the degree and importance of this axis in severe liver injury needs further study and this may require the development of models where hepatocyte replication can be inhibited to model the severe human liver disease.

Zebrafish

Zebrafish have recently been developed to model many diseases and understand pathophysiological processes (**see figure 1**)[37]. Their small size and optically translucency brings the advantages of low cost, rapid analysis. Because they grow in water zebrafish have been used as model system for in vivo chemical screening. To date, their use has shown that many of the biological processes and signalling pathways seen in the mouse and rat are recapitulated in zebrafish.

There are a number of ways of provoking liver generation in the zebrafish including surgical partial hepatectomy, drug induced liver injury and nitroreductose-mediated hepatocyte ablation[38]. The zebrafish has a trilobar structure and the one-third partial hepatectomy model has been established in the zebrafish by removal of one lobe[39]. Clearly this is currently a more limited resection than performed in rodents. These studies have established signals such as Wnt[40], BMP and FGF as important for liver regeneration in zebrafish[41]. Interestingly zebrafish exhibit cellular plasticity in that bile ducts can convert to hepatocytes following large-scale hepatocyte loss. Two independent reports found, using hepatocyte ablation and lineage tracing, that following extensive hepatocyte loss the biliary cells are able to regenerate the hepatocytes[42-44]. Interestingly, in an ethanol induced model of liver fibrosis Huang et al. found that Wnt and Notch has opposing roles in directing HPCs in their regeneration of hepatocytes. Low levels of Notch stimulation stimulated HPC proliferation and hepatocyte differentiation, high levels of Notch suppressed this pathway. Wnt ligands were found to suppress Notch signalling via Numb a protein inhibitor of Notch[45]. Importantly this helps to validate the zebrafish model in the liver regeneration setting, as the same opposing signals Wnt and Notch acting via the node Numb signals have previously been shown to control the behaviour of HPCs in mouse and are differentially expressed in hepatocellular versus biliary injury in human liver[31]. Zebrafish are an ideal model for “forward genetic” due to their small size and ability to screen large numbers of organisms following exposure to a chemical mutagen. Phenotypes can be screened and the actual gene/s responsible then mapped, an approach that has already yielded results in the setting of liver development[46].

This exciting new model system looks set to make important inroads especially into the area of screening compounds and drugs for their effects upon liver regeneration. However we should still express some caution and there is an important need to show that the signals and targets identified translate through into mammalian systems including human liver regeneration.

The cellular sources and mechanisms controlling epithelial regeneration in various model systems

As discussed above, mouse models of liver injury recent lineage tracing experiments have failed to show convincing regeneration from non-hepatocyte sources[34, 47] unless there is significant liver injury and hepatocyte proliferation is strongly inhibited[36]. Furthermore, there is evidence that hepatocytes can undergo a ductular change and at least partly contribute to the ductular cell population[48, 49]. However as outlined above, in zebrafish there is strong evidence using lineage tracing systems that following significant liver injury ductular cells/HPCs can give rise to significant hepatocyte regeneration[42-44]. In the rat there is some

circumstantial evidence suggestion oval cells/HPCs can regenerate hepatocytes when hepatocyte regeneration is compromised;[17] however, opposing data does exist suggesting that this regenerative pathway is not significant[19]. A common theme is the need for reliable lineage tracing systems to provide proof of the regenerative lineages in the models commonly studied, a further issue is the whether the liver injury systems reliably recapitulate the severity of liver injury seen in human disease. Morphological studies have recently claimed that hepatic progenitor cells regenerate hepatocyte "buds" in areas of liver parenchyma has been obliterated[50]. However, performing lineage tracing experiments in humans is not possible and caution is required in interpreting such studies. The nearest approach to lineage tracing in the human liver is the use of mitochondrial DNA mutation analysis to show that regenerative nodules and adjacent ducts can be clonal[51]. Although an important observation and a technical "tour de force" this does not conclusively prove the any precursor- product relationship. Understanding the cellular contributions to hepatocyte and biliary regeneration may seem an academic exercise and remote from clinical practice, however, defining the regenerative cells in clinically relevant models of liver injury and regeneration will considerably aid the development of strategies to promote liver regeneration, either through cell therapy or through the stimulation of endogenous repair and regeneration.

Bile acids and liver regeneration

Bile acids (BAs) have recently been recognised as important for liver regeneration. Ueda et al. showed that liver regeneration is impaired in rats in the absence of intestinal bile[52]. Following this report, it was shown that increased bile acid levels accelerate regeneration, whilst low levels of BAs impair regeneration as does absence of the BA receptor Farnesoid X Receptor (FXR)[53]. BAs are rapidly increased following partial hepatectomy and signal via the receptors FXR and G-protein-coupled BA receptor 1 (GPBAR1). FXR signalling reduces liver injury and promotes liver regeneration following CCl4 induced liver injury[54]. The potential clinical application of these basic studies was indicated by Otato et al. who analysed liver regeneration in patients following major hepatectomy and found that patients who had external biliary drainage had lower levels of liver regeneration than those patients without external biliary drainage[55]. This was a retrospective study and there may be confounding factors that could explain the striking results however further studies into this area are warranted. This interesting study highlights a general point, that prospective trials are warranted in the clinical setting where there is strong animal data indicative of efficacy and where there is an acceptable risk/benefit ration from a new intervention.

2. The gap between animal models and the clinical experience of liver regeneration

Both animal models and clinical studies are informative but there still remains a gap that in poorly bridged between these two disciplines (**see table 1**). Ideally to maximise the development of understanding liver regeneration and develop new techniques to enhance liver regeneration observations made using in vivo models should inform human studies and the human studies should feed-back to refine the in vivo models.

At one end of this "bridge" animal models have been highly informative regarding the drivers of liver regeneration, the timing of response and cellular sources of the regenerative cells in the liver. Through the use of modern cell and molecular biology techniques, combined with modern transgenic mouse and now zebrafish technology, the signalling and cellular mechanisms underpinning liver regeneration are rapidly being described.

At the other side of the bridge human clinical studies of liver regeneration have often followed patients who have has a heterogeneous collection of liver insults. The studies have primarily looked at clinical outcome and sought to define associations of poor outcome and pre-operative markers of poor outcome. The study techniques have often revolved around whole organ imaging and serum analysis.

To date there is still a marked gap between these areas of study with the signalling mechanisms rarely translating to clinical trials or indeed human observational studies, and thereby rarely having any clinical impact. Likewise, the human observational studies providing little data to support further discriminatory *in vivo* studies. Non-invasive measurements during liver regeneration should be particularly insightful in the future. Modern functional imaging techniques, such as MRI spectroscopy[56-58], where distinct metabolic signatures are seen in patients regenerating liver should impact on. Likewise proteomic analysis of patients' blood during regeneration seems an obvious future development; studies in mice have already shown a distinct proteomic signature in plasma following hepatectomy and during liver regeneration that were strongly associated to metabolims.[59] Another non-invasive method analysis is the 13C-breath tests which can measure hepatic mitochondrial, microsomal, and cytosolic function.[60] The 13C-phenylalanine breath test has been used in a rat model of 70% hepatectomy and showed good discrimination between 70% hepatectomy rats and controls at 24 hours post-surgery, indicating possible future clinical utility[61]. The above techniques all show promise and may help to build strong links between the in vivo models and human studies and indeed allow further refinement of the current in vivo models.

3. Regeneration in the "abnormal liver"

In the clinic the regeneration of normal liver is relevant- for example when a well relative donates part of their liver to a recipient with liver disease- so called living donor liver transplantation". Here, the donor will have been specifically screened to exclude significant liver disease[62]. However, in the majority of clinical scenarios the abnormal- damaged liver is the one required to perform the feat of regeneration. The challenges to regeneration are very different across the different clinical scenarios and some of these are detailed below:

Severe acute liver damage resulting in fulminant liver failure

Common causes of acute liver failure include viruses such as hepatitis A, B and E, drugs such as acetaminophen, and auto-immune liver disease[63]. By definition the liver was previously normal and the damage acute- often with widespread hepatocyte apoptosis and necrosis. Following moderate liver injury

and necrosis, there is proportional hepatocyte proliferation until homeostasis is achieved. However, with increasing injury a threshold is reached beyond which the remaining liver fails to regenerate adequately. This has been clearly shown by Bhushan et al. in mice that, compared to a moderate dose of acetaminophen (300mg/kg), a higher dose (600mg/kg) actually resulted in poorer liver regeneration in the non-necrotic parts of the liver[64]. There have been attempts to stimulate liver regeneration following acute liver injury in humans. In mice, using the acetaminophen model of liver injury, loss of β-Catenin activation prevents liver regeneration. In patients with APAP mediated liver injury the degree of β-Catenin activation correlated with the degree of liver regeneration, indicating that β-Catenin activation could be a possible therapeutic strategy in patients with acute liver injury[65].

In the setting of acute liver injury, the innate immune system is critical for coordinating and stimulating regeneration, as well as for maintaining immunity[5]. In particular macrophages are important for the phagocytosis or the necrotic tissue and the stimulation of liver regeneration. Following acetaminophen induced liver injury macrophages are rapidly recruited to the areas of liver necrosis[66]. Mice deficient in CSF-1 have reduced numbers of tissue macrophages and an impairment in liver regeneration, which can be overcome by the addition of exogenous CSF-1[67]. As well as helping to co-ordinate a liver regeneration response the hepatic macrophages are important in controlling sepsis, a major complication of acute liver failure that is associated with clinical deterioration, systemic inflammatory response and multi-organ failure. Hepatic macrophages, so-called Kupffer Cells (**Figure 2**), are a major filter of portal blood and are particularly important when the gut barrier function is compromised in liver failure. CSF-1 levels are related to prognosis in acetaminophen induced fulminant liver failure and in experimental models of acute liver injury the exogenous administration of CSF-1 has been used to boost immunity and hepatic macrophage phagocytic function[68].

Fatty liver

Given the increasing levels of fatty liver in the West it is not surprising that the impaired regeneration of fatty livers is an increasingly important clinical question. Many fatty liver grafts have to be discarded as above a threshold the liver often fails upon transplant. Obese patients regenerate their livers more slowly than non-obese controls[69]. Dietary induced hepatic steatosis reduces liver regeneration following 70% hepatectomy in rats[70]. In the setting of liver regeneration of fatty liver, growth arrest and DNA damage-inducible 34 (Gadd34) inhibition was shown to be important, and Gadd344 overexpression through gene therapy increased liver regeneration in mice with fatty liver[71].

Clinically, fatty livers are the subject of potential hepatectomy when the resection of colorectal metastasis is being contemplated in patients with fatty liver due to NAFLD or as a secondary response to chemotherapy.

Interestingly in a recent analysis of patients who had undergone liver resection for colorectal liver metastases, in over 5800 patients that had not received pre-operative chemotherapy, and 4000 patients that had received pre-operative chemotherapy, the presence of hepatic steatosis did not worsen their 90 day or 5 year overall survival rates[72, 73].

In transplantation the presence of macroscopic hepatic steatosis can have serious consequences and increase the risk of primary graft non function[74], the more severe the steatosis then the greater the risk of hepatic dysfunction. The assessment of steatosis can be made by the surgeon at the time of organ procurement but has an inbuilt subjective element. A pathologist can quantify hepatic steatosis on biopsy but this is not always convenient and of course is a limited sample area. Imaging methods are therefore being trialled as a way of objectively quantifying hepatic steatosis. A CT assessment of the donor liver was tested in 109 consecutive cadaveric donors and the liver/spleen attenuation ratio determined (used in liver donor imaging). All graft had a biopsy and blinded pathological assessment. The CT scan was able to predict significant steatosis (defined as >30%) with a sensitivity of 79% and a specificity of 97% [75] indicating its potential future utility.

Small liver grafts, ischaemia-reperfusion injury and RAGE

The transplantation of small grafts relative to the recipient can result in so-called “small for size syndrome”-SFSS. There are several putative pathological mechanisms thought to be causative including intrahepatic vascular shear stress. SFFS results in a combination of injury in the liver and poor regeneration of the graft[76]. The clinical presentation often includes liver failure, coagulopathy, ascites, cholestasis and encephalopathy. Whilst the mainstay of management is prevention by careful volume analysis, surgical techniques are developing to reduce the incidence of this serious but thankfully rare condition[77].

Ischaemia –reperfusion injury is a major issue affecting transplanted livers. Reactive oxygen species increase in the graft during removal of the graft and cold storage due to anaerobic metabolism. Receptor for advanced glycation endproducts (RAGE) is markedly increased in mice subject to hepatic ischaemia-reperfusion injury. Two important studies have shown that RAGE is a potential therapeutic target. In a murine model of ischaemia-reperfusion, blockage of RAGE signalling reduced liver injury and increased regeneration[78]. In a murine model of hepatectomy, RAGE was increased in 85% hepatectomy mice, compared to 70% hepatectomy in mice. RAGE was expressed in dendritic cells mononuclear phagocyte - derived dendritic cells. Blockade of RAGE reduced hepatocyte death, increased regeneration and increased survival in the 85% hepatectomy mice from 30% to 90%[79]. In patients with acetaminophen induced acute liver failure, increased circulating levels of soluble receptor for advanced glycation end products (sRAGE) was found to be associated with liver transplant or death rather than spontaneous recovery[80]. Future therapy may be indicated by a study in mice which reduced hepatic IR injury by prior treatment with the drug

Losartan which increased PPARG signalling and reduced RAGE activation[81]. Clearly the early detection and quantification of the livers metabolic status to predict post-transplant ischaemia-reperfusion may allow therapeutic trials and interventions. It has been suggested, that ex vivo spectroscopy of the organ could be performed to gain a real time assessment of the metabolic status of the graft and facilitate possible pre-conditioning interventions[56]. Whilst this is an interesting future technique it is technology and operator intense and at present time this may limit widespread uptake.

The issue of abnormal and excessive extracellular matrix

There is activation of the quiescent stellate cells (pericytes into activated scar forming myofibroblasts leading to excessive deposition of extracellular matrix (**Figure 2B**). This aberrant scar formation in the liver has been shown to inhibit hepatocyte proliferation[23]. The collagen scar also needs to be remodelled for the formation of a ductular response[26]. The relationship between the "ductular reaction", which occurs during times of impaired regeneration, and fibrosis is complex as a florid ductular response is also commonly associated with a rapid fibrotic response[29]. Following cessation of injury the scar tissue can be degraded, the hepatic macrophages are thought to be key cellular mediators of this action[24], secreting matrix metalloproteinases that can degrade scar tissue[24, 82]. If liver injury and scarring progresses, then eventually bridging fibrosis and the appearance of regenerative nodules occur. In this setting vascular abnormalities develop and the blood flow to the liver switches from being predominantly from the portal vein to predominantly from the hepatic artery-i.e. becomes "arterialised". The livers epithelial cells- hepatocytes and biliary epithelial cells- become increasingly senescent (unable to divide)[83]. In this setting removal of the injurious agent is the key goal to promote endogenous liver repair but there is evidence that the addition of additional ex vivo cell therapies such as macrophages may promote the endogenous repair by increasing the resolution of liver fibrosis and promoting liver regeneration[84, 85].

Clinical evidence of regeneration in the setting of cirrhosis and chronic liver injury

Liver disease is often silent and patients may present to liver physicians with established cirrhosis. Often the insult can be removed such as the treatment of hepatitis C, cessation of alcohol or treatment of an auto-immune disease, however patients are on a precarious tightrope with very small changes leading to decompensation and frequent hospital admissions with decompensated liver cirrhosis- likewise natural history studies clearly show that some patients can re-compensate at this stage and not require further inpatient hospital treatment. By providing a stimulus to the natural regenerative process this treatment is targeted to a patient group who will benefit hugely from a successful strategy to improve liver regeneration.

Even in cirrhosis, if the injurious insult can be treated the liver can regenerate and remodel to some degree. D'Ambrosio et al. showed in a paired biopsy study in patients with hepatitis C induced liver cirrhosis who had been successfully treated for the hepatitis C infection that after 61 months from viral eradication (SVR),

cirrhosis regression was observed in 61%, and the collagen content decreased in 89%[86]. Critically, regression of cirrhosis can lead to a reduction in hard clinical endpoints (complications, death)[87]. This clearly indicated that even in the context of liver cirrhosis the natural history can be modified and patient outcome improved. With the new, exciting and effective anti-viral treatments appearing for hepatitis C there will be many patients with hepatitis C induced cirrhosis, who have been cleared of virus and have non-progressive disease but who are still at high risk of clinical events and decompensation, who will benefit greatly from treatments which improve liver regeneration and background liver function.

The requirement for regeneration is even greater when considering liver resection for HCC in the setting of a patient with cirrhosis. Whilst regeneration can still occur in this setting, there is a risk of developing liver decompensation which is often guided by clinical features such as the presence of portal hypertension.

The changes in liver function can be clinically measured using the MELD scoring system using objective variables that are readily obtained namely; serum bilirubin, serum creatinine and INR. MELD has been validated in outpatients with compensated cirrhosis and across a broad spectrum of liver disease. It is highly accurate in predicting one week, three month and one year mortality. MELD independently predicts clinical decompensation in patients with compensated cirrhosis. MELD score has been used by all the major Western regulatory authorities involved in liver transplantation (UK Transplant, Eurotransplant and UNOS) to help prioritise the allocation of liver transplants. This indicates that simple measurements can have predictive power.

4. Clinical measurement of liver regeneration

Much of the literature around measurement of human liver regeneration relates primarily to liver resection and liver cancer and to a lesser extent to acute liver failure. Consequently there has been a greater focus on measures of volume replacement or recovery from very low levels of hepatic functionality as seen in acute liver failure. The advent of new therapeutic strategies, and in particular their use in the setting of chronic liver damage, will require additional measures of liver regeneration that more appropriately reflect the less profound changes, albeit still clinically relevant, that may occur (**Figure 3**).

Clinical symptoms and signs of liver dysfunction:

For the patient with compensated liver cirrhosis it is unlikely that there will be any significant symptoms or signs that can be used as a measure of regeneration. Symptoms that exist in this setting are often less precise such as fatigue, sub-clinical hepatic encephalopathy or muscle weakness and can sometimes be multi-factorial in origin. Nevertheless such parameters can be measured using validated questionnaires and may provide clues to the effect of an intervention[88-90]. For patients with more advanced chronic liver disease

there will be more over features of liver dysfunction (hepatic encephalopathy, jaundice, ascites) that can serve as a point from which to measure improvement in liver function (regeneration). This evaluation can range from a categorical assessment (clearance of ascites/encephalopathy), to subjective semi-quantitative scores (grades of ascites or encephalopathy) through to a more formal model such as the modified Child-Pugh score (CPS). The CPS which encompasses both objective (bilirubin, albumin, INR) and subjective (hepatic encephalopathy & ascites) assessments of liver dysfunction generates a numerical value to reflect the state of liver dysfunction. This scoring system was originally validated as a prognostic tool to predict mortality during surgery for patients with liver cirrhosis[91], although it is now more commonly used to determine their overall prognosis.

Blood measures of liver regeneration:

Simple measures of regeneration include measurement of the cancer neo-antigen alpha-fetoprotein (AFP) in serum which is also released in response to [92]hepatocyte turnover. The rate of increase of serum AFP has been shown to correlate with survival of patients with acute liver failure, although its utility in the setting of chronic liver disease is less well established. Higher serum levels of miR-122, miR-21 and miR-221 have been reported in patients spontaneously recovering from acute liver failure due to a range of aetiologies as compared to patients that did not recover. Additionally, patients with elevated serum miR levels displayed increased hepatocyte proliferation and down-regulation of hepatic miRNA target genes that impaired liver regeneration. Recently the Acute Liver Failure Study Group [ALFSG] index was established and compared with the long-standing King's College criteria (KCC) and Model for End Stage Liver Disease (MELD). Hepatic coma grade, INR, serum bilirubin, serum phosphorus and serum M30 value accurately identified patients that would require liver transplantation or die. The ALFSG index identified these patients with 85.6% sensitivity and 64.7% specificity. The ALFSG Index was superior (AUROC 0.822) to KCC (AUROC 0.654) or MELD (AUROC 0.704) (p=0.0002 and p=0.0010 respectively) in identifying patients that would require LT.

Recognising the potential limitation of scoring systems such as CPS which include subjective assessments the MELD score was developed in 2000 and consisted of bilirubin, creatinine and INR[93, 94]. Its ability to predict prognosis of patients with liver cirrhosis has been validated in many studies. Prognosis can be deduced from the absolute value as well as a change in MELD over a defined time-period such as 3 months[95], which may be of particular relevance for the assessment of new therapies. Studies using MELD as an outcome measure will need to determine the durability of any observed change as well as establishing if it has the same clinical prognosis.

Comment [PN1]: PMID 24913549 R3P3

Comment [PN2]: PMID 22885329

Less often used tests for liver function include those that test the ability of the liver to metabolise administered chemicals such as Erythromycin breath test (EBT)[96] and Caffeine elimination rate (Caff kelim) and clearance (Caff Cl)[92]. Other tests measure hepatic circulation using test compounds with flow-dependent, high first-pass hepatic extraction such as galactose (Galactose elimination capacity; GEC)[97] and cholate clearances (CA Cl) and shunt (CA shunt)[98]. Whilst attractive candidates to assess response to a treatment the clinical validity of these parameters have yet to be established and thus the clinical significance of any change remains uncertain.

Imaging assessment of liver regeneration:

In the setting of liver resection the rate at which liver volume recovers is commonly used as a measure of regeneration, and can be undertaken with a range of imaging modalities including CT, MRI and SPECT scanning **(See table 2).** The relevance of this relatively crude measure of regeneration is not entirely clear in the setting of more subtle interventions and thus new approaches are needed. In the setting of chronic liver disease imaging modalities are being increasingly used to determine the extent of liver fibrosis through assessments of liver stiffness. Resolution of liver fibrosis is likely to be an important therapeutic target when trying to promote liver regeneration. These include ultrasound based modalities (Fibroscan) which are widely used in clinical practice[99] as well as CT and MRI approaches which may be superior in their ability to provide a more global assessment of fibrosis and hence allow for identification of more subtle changes. Dynamic contrast enhanced CT (DCE-CT) and MRI (DCE-MRI) imaging allow[100] for measurement of hepatic perfusion, and in the setting of liver cirrhosis may be able estimate the extent of portal hypertension which is commonly elevated in this setting. A commonly used measure is the hepatic perfusion index which is calculated by estimating the slope of arterial perfusion divided by the sum of the slope of the arterial and portal perfusions[101]. The ability to non-invasively measure portal hypertension would be an important outcome although as yet this has not been achieved. It represents a clinically relevant parameter against which the effectiveness of new therapies can be judged and is accepted as a licensing end-point by regulatory authorities.

The future:

The most immediate developments will likely focus on non-invasive assessment of portal hypertension which will represent a significant step forward. There is also a requirement however for the development and validation of new non-invasive tests to inform on early signals of hepatocyte turnover and fibrosis re-modelling.

5. The management and therapeutic targeting of liver regeneration

Regenerative mechanisms are present during liver injury, even after chronic damage, but in many cases they are insufficient to overcome the ongoing insults, necessitating additional measures to be explored (**see table 3**).

Reduction or removal of injurious process:

In many cases the optimal method to promote liver regeneration is to either stop or interfere with the injurious process. In many cases this involves stopping or reducing the aetiological factors such as alcohol cessation or losing weight, whilst in the case of viral hepatitis the use of new anti-viral medications has had a major impact on disease progression/resolution[102, 103]. In many situations though there are either no effective treatments (non-alcoholic fatty liver disease[104]/autoimmune liver disease[105]) or patients continue to consume alcohol, and thus in these settings additional interventions to promote liver regeneration. Moreover, there are occasions where even after the injurious agent has been removed the residual liver damage is so advanced, and/or continues to progress such that adjunctive measures are needed.

In many forms of liver disease there is a superimposed immune-driven component to the ongoing liver damage, leading to strategies to modulate immune responses in this setting. This has taken the form of either pharmacological approaches to reduce lymphocyte ingress to the liver as well as cell therapy approaches to reduce the activity and ingress of inflammatory cells[106].

Encouraging endogenous liver regeneration:

In the setting of chronic liver disease hepatocyte proliferation is impaired, and the presence of liver fibrosis is recognised as a major factor inhibiting hepatocyte proliferation[107]. Thus approaches to reduce liver fibrosis as discussed below may be effective in promoting liver regeneration. Other strategies include the use of pharmacological agents or cytokines to stimulate hepatocyte proliferation. Granulocyte colony stimulating factor (GCSF) has been used extensively in pre-clinical models where it has been demonstrated to stimulate proliferation of endogenous hepatocytes resulting in both less liver damage and less fibrosis[108]. GCSF has also been demonstrated to increase both the proliferation and motility of hepatic progenitor cells[109], which may in turn also aid regeneration. However, there still remains uncertainty about the efficacy of GSCF in liver disease with the majority of clinical studies being small in nature and not powered to confirm efficacy[110]. A notable exception to this relates to a randomised controlled trial in acute on chronic liver failure, where GCSF administration was demonstrated to improve survival of patients. The mechanism of this effect was not established although the authors speculated that GCSF may improve neutrophil

function, which is commonly diminished in the setting of chronic liver disease. Thyroid hormone (T3) has been demonstrated by several groups to be a strong inducer of liver cell proliferation in rats and mice[3], and recent studies have shown that the hepatocyte mitogenic response is mediated by PKA-dependent β-catenin activation[111]. Enthusiasm for T3 is reinforced by the observation that its administration helps inhibit/reverse non-alcoholic fatty liver disease[112] and lowers the risk of hepatic tumour development[113]. An important concern when the promotion endogenous regeneration is considered is the potential development of hepatocellular carcinoma (HCC). This is pertinent in the context of cirrhosis when the risk if HCC is raised and there is frequently activation of the ductular compartment. The cellular origin of the HCC is therefore a consideration. Recent data from the Schwabe group convincingly found that in 2 mouse models of HCC that the cancers arose from lineage traced hepatocytes rather than the biliary/progenitor compartment[114]. The important study, reviewed above, by Ninomiya et al. in the 90% hepatectomy model raises the idea that when there is a small liver remnant and significant liver volume gain is required, then controlling the rate of liver regeneration and thus minimising architectural and sinusoidal disorganisation is a valuable concept that may be worth translating toward the clinic[14].

Degradation of fibrosis:

There has been extensive investigation of potential effective anti-fibrotic agents in pre-clinical models of liver disease, predominantly in the carbon tetrachloride (CCl_4) model. Resolution of liver fibrosis is known to be more difficult in its more advanced stages, and thus there are uncertainties about the generalizability of pre-clinical models such as CCl_4 to the clinical situation of liver cirrhosis. One of the other major challenges in the translation of such agents into the clinical arena, is the lack of any satisfactory non-invasive methods to quantify liver fibrosis, and hence the reliance on liver biopsy for measurement of fibrosis which poses significant logistical issues. Nevertheless, several anti-fibrotic drugs have been studied in early phase clinical trials[115] with no compelling signal for efficacy seen with Colchicine[116], IL10[117], IFNγ[118] and a possible signal with Losartan[119].

Cell therapies have also been studied in pre-clinical models of liver fibrosis, with macrophages[120], bone marrow[121] and mesenchymal stromal cells[122] all having demonstrated efficacy in models of CCl_4-induced liver fibrosis (**see table 3**). Smaller scale clinical studies in patients with chronic liver disease have also suggested reductions in liver fibrosis alongside improvements in liver synthetic function[123]. These studies have predominantly used haematopoietic stem cells or mononuclear preparations which have either been harvested from bone marrow or mobilised into the circulation by the use of GCSF, which as indicated earlier, may have additional beneficial effects on liver regeneration. Whilst these improvements in synthetic function came from small non-powered studies there was little to suggest any significant safety concern, with the exception of when cells were infused intra-portally[124]. In that setting there was an increase in portal hypertensive bleeding, which serves as a caution for such routes. Indeed, homing of stem cells to the liver is

enhanced after liver injury and given that trial data do not suggest superior efficacy with liver-directed infusions logistically easier routes can be used. To determine the mechanisms by which these potential effects are achieved in the clinical setting requires further investigation, as does their confirmation in larger clinical trials. To date there are no adequately powered randomised trials of cell therapy that show a positive effect[125].

6. Conclusions

Liver regeneration in the normal liver is well described in validated model systems such as the rat and mouse partial hepatectomy models. The cellular and signalling mechanisms described using these models have provided a general template for understanding liver regeneration and to plan therapeutic interventions. New models such as the zebrafish are bringing the ability to rapidly screen compounds for their ability to improve liver repair and regeneration following injury.

In the clinical setting, the deficiencies of regeneration usually impact when there is a grossly abnormal liver architecture or when normal liver regeneration is severely impaired. Understanding the abnormal regenerative responses, and how they differ from “normal healthy regeneration” will be critical to accurately targeting new therapies. Such strategies may have several broad targets such as the excessive fibrosis, abnormal ductular responses and the impaired innate immunity which is a feature of liver dysfunction. Advances in imaging technology such as MRI combined with liver spectroscopy may provide more complete picture of the liver volume and anatomy, liver blood flow data, measures of whole liver fibrosis and whole liver signatures of metabolic function that could provide a “whole liver” picture of structure and function to guide surgical resection and other therapeutic decisions. Overall, we conclude that where there is good animal data of efficacy for a particular intervention, and there is an acceptable risk-benefit ration, then the time is right to translate this knowledge and perform appropriate prospective clinical studies.

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Schemata of Normal and Abnormal liver regeneration

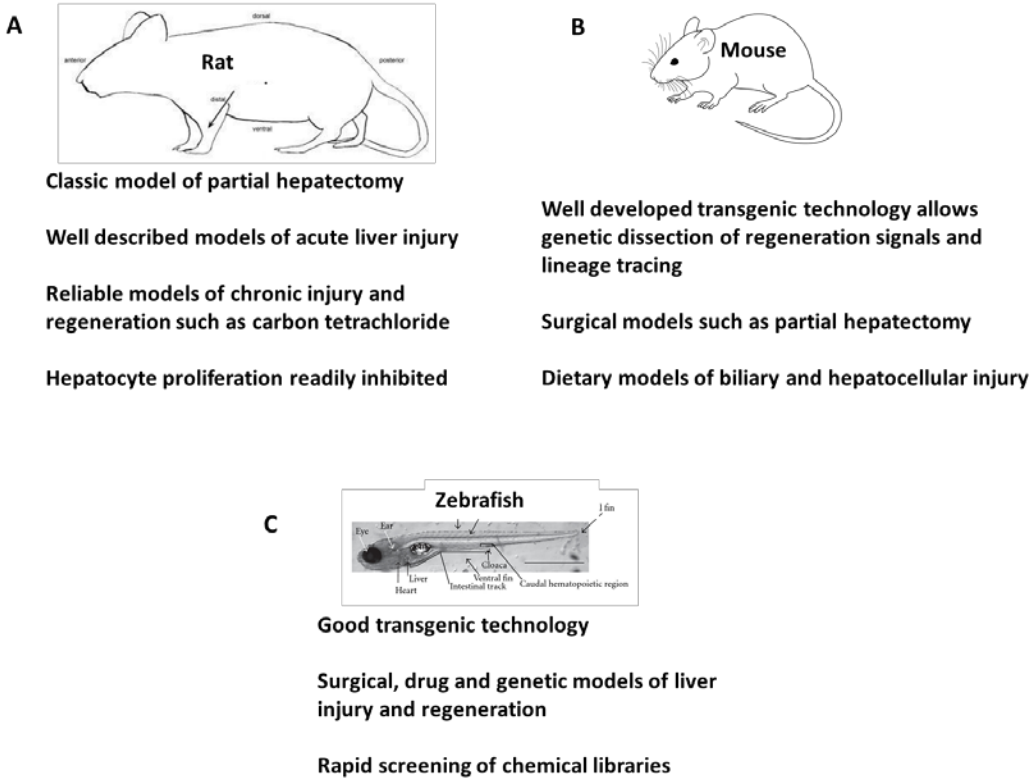

Figure 1: Animal models of liver regeneration: A. the rat is the classic model for studying liver regeneration following 70% hepatectomy which results in rapid activation of regenerative signals peaking at 24hours. Hepatocyte proliferation can be inhibited efficiently by toxins, when additional injury such as partial hepatectomy is added then a widespread "oval cell response" is activated. B. The mouse has similar properties to the rat but with the additional advantage of good transgenic technologies. Hepatocyte proliferation is not readily inhibited using standard methodologies. C. The zebrafish is rapidly gaining favour because it is rapid to use, is cost effective, allows excellent imaging and permits chemical screening.

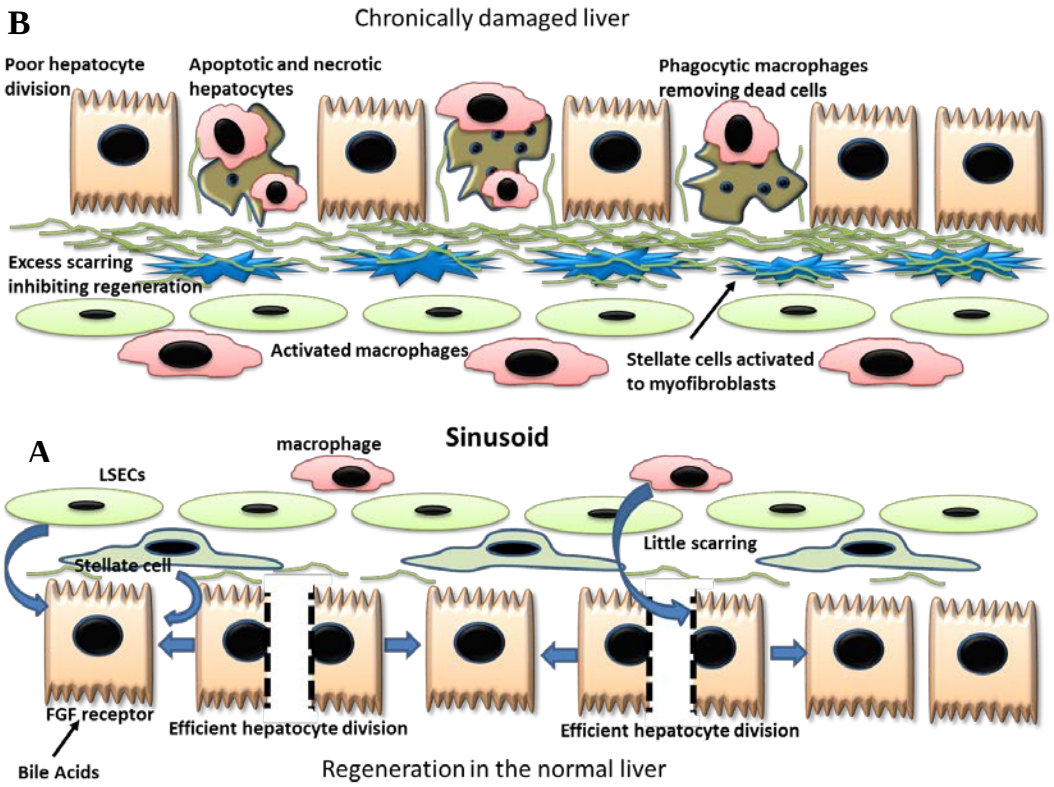

Figure 2: A: Regeneration in the normal liver follows partial hepatectomy or moderate liver injury. In this setting bile acids are rapidly upregulated, and serum factors are able to rapidly induce regeneration in the liver. Non-parenchymal cells; macrophages, stellate cells and LSECs signal to hepatocytes to leave their mitotically quiescent state and enter mitosis. Stellate cells are not activated to myofibroblasts and there is little or no scar tissue. **B:** Regeneration in the abnormal chronically damaged liver is hampered by several factors. Hepatocytes are increasingly senescent and unable to divide efficiently, the stellate cells are activated to myofibroblasts and excessive scar tissue inhibits regeneration. Excessive cellular debris inhibits efficient liver regeneration.

Strategies to improve liver regeneration

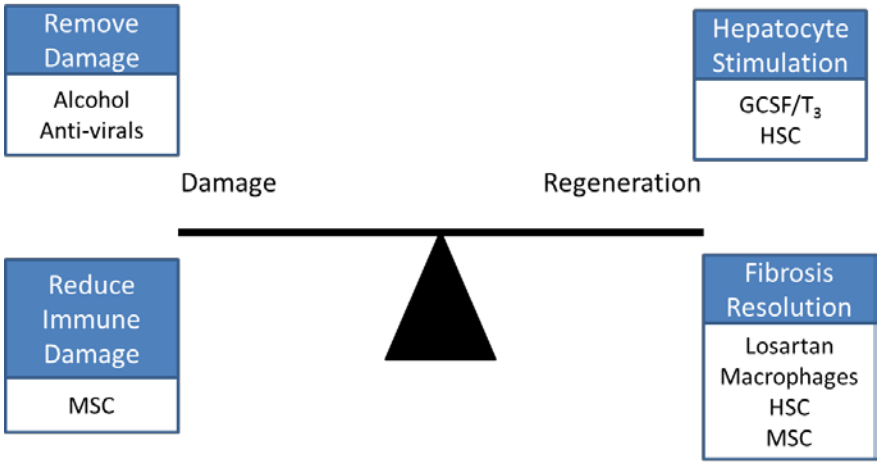

Figure 3: Strategies to improve liver regeneration include the removal of injurious agents, the promotion of fibrosis resolution and the direct stimulation of hepatocyte proliferating. The balance between these two sets of processes is key in determining the clinical outcome.

Animal Models	Clinical Studies
Have helped to define many signals and cellular sources controlling liver regeneration Transgenic technology enables cell specific and temporally controlled gene deletion to define gene function during liver regeneration Lineage tracing studies help define the cells that actually regenerate the liver	Liver pathology studies show static pictures of cellular and extracellular architecture during regeneration Gene expression studies complement the pathological studies
Studies of injury and regeneration in animals are often relatively short lived and mild	Chronic injury can develop over decades in humans and produce very abnormal liver architecture
Animal models usually study one form of injury	Patients often have multiple factors affecting their liver physiology and regeneration
Imaging modalities are developing rapidly but are often not widely clinically applicable	Clinical studies rely heavily on serum markers and non-invasive markers of liver function

Table 1: the differences between animal models of liver regeneration and clinical studies of liver regeneration

Modality	Mode of action	Purpose
Elastography	Measurement of shear wave velocity after stimulus	Measure liver stiffness (fibrosis)
Computed tomography (CT)	2-D reconstruction of x-ray imaging	Information on liver structure and size
Magnetic resonance imaging (MRI)	2-D/3-D reconstruction based on radiofrequency wave detection	Information on liver structure and size
Dynamic contrast enhanced CT (DCE-CT) and MRI (DCE-MRI)	Measurement of changes in contrast enhancement over time in vascular beds/organs	Measurement of hepatic perfusion and guide to portal pressure
Single-photon emission computed tomography (SPECT)	Measurement of uptake of radioactive tracer by metabolically active cells	Visual measure of hepatic metabolic function

Table 2: non-invasive methods of assessing liver injury, structure, volume, and function

Cell type	Indication	Advantages	Disadvantages
Haematopoietic stem cells	Promote hepatocyte proliferation; Reduce liver fibrosis	Safe; Supportive pre-clinical and early phase clinical data	Autologous therapy; Cost; Still unproven
Macrophages	Reduce liver fibrosis	Supportive pre-clinical data	Autologous therapy; Cost; Possible off-target effects; Still unproven
Endothelial progenitor cells	Reduce liver fibrosis; Promote revascularisation	Supportive pre-clinical data	Autologous therapy; cost; Possible off-target effects; Still unproven
Regulatory T cells	Reduce immune-mediated damage	Supportive pre-clinical data; efficacy in renal transplant clinical studies	Autologous therapy; cost; still unproven
Mesenchymal stromal cells	Reduce immune-mediated damage; Reduce liver fibrosis	Allogeneic; Supportive pre-clinical data; Efficacy in non-liver transplant clinical studies; Safety profile encouraging.	Still unproven; Phenotypic characterisation of infused cells poorly defined

Table 3: different types of potential cell therapies for liver injury and regeneration