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Review

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Cellular and Molecular Cascades during Liver Regeneration

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ABSTRACT

The demand for organs such as the liver for patients with end stage disease is greater than what is currently available. Thus, there is a dire need to have alternative solutions, for which none exist at the moment. Investigating the key underlying mechanisms involved not only in liver regeneration and repair, but also in development, can give us a better understanding of how to promote a pro-regenerative phenotype in the liver. This review will focus on the cellular and molecular aspects of liver regeneration and address signaling mechanisms involved in liver development and how they are recapitulated in regeneration after a partial hepatectomy.

KEYWORDS: Liver; Regeneration; Hepatectomy; Stem cell; Healing; Inflammation.

ABBREVIATIONS: PHx: Partialhepatectomy; HSC: Hepatic Stellate Cells; BECs: Biliary Epithelial Cells; STM: Septum Transversum Mesenchyme; IL-6: Interleukin-6; FSCs: Facultative Stem Cells; Ang2: Angiopoietin 2; GFAP: Glial Fibrillary Acidic Protein; GFP: Green Fluorescent Protein; Hh: Hedgehog.

PREFACE

The liver's remarkable regenerative capacity was first described by the Greeks in the legend of Prometheus, a Titan who was banished by Zeus to eternal punishment. He was chained to a rock on a mountain, where an eagle would eat his liver daily, only to have it regenerate every night. To this date, we still do not have a clear idea how the liver recovers following injury.

INTRODUCTION

The liver is known for its imperative roles in metabolic homeostasis, immune regulation, bile secretion, serum protein synthesis and detoxification properties. The majority of blood flow that enters the liver is from the spleen, pancreas and intestines via the portal vein. This blood gets filtered from toxins and drugs before entering the heart to be circulated to the rest of the body. Thus, the liver is subjected to routine exposure to damaging agents. It has been hypothesized that the liver has evolved to become a highly regenerative organ to counter these toxins,[1] because liver dysfunction and failure can ultimately lead to death. It is yet to be demonstrated whether the liver's remarkable regenerative capacity is due to several cell types or a single cell of origin.

One of the most studied models of cell organ and tissue regeneration is liver regeneration after a 2/3 Partialhepatectomy (PHx). Different methods of liver resection are used to obtain the desired amount of liver mass loss. When performing a PHx, the vessels and ducts at the pedicel of the particular lobe must be ligated prior to cutting the lobe. Typically, the left lateral

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lobe and median lobes are removed, which equates to 67% of the liver mass.[2] There is an impressive increase in hepatocyte proliferation, which peaks at 36 hours,[2] followed by reconstitution of non-parenchymal cells after surgical liver resection as seen in animals. This surgical model has become popular over the last few years and gained acceptance by the majority of the research community for numerous reasons. The first reason being due to the multi-lobe structure of the liver, resection of different segments can be done without disturbing the remnant lobe(s). Thus, regeneration of the remaining lobes is accomplished through liver specific mechanisms and not due to acute inflammation or necrosis,[3,4] which is observed during liver laceration. Second, the procedure can be done in 10-15 minutes and regeneration is triggered almost instantly, which can be tracked temporally through different phases. Third, the procedure is easily reproducible and if done correctly, all animals will survive.[2]

Due to the absence of any significant inflammation or injury to the remaining lobes after a PHx,[4] there is no reported observation of stem cell activation or cellular reprogramming. In fact, after a PHx, the liver does not regrow the resected lobes but the remaining lobes, compensate for the loss via proliferation and increase in hepatocyte size. This process is referred to as "compensatory hypertrophy"[5,6] but we will continue to use "liver regeneration" as it is still a widely used term in this field. Previous studies have shown that during liver regeneration, almost all the hepatocytes undergo1-2 rounds of replication to restore normal liver mass.[6,7] However, more recent findings using modern lineage tracing and imaging techniques demonstrate that cellular hypertrophy is a significant contributor to the compensatory response and that hepatocytes undergo on average only 0.7 rounds of cell division in mice. The first 4 hours after a PHx is known as the "priming phase" as hepatocytes prepare to respond to various cytokines by substantially changing their gene expression, including up-regulation of anti-proliferative genes.[8] It is speculated that it is during this phase that hepatocyte hypertrophy is initiated.

Considering that healing involves several stages starting with inflammation, it is not clear whether the regenerative capacity of liver is mainly due to the absence of significant inflammation or the internal capacity of liver by itself to deliver the regeneration capacity. Part of this might be due to its unique histology and anatomical position, which we will discuss here.

LIVER ANATOMY

The liver is made of liver lobules, which are hexagonal in shape with a portal triad in each corner and a central vein in the center[9] (Figure 1A). The portal triad consists of a bile duct, portal venule and portal arteriole. Hepatocytes work to absorb metabolites and toxins, which have entered the liver through the portal vein. Bile is secreted from hepatocytes into the bile ducts, which will eventually enter the gall bladder for storage and released into the duodenum. Sinusoids are lined with endothelial cells forming the blood vessels. They drain the blood from the portal venules and arterioles into the central vein to be taken back to the heart. Inside the sinusoids are Kupffer cells, which are the resident macrophages of the liver. These cells work to cleanse the blood before it enters the central vein. Hepatic Stellate Cells (HSC) are located in the area between the sinusoids and hepatocytes, known as the space of Dissé[10] (Figure1B).

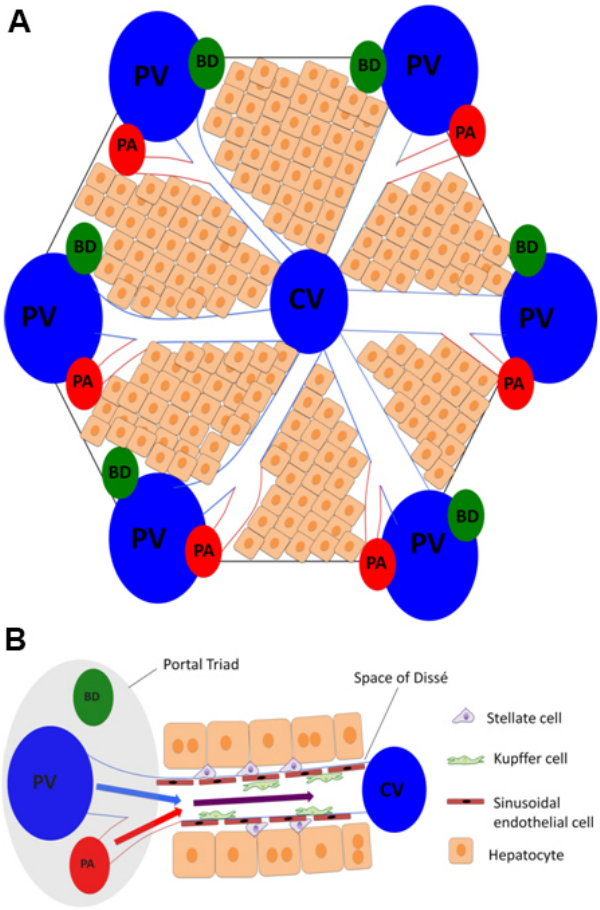

Figure 1: The functional unit of the liver. (A) The liver lobule. (B) The cell populations between the portal triad and central vein.

OVERVIEW OF LIVER DEVELOPMENT

Hepatocytes make up approximately 70% of the mass of the adult organ and are derived from embryonic endoderm, as are Biliary Epithelial Cells (BECs), also known as cholangiocytes. Other cells populating the liver include stellate cells, Kupffer cells and endothelial cells, which are of mesodermal origin. Through developmental studies on various animal models such as mouse, chicken, zebrafish, and Xenopus, many genes and molecular pathways have been identified that regulate embryonic development. These studies have enabled scientists to identify pathways implicated in liver regeneration in adult animals and humans. The regenerative mechanisms appear to recapitulate what is observed during development.

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The endoderm germ layer develops during gastrulation and forms a primitive gut tube that is subdivided into foregut, mid-gut, and hindgut regions. Fate mapping studies have demonstrated that the embryonic liver originates from the ventral foregut endoderm at embryonic day 8.0 of gestation (e8.0).[11] The thickening of the ventral foregut epithelium at e9.0 results in a hepatic diverticulum, which is the first indicator of liver development. The anterior segment of the hepatic diverticulum gives rise to the liver and intrahepatic biliary tree, while the posterior segment forms the gallbladder and extra-hepatic bile ducts. Preceding vascularization of the liver bud, at e9.0 endothelial precursor cells are situated between the epithelial cells and the Septum Transversum Mesenchyme (STM). Expression of vascular endothelial growth factor receptor 2 (Vegfr-2) has been shown to be essential as embryos that lack this gene fail to produce endothelial cells and hepatoblasts cannot go on to occupy the STM.[12]

During e9.5, the liver bud forms through the hepatic endoderm cells, known as hepatoblasts, and occupying the STM.[13,14] The STM provides the hepatic fibroblasts and stellate cells.[15] Starting at e10 until e15, liver bud gets invaded by hematopoietic cells as its development accelerates in order to become the main hematopoietic organ of the fetus. Thus, liver development involves contributions from tissues of endoderm and mesoderm origin. Hepatoblasts have bi-potential properties. Hepatoblasts that surround the portal vein differentiate to cholangiocytes, which form the primitive bile ducts also known as ductal plates. Primitive cholangiocytes express markers: Sry box containing gene 9 (Sox9), Ostepontin (OPN), and EpCAM. The remaining hepatoblasts in the parenchyma differentiate into hepatocytes.[16]

ESSENTIAL FACTORS DURING LIVER DEVELOPMENT

The regional identity of the endoderm seems to be contingent upon the spatial gradients of FGF, Wnt, BMP and retinoic acid secreted from the adjacent mesoderm.[17] However, it is still not understood how these pathways specify regional identity. Studies on chick and Xenopus suggest that FGF and Wnts released from the posterior mesoderm suppress foregut fate and promote hindgut development.[18] To establish foregut identity Wnt and FGF4 signaling needs to be inhibited in the anterior mesoderm. Inhibiting β-catenin, a downstream effector molecule in Wnt signaling, results in activation of Hhex, leading to ectopic liver buds in the intestine.[17] Interestingly, by e10, β-catenin has the opposite effect and promotes hepatic growth.[19] The specific Wnt ligands that effect hepatogenesis are still unknown. Experiments on chick embryos show that Wnt9a expressed in the sinusoidal wall is essential for liver bud growth through proliferation of hepatoblast and hepatocytes in culture.[12] In zebrafish, Wnt2b expression in the lateral plate mesoderm has been shown to be necessary for liver development. Wnt2 is also expressed in the lateral plate mesoderm and cooperates with Wnt2bbto control liver specification and proliferation in zebrafish.[20] The combined role of these signaling molecules is essential for liver specification because blocking them causes liver agenesis.[21]

In terms of hepatoblast proliferation and differentiation, hedgehog signaling is involved in promoting the proliferative response and subsequently needs to be shut off for differentiation to occur in a timely manner.[22]

Jagged-1, a Notch ligand is known to be expressed in the portal mesenchyme, which activates Notch-2 in neighbouring hepatoblasts, to promote differentiation of hepatoblasts into bile ducts.[23] Loss of Jag1 expression in the portal vein mesenchyme causes duct development to stall midway during ductal plate morphogenesis, leading to a paucity of bile ducts.[24]

Despite advancements in system biology and cell lineage studies, the cellular and molecular mechanisms of liver regeneration are still not clear. The information we learn and gather from regeneration of the liver may be used and applied to enhance regeneration of other organs. Here, we summarize the molecular and cellular mechanisms of liver regeneration after a PHx.

THE CELLULAR RESPONSE AFTER A PHx

Proliferation is the main method of liver regeneration after a PHx.[25] In mice it takes one week for the liver to return to 75% of its original size. The regenerative response involves constitution of hepatocytes first followed by biliary epithelial cells and then non-parenchymal cells.[26] Although cellular proliferation is the key regenerative mechanism, cellular hypertrophy is also observed.[6] Impaired hepatocyte proliferation is observed in aged mice, which is reversed in pregnant mice. Pregnant mice recover from a PHx at rates comparable to younger mice through hepatocyte hypertrophy.[27] This highlights the role of systemic factors contributing in hepatocyte hypertrophy.

The liver's response to a PHx is divided into two main phases. The first phase occurs between days 1-3 and is termed the "inductive phase" (Figure 2A). During this phase hepatocytes undergo proliferation. This proliferative response peaks at 36 hours and goes back down at 72 hours.[28-30] The "angiogenic phase" is the next phase which occurs, from day 4 to 8, where non-parenchymal cells proliferate, returning the liver to its normal mass and function (Figure 2B). Non-parenchymal cells have an essential role during these phases of regeneration, which will be discussed in more detail below.

THE MOLECULAR RESPONSE AFTER A PHx

The ability of the liver to know when to start and stop regeneration has puzzled scientists for years. However, certain factors have been shown to be necessary for regeneration post PHx. For example, Interleukin-6 (IL-6) and the bile acid receptor, FXR, have been shown to be essential for regeneration.[31,32]

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When the genes for IL-6 or FXR are knocked down, there is a higher mortality rate post PHx compared to their respective wild-type counterparts. In addition, assessment of proliferation through BrdU staining shows a poor proliferative response in hepatocytes. However, there is no change in non-parnechymal cells such as Kupffer cells, and endothelial cells, suggesting that non-parenchymal cells do not need IL-6 for this response.

According to transplantation studies, hepatocytes appear to have intrinsic regenerative mechanisms that are species-specific. For instance, transplantation of rat hepatocytes into mice liver, which later are subjected to PHx, has shown irregular proliferation kinetics. Rat hepatocytes become BrdU+ 24 hours later, as expected while mouse hepatocytes express BrdU 32 hours later.[33] Thus, even with the change in cellular environment, rat hepatocytes stay true to their typical response to a PHx. This suggests that hepatocytes have a certain level of autonomy when it comes to regeneration and highlights the intrinsic capability of hepatocytes rather than micro-environmental niche effects.

An alternative mechanism to liver regeneration involves a group of cells termed "Facultative Stem Cells" (FSCs) or "oval cells". FSCs were first described in rat studies that involved exposure to several carcinogens that are known to be toxic to the liver.[34] In rats it has been shown that these cells appear when hepatocyte proliferation is impaired but they are also observed in mice even with hepatocyte proliferation. However, the appearance of oval cells or impaired proliferation is not observed when rodents undergo a PHx without any chemical intervention. Further discussion of FSCs is beyond the scope of this review. Although, it is evident that the liver's resiliency comes from the multiple avenues of regeneration at its disposal.

LIVER SINUSOIDAL ENDOTHELIAL CELLS (LSECs)

LSECs are shown to regulate the temporal response of liver regeneration post-PHx. Angiopoietin 2 (Ang2), is an angiogenic protein that is down-regulated during the inductive phase,[30] which is associated with decreased TGF-β, an anti-proliferative factor, and increased expression of cyclin D1, thus boosting hepatocyte proliferation (Figure 2C). In the angiogenic phase, Ang2 levels increase, and subsequently promotes increased VEGFR2 and Wnt2 expression and proliferation of LSECs initiates[29] (Figure 2D).

The liver vasculature has varying responses to whether there is an acute or chronic injury. During an acute insult, there is up-regulation of CXCR7 by LSECs and increase in CXCR4, which together induce transcription factor inhibitor of DNA binding 1 (Id1).[28] This induces production of Wnt2 and HGF, which are pro-regenerative angiocrine factors and triggers regeneration. The essential role of CXCR7 was shown when deletion of CXCR7 in LSECs through an inducible system resulted in a poor regenerative response due to an impaired ID1 mediated production of angiocrine factors.[28] (Table 1)

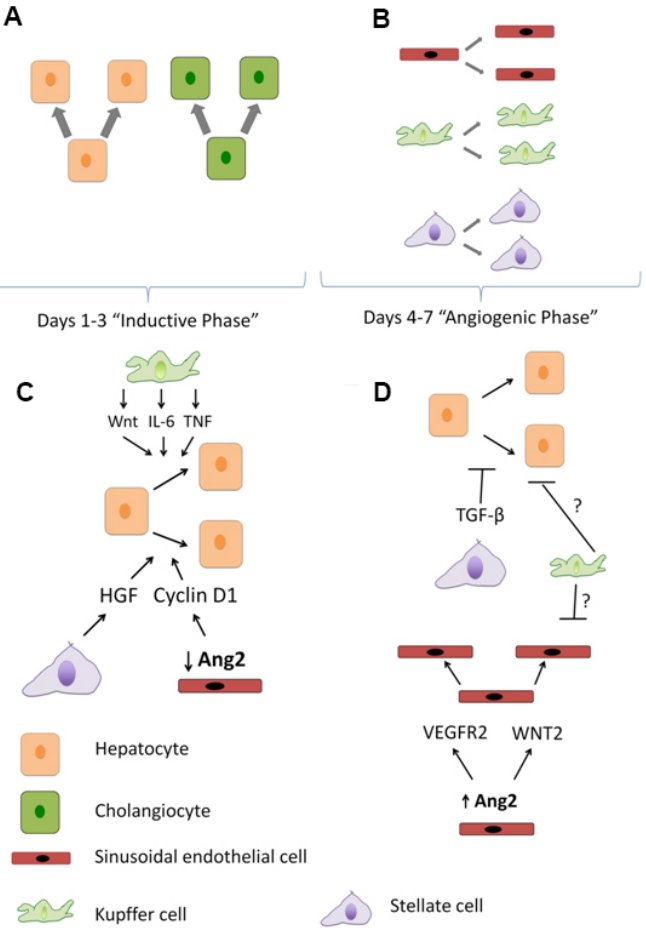

Figure 2: The proliferation kinetics and main signalling pathways involved in liver regeneration after a PHx. (A) Proliferation of non-parenchymal cells occurs during the inductive phase. (B) The angiogenic phase involves proliferation of non-parenchymal cells. (C) Role of non-parenchymal cells during hepatocyte proliferation in the inductive phase. (D) Role of non-parenchymal cells in inhibiting hepatocyte proliferation arrest and regeneration in the angiogenic phase.

Liver Lineage	Signalling pathway	Reference
Foregut Endoderm	Wnt/β-catenin and FGF4 suppressed	18
Hepatoblast	FGF, BMP	35,36
Hepatocyte	Wnt/β-catenin	20,37
Cholangiocyte (Bile duct cell)	Notch	38,39

Table 1: Signalling pathways involved in liver development.

MACROPHAGES

The powerful role macrophages play in regeneration has been shown in organisms such as zebrafish, which depend on these cells to regenerate their fins, and portions of the heart. In addition, macrophages are required for limb re-growth in salamanders.[40] The liver is known to have the highest concentration of resident macrophages of any organ. Both Kupffer cells and recruited monocyte-derived macrophages have been impli-

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cated in liver regeneration after a PHx.[41] When macrophages are ablated using liposomal clodronate followed by a PHx there is a delayed proliferative response from hepatocytes and the size of the remnant liver at 96 hours post-surgery is significantly less in Kupffer cell depleted rats.[41] This suggests that cytokines and growth factors secreted by macrophages are important for proliferative responses. Expression of key cytokines involved in liver regeneration are also down regulated at the mRNA level, this includes, IL-6, IL-10, TNF, HGF, and TGF-β1 at 4 hours post-PHx. The temporal defect in liver regeneration due to the absence of Kupffer cells may be associated with a lack of Wnt ligands that promote Wnt/β-catenin signaling in hepatocytes. When there is macrophage specific knockdown of the gene Wntless and PHx is performed a temporal deficiency in liver regeneration is observed.[42] There is a 1/3 drop in S- phase hepatocytes and hepatocyte mitosis was observed in Wls-MKO mice 40 hours after PHx. This was associated with a reduction in β-catenin-TCF4 complex and Cyclin-D1 expression at 40 hours, highlighting a role for β-catenin mediated TCF transcription factor in this process.[43] These findings suggest that Kupffer cells are essential for initiating hepatocyte proliferation in a timely manner through secretion of Wnt ligands. Other factors thought to be important for hepatocyte proliferation is interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Mice deficient in either IL-6 or TNF-α receptor type 1 showed impaired hepatocyte proliferation 40 hours post-surgery and higher mortality. (Table 2)

STELLATE CELLS

In a healthy liver, Hepatic Stellate Cells (HSCs) are in a quiescent state and store lipids such as Vitamin A. HSCs encompass approximately 5-8% of cells. Upon chronic liver injury, impaired hepatocytes and immune cells secrete factors that cause HSCs to become proliferative and differentiate into myofibroblasts.[10] These myofibroblasts are well known to be key producers collagen 1 and promote fibrosis.[49] Thus, it seems they are associated with an undesirable outcome in liver injury. However, it is also suggested that HSCs may have pro-regenerative properties as well. Spatially, the majority of HSCs reside in the Canals of Hering, a suggested stem cell niche in the adult liver.[46] More importantly, they are known to produce factors associated with regeneration such as HGF, Notch and hedgehog ligands. HSCs isolated from the early phase of regeneration in rats showed high levels of HGF in conditioned media. Furthermore, it has been argued that HSCs express the stem/progenitor cell marker CD133+ and are able to differentiate into hepatocyte-like cells with certain cytokines.[47] A lineage study was done on HSCs using a Glial Fibrillary Acidic Protein (GFAP) promoter and a Green Fluorescent Protein (GFP) reporter gene showing that after a diet-induced injury GFP+ cells proliferate and express progenitor markers cytokeratin 7 and 19.[48] Afterwards, GFP+ hepatocytes were observed suggesting that HSCs gave rise to progenitor cells that went on to differentiate into hepatocytes. They show that HSCs may produce hepatocytes *via* mesenchymal to epithelial transition.

HSCs play an essential role during liver regeneration as their regulatory effect includes stopping regeneration. They secrete factors that arrest regeneration once the appropriate mass is achieved. The dominant arresting factor is TGF-β, which HSCs are the main producers of in the liver. In mice with the gene Foxf1 knocked down, the stellate cells were unable to become activated and impaired liver regeneration ensued along with diminished notch-2 production, which promotes regeneration of biliary epithelial cells.[50] Furthermore, in rats with 2-AAF/PHx injured livers and given L-cysteine in their diets, to impair stellate cell activation, there was abnormal regeneration due to poor progenitor cell response.[51] Thus, HSCs appear to have a temporal role in regulating the regenerative response of the liver. Initially, they promote regeneration through secretion of growth factors and then put on the brakes once the normal weight and function is achieved.

THE CRITICAL ROLE OF HEDGEHOG SIGNALING

The importance of Hedgehog (Hh) signaling goes beyond just development as it is up regulated during regeneration after PHx. When Hh signaling is blocked after a PHx, *via* cyclopamine, there is reduced expression of numerous progenitor markers such as α-fetoprotein (AFP), Factor-inducible 14

	Role in liver regeneration	Reference
Hepatocyte	Hyper proliferative response post-PHx	25
Cholangiocyte (Bile duct cell)	Hyper proliferative response post-PHx	38,44
Sinusoidal endothelial cell	Spatiotemporal regulation in proliferation kinetics of hepatocytes and endothelial cells	28-30
Kupffer cell	Secrete wnt ligands that control hepatocyte proliferation in a timely manner, wnt3a secretion promotes differentiation of hepatic progenitor cells into hepatocytes.	41,45
Stellate cell	Secrete factors that promote and stop hepatocyte proliferation. May give rise to hepatocytes through MET, Secretion of Notch ligands promotes differentiation of hepatic progenitor cells to cholangiocytes.	46-48

Table 2: Contribution of different cellular components of the liver during liver regeneration.

(Fn14), and cytokeratin 19 at the mRNA and protein level.[52,53] Furthermore, proliferation of hepatocytes was impaired as BrdU incorporation decreased by 90% in hepatocytes and 40% in ductular cells.[52,53] The final outcome of this treatment shows a higher mortality in comparison with the control treated group. This highlights the importance of Hh signaling pathway in liver regeneration. It is still not clear which cell type needs activation of the Hh signaling pathway during liver regeneration, which can be further elucidated in cell lineage studies.

CLINICAL INSIGHTS INTO LIVER REGENERATION

The human liver, like in rodents, undergoes a hyperproliferative response after a PHx.[54,55] However, because the PHx model in animals, when done with precision, is relatively "clean" it does not fully recapitulate what is observed in the context of human liver disease, where significant inflammation, necrosis, and fibrosis are commonly observed.

In humans, outcomes of hepatectomy have improved over time. However, post-hepatectomy liver failure is still one of the most fatal complications of hepatectomy and occurs in up to 10% of cases. The ability of the remnant liver to regenerate after hepatectomy is the main factor in determining morbidity and mortality. If the remnant liver is less than 20%, liver function is impaired and could lead to post-resection liver failure.[56,57] Due to a scarcity in treatments for numerous liver conditions, liver resection remains the sole remedy,[54-57] despite the high concern for morbidity and mortality.[58,59] Investigating the pro-regenerative aspects of the cell types discussed above may assist in enhancing the recovery and survival of patients' post-hepatectomy and possibly after trauma, such as a severe burn.[60] Thus, despite the divergence, the compensatory response after liver resection is clinically essential and provides a great model to learn about growth and regeneration. A better understanding of how cells in the liver interact and respond to their microenvironment will give us the ability to pinpoint aberrant healing and develop novel therapies to treat liver disease.

FUTURE OUTLOOK

The regenerative capacity of the liver is unquestionable. Whether a single or several cell type(s) give rise to new hepatocytes during liver regeneration is not yet well defined. While it is believed that hepatocytes undergo hypertrophy and proliferate to regenerate the liver, it is not clear whether all hepatocytes are able to proliferate. Can a group of hepatocytes have higher capacity to proliferate? Are these hepatic progenitor cells? In addition, the majority of liver regeneration studies using the PHx model focus on how regeneration is initiated and what factors promote it while missing out on how it is stopped once regeneration is complete. Thus, future studies need to focus more on cell specific studies through lineage tracing to address the plasticity of liver cells and their fate during regeneration. Furthermore, a better understanding of how liver regeneration is terminated and the discrepancies between the PHx model in rodents and what is observed in the clinic need to be taken into consideration.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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