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Revisiting the liver: from development to regeneration - what we ought to know!

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ABSTRACT The liver is structurally and functionally heterogeneous and complex, and it accomplishes crucial functions for the organism. Its most remarkable potential is its capacity to regenerate after injury in order to maintain whole body homeostasis and guarantee the survival of the individual. Under normal conditions, liver regeneration (LR) is attributed to adult hepatocytes, the main cells in the liver which are able to proliferate in response to different stimuli or injuries. Nevertheless, when liver injury is severe and/or hepatocytes are prevented from proliferation, liver stem/progenitor cells (LS/PCs) participate directing LR to maintain liver mass and functions. Different mechanisms have been shown to guide this second line of LR, such as intrahepatic and extrahepatic liver progenitor cells, as well as transdifferentiation processes between hepatocytes and other liver cells. For this reason, many efforts have been made to elucidate the specific molecular mechanisms which orchestrate this process; this in turn would improve the prognosis and treatment of liver diseases. In this review, we revisit the fascinating process of LR, also with a short overview about liver development, the process from which arises the concept of LS/PCs participating in LR, and very important nowadays when considering cell therapy and tissue bioengineering for the treatment of patients suffering from liver disease.

KEY WORDS: *liver regeneration, hepatocyte, proliferation, liver stem/progenitor cell (LS/PC), growth factor*

The liver has been shown to be a master central regulator of metabolism and detoxification. Thus, it uncovers a major role controlling part of body homeostasis, and liver failure would constitute a major health problem if this function is not properly carried out by the liver. Understanding this complex organ from its development during embryogenesis until the development of a liver disease will permit to pomp out with new targeted therapies and tissue engineering technologies to avoid liver failure or to recover liver functions crucial for life. More interesting for this review is the special ability of the liver to regenerate after toxic insults, tissue damage or tissue loss. Liver transplant is the only curative treatment for end-stage liver disease, which is the consequence of many chronic hepatic diseases. For instance, patients with hepatocellular carcinoma (HCC), the most often primary liver cancer and the second leading cause of cancer-related mortality worldwide, undergo surgical resection. However, remnant liver can exhibit poor regenerative capacity because of the underlying pathologies. These cases of a defective process explain the huge interest to better understand the machinery of liver regeneration to find new biomarkers that could predict the response and to develop improved therapeutic options for patients with liver diseases.

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The origin: an overview of liver development

To better understand the origin of this organ, it is worthy to start emphasizing its importance within the organism. The liver carries out numerous functions: glycogen storage, drug detoxification, plasma proteins secretion (Albumin (ALB), Transferrin and Apolipoproteins) and, importantly, the control of metabolism (glucose, fatty acids and triglycerides, cholesterol, urea and non-essential amino acids). Although hepatocytes account for about 80% of liver weight, other biologically important cell types are present: Biliary Epithelial Cells (BECs or cholangiocytes), Sinusoidal Endothelial Cells (SECs), Kupffer cells (KCs, resident liver macrophages), Pit cells (resident liver natural killer cells) and Hepatic Stellate Cells (HSCs) (Si-Tayeb *et al.*, 2010).

Liver development in the embryo occurs in several stages (Fig. 1). In general terms, first, cells become "competent" and can restrict their fate. Then, competent cells subsequently become "committed" to a specific lineage, exhibiting morphological changes and express-ing genes associated with cell commitment. Eventually, cells then "differentiate" along that lineage and are ultimately able to carry out the function of a terminally differentiated cell (Hata *et al.*, 2007).

In mice, at early stages of development, epithelial cells of the foregut endoderm express the transcription factors (TFs) *Foxa1/2* and *Gata4-6,* which permit to establish the "competence" of these cells to develop into the liver. At E8.5, this "competent" foregut endoderm interacts with the cardiac mesoderm and "commit" to induce hepatic fate in the ventral foregut endoderm. This is due to the coordinated signaling of Fibroblast Growth Factors (FGFs) from the cardiac mesoderm, and Bone Morphogenetic Proteins (BMPs) from the septum transversum mesenchyme (STM) that facilitate the "commitment" of "competent" foregut endoderm cells to become hepatoblasts. On the contrary, Wnt/β-catenin signaling appears to repress liver fate (Rossi *et al.*, 2001; Zorn, 2008; Kung *et al.*, 2010; Si-Tayeb *et al.*, 2010).

Shortly after hepatic specification (E8.5 to E9), the epithelium begins to express liver genes such as *Alb*, α-fetoprotein (*Afp*), transthyretin (*Ttr*), *Hnf1a* and high expression of Cytokeratins (*CKs*) 8/18 while low expression of CK-19. In addition, the ventral foregut

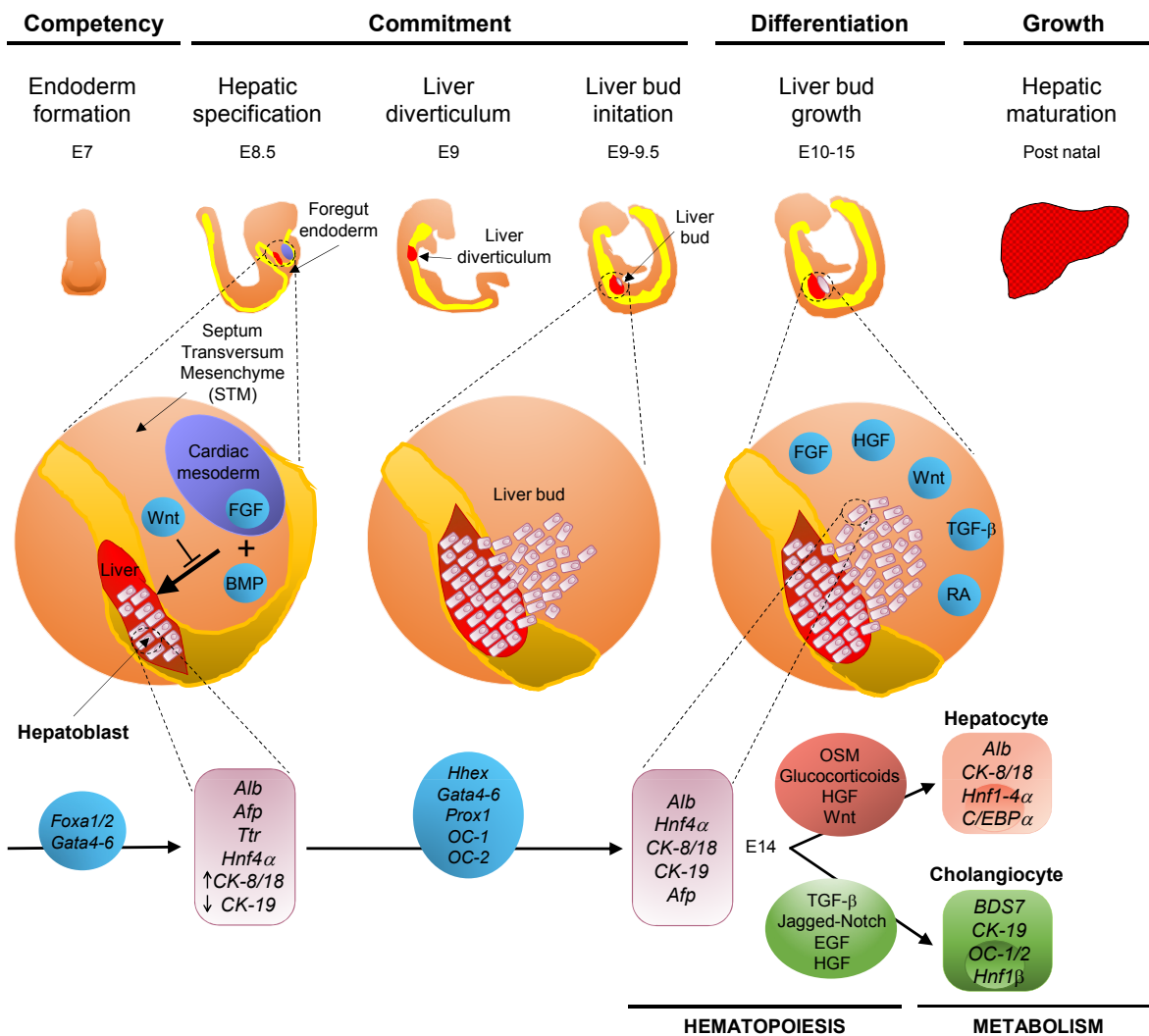
Fig. 1. Schematic overview of murine liver development. *Stages of liver development are depicted, from a macroscopic and molecular point of view. Important factors are represented in each step. See text for further details.*

epithelium thickens finally forming the liver diverticulum. Between E9 to E9.5, the basal layer surrounding the hepatic endoderm breaks down and hepatoblasts delaminate from the epithelium, migrate to invade the adjacent STM and continue to proliferate and differentiate, to give rise the nascent liver bud. Several TFs and signals from endothelial cells are required for this process, such as *Hhex*, *Gata4-6, Prox1*, *Onecut-1* (*OC-1*, also known as *Hnf6*) and *Onecut-2* (*OC-2*) (Shafritz and Dabeva, 2002; Zorn, 2008; Kung *et al.*, 2010; Si-Tayeb *et al.*, 2010) (Fig. 1).

Between E10-15, the liver bud undergoes a period of accelerated growth and it is colonized by hematopoietic cells to become the major fetal hematopoietic organ. This is regulated by paracrine signals from STM and hepatic mesenchyme that promote proliferation and survival, including FGF, BMP, Hepatocyte Growth Factor (HGF), Transforming Growth Factor-beta (TGF-β), retinoic acid (RA) and Wnt, the last having the opposite effect than in previous stages. Around E14, one of the major stages occurs, when these bipotential hepatoblasts "differentiate" into hepatocytes and cholangiocytes. Initially, hepatoblasts express genes associated with both adult hepatocytes (*Hnf4a, Alβ, CK-8/18*) and BECs (*CK-19*), as well as fetal liver genes such as *Afp*. Those hepatoblasts residing next to portal veins adopt a specific structure together with the expression of BDS7, an increase in *CK-19* and BEC TFs (OC-1/2 and HNF1β), concomitant with downregulation of hepatic genes, finally becoming cholangiocytes. Among the mesenchyme signals involved, it is found TGF-β and Jagged-Notch, which stimulates the expression of EGF, and together with HGF, participate in the differentiation towards a biliary epithelial phenotype. On the contrary, most hepatoblasts localized in the liver parenchyma differentiate into hepatocytes, acquiring characteristics of epithelial cells arranged in hepatic chords, and expressing *Alb* and *CK-8/18*. Signals such as oncostatin M (OSM), glucocorticoids, HGF and Wnt promote hepatocyte differentiation, most of them through the regulation of the TFs *C/EBPa, Hnφ1a* and *Hnf4a*. Maturation into hepatocytes and cholangiocytes continue until several weeks after birth. At this point, the organ's main function switches from hematopoiesis to metabolism, a capacity that dominates in the adult liver (Fig. 1) (Shafritz and Dabeva, 2002; Hata *et al.*, 2007; Zorn, 2008; Kung *et al.*, 2010; Gordillo *et al.*, 2015).

Liver regeneration: a well studied and still unknown process

Although function, structure and development clearly differentiate the liver from other organs, the outstanding physiological characteristic that makes it special is the unique ability to fully regenerate. This process reminds the ancient greek myth of Prometheus, a titan who was bond to the mountain Caucasus by Zeus to get his liver eaten by an eagle daily, which regenerated during the nights, as a punishment for stealing the fire from gods and giving it to the mankind. Liver regeneration (LR) is the development of new hepatocytes during adulthood. Adult hepatocytes can replicate in a highly-regulated manner, regenerating the liver in response to surgical ablation, toxic injury, infections, exogenous stimulus, massive hepatocyte necrosis or apoptosis. In this section, we will try to give a vast overview of LR.

Experimental models of liver regeneration (LR)
Several models, considering animal species and methodologies, have been proposed for the study of LR. On the one hand, among

the animal models traditionally used, rodents (rats and mice) are on the top, and almost all the studies and the current knowledge about LR come from them (Forbes and Newsome, 2016). However, new animal models have recently emerged, such as zebrafish. On the other hand, several methods have been described to induce loss of liver mass. It can be induced in pharmacological models by administering hepatotoxic chemicals (carbon tetrachloride (CCl_4), D-galactosamine or acetaminophen), bacterial particles (lipopolysaccharides (LPS)) and virus, among others. But there are also surgical models, and the most commonly and best-studied is the surgical procedure which removes 70% of the liver mass in rodents, known as 2/3 partial hepatectomy (PHx), and first described by Higgins and Anderson (Higgins and Anderson, 1931). Taking advantage of the multi-lobe structure of the liver, three of the five lobes (left lateral and median lobes) are removed by an easy surgical procedure, causing minimal tissue damage to the residual two lobes, which grow in size to restore the mass. The process, in rats and mice, is almost complete within 7-14 days after surgery.

What is the reason for regeneration?
The words "liver regeneration" could indicate that the liver is able to recover exactly the same disappeared lobules after resection. Nevertheless, this process is actually a "compensatory hyperplasia and hypertrophy" rather than a true restoration of the liver's original gross anatomy and architecture, which takes place in amphibians or Salamander in some parts of their bodies. Thus, during liver "regeneration" after PHx, the excised parts do not grow back. Instead, the remaining liver increases the mass to compensate for the lost tissue. Regarding terminology, *hyperplasia* would account for an increase in cell number, while *hypertrophy* refers to an increase in cell size. Although it has been generally accepted that hepatocytes undergo one to two rounds of cell division after PHx, several studies point to the hypertrophy of hepatocytes during LR (Nagy *et al.*, 2001; Minamishima *et al.*, 2002; Miyaoka *et al.*, 2012).

The reason of this special process is that liver functions are extremely essential for survival of the organism. Liver mass is maintained within a very narrow range in relation to the overall body mass, known as Liver Index (liver weight/body weight) x100 ~ 4-5%). The liver-to-body-weight ratio must be maintained always at 100% of what it is necessary for body homeostasis, so the degree of grow is precisely controlled. If there is loss or gain of liver mass, such as after liver injury or pregnancy, respectively, compensatory proliferation or apoptosis of cells restore this ratio once the stimulus is removed (Riehle *et al.*, 2011; Kang *et al.*, 2012; Cienfuegos *et al.*, 2014). This unique relationship is termed "hepatostat", and it has been well-defined and studied by many groups (Avila and Moschetta, 2015; Naugler *et al.*, 2015) and recently extensively reviewed (Michalopoulos, 2017). Therefore, liver homeostasis is of huge importance for the organism to survive, and "hepatostat" permits to drive LR, to control its termination up to a right liver size, as well as to maintain the liver weight even under normal physiological conditions.

First line of liver regeneration: hepatocytes as the main player
It is now well accepted that two physiological forms of LR exist in response to different types of liver injury. The first one, in the case of PHx and some chemical liver injuries, is when liver mass is replaced by replication of existing hepatocytes, considered the quickest and most efficient way of LR.

Mainly three networks mediate LR after PHx: cytokines, growth factors (GFs) and metabolic signals. Due to the high redundancy among their intracellular components, loss of an individual gene rarely leads to complete inhibition of LR. It proceeds along sequential and overlapping steps: an initiation "priming phase", rendering hepatocytes in a replicative competence state; a "proliferation/progression phase", with the expansion of the entire hepatocyte population; and a "termination phase", where cell proliferation is suppressed to terminate regeneration at a defined point (Fig. 2). In addition, proliferation in the expansion phase requires a complex re-design of the liver, a remodeling process representing a "fourth phase".

Priming phase

Hepatocytes resting in proliferative quiescence (G_0 phase) can rapidly and synchronously enter into cell cycle upon stimulation, undergoing one to two rounds of replication before returning to quiescence. However, in quiescent state, hepatocytes do not fully respond to GFs and need to be "primed" to enter the cell cycle (G_1 phase). The cytokine network acts as the "priming phase", which occurs during the first 4h and begins with the recognition of the Pathogen-Associated Molecular Patters (PAMPs) and the Damage-Associated Molecular Patterns (DAMPs) released from necrotic cells after tissue injury. They trigger the natural immune response, Tumor Necrosis Factor-alpha (TNF-α) secretion by KCs, and interleukins IL-6, IL-1β and IL-8 synthesis. In brief, there is an initial activation of Nuclear Factor-kappa B (NF-κB) in KCs via TNF, lymphotoxin (from T cells), and/or complement components, with downstream secretion of IL-6. In turn, IL-6 binds its receptor on hepatocytes, leading to activation of the TF Signal Transducer and Activator of Transcription 3 (STAT3), among other pathways. Then, several immediate-early genes related to hepatocyte proliferation are induced within 2h, such as *c-Fos*, *c-Jun* and others (Michalopoulos, 2010; Riehle *et al.*, 2011; Cienfuegos *et al.*, 2014).
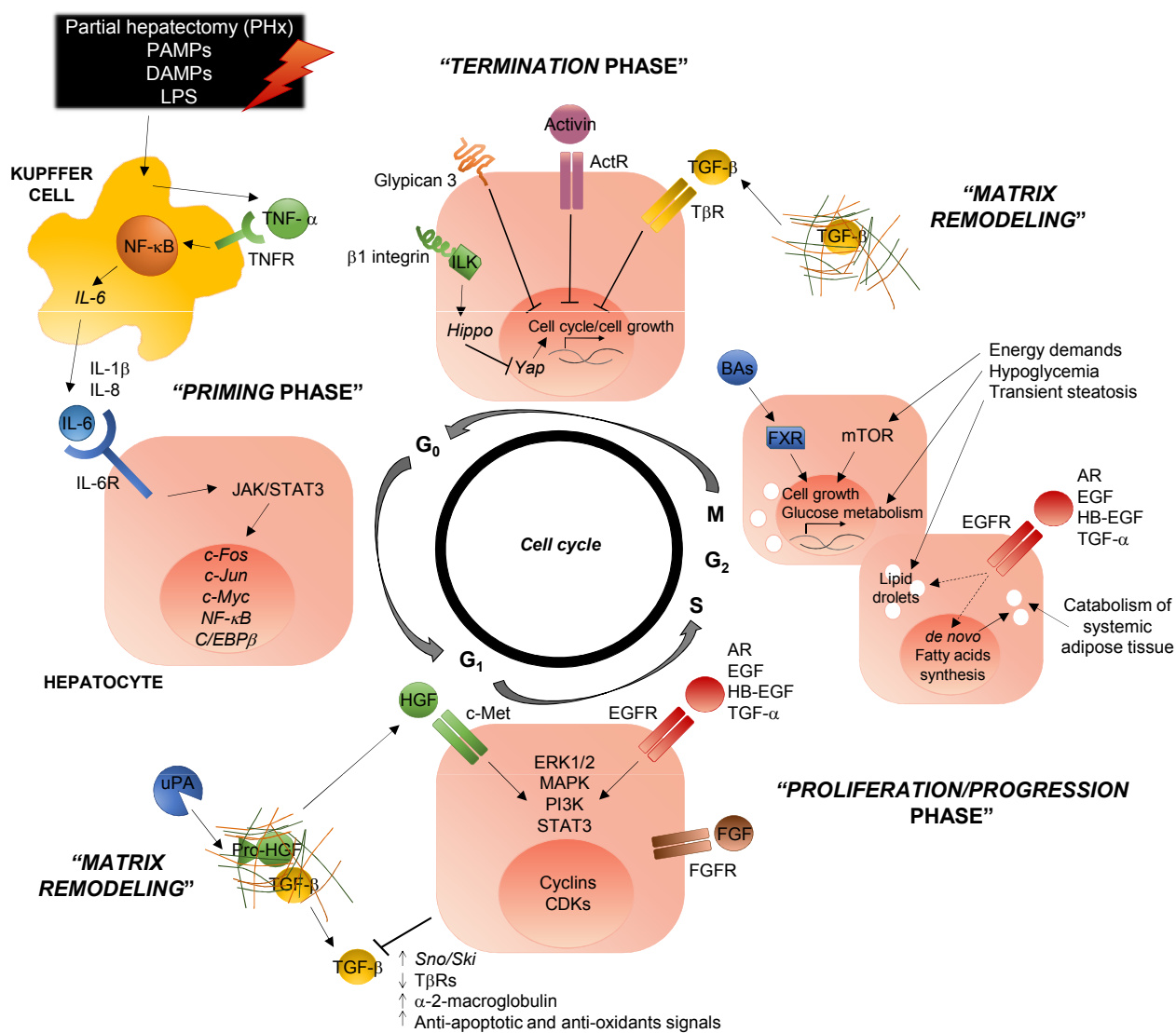

Fig. 2. Liver regeneration based on hepatocyte proliferation. *Four phases are represented: Priming, Proliferation/progression, Termination and Matrix remodeling. Cytokines, growth factors and metabolic signals involved are also indicated. See text for further details.*

Although these cytokines are not directly mitogenic to hepatocytes, they are crucial to orchestrate and optimize the process: delayed LR was observed in TNFR1-/- mice due to inefficient activation of NF-κB (Yamada *et al.*, 1998) and in mice lacking IL-6 due to loss of STAT3 activation (Cressman *et al.*, 1996).

Proliferation/progression phase

After cytokines have triggered the G_0/G_1 transition, cell cycle progression is driven by GFs, which override the G_1 restriction point denominated "R", allowing hepatocytes enter S phase (DNA synthesis) 24 and 36h post-PHx in rat and mouse, respectively. After, hepatocytes enter into mitosis 48h post-PHx. The mitogenic GFs Epidermal Growth Factor (EGF) and HGF activate their respective receptors, EGF Receptor (EGFR) and c-Met, stimulating hepatocytes progression through the cell cycle. Both are tyrosine kinase receptors, able to activate multiple intracellular signaling pathways. Among them, Mitogen-Activated Protein Kinase (MAPK), STAT3, Phosphatidylinositol-3-Kinase (PI3K)/Akt and Extracellular signal-Regulated Kinase 1 and 2 (ERK1/2) are the most important for LR, which in turn regulate a multitude of TFs including c-Jun, c-Fos, c-Myc, NF-κB, STAT3 and C/EBPβ. Together with the induction of intermediate and early-delayed genes, such as cyclins and Cyclin Dependent Kinases (CDKs), they facilitate the transition to DNA synthesis and mitosis (Riehle *et al.*, 2011; Kang *et al.*, 2012; Mao *et al.*, 2014; Cienfuegos *et al.*, 2014). Finally, FGF family is also implicated in LR. Models expressing a dominant-negative mutant of FGF receptors (FGFR) signaling pathways presented delayed proliferation after PHx (Steiling *et al.*, 2003). However, not all FGFRs play the same role: while loss of FGFR4 in mice did not affect the process (Yu *et al.*, 2000), lacking FGFR1 and FGFR2 provoked an increased mortality showing a cytoprotective role of these receptors in regenerating liver (Böhm *et al.*, 2010).

However, c-Met and EGFR are key factors in LR, which deserve a detailed discussion.

HGF/c-Met pathway - The receptor c-Met is activated by the mitogen HGF, produced by non-parenchymal cells, such as HSCs and SECs. It is produced by HSCs as single peptide (pro-HGF) and deposited in the extracellular matrix (ECM). Interestingly, one of the earliest changes is the increase in the activity of Urokinase-type Plasminogen Activator (uPA) within 1min after PHx. uPA mediates the ECM remodeling that takes place soon after PHx, involving metalloproteinases (MMPs) activation, such as MMP-2 and MMP-9 (Kim *et al.*, 2000). Then after, there is the subsequent activation of this pro-HGF, finally releasing active HGF in the environment of hepatocytes as well as in blood circulation. In fact, uPA deficiency has been shown to retard LR (Shimizu *et al.*, 2001).

EGFR pathway - This receptor is activated through different sources: in an autocrine manner by Amphiregulin (AR) and Transforming Growth Factor-alpha (TGF-α); by Heparin Binding EGF-like growth factor (HB-EGF) derived from KCs and SECs; and by EGF, secreted from salivary glands and Brunner's glands in the duodenum (Berasain and Avila, 2014). EGFR ligands have different but often overlapping functions during LR. Thus, TGF-α null mice have no deficiency during LR (Russell *et al.*, 1996), whereas the removal of the submandibular glands in mice (source of EGF) or AR and HB-EGF knock-out mice promoted an impaired or delayed LR (Noguchi *et al.*, 1991; Berasain *et al.*, 2005; Mitchell *et al.*, 2005).

It seems that both receptors have unique and potentially overlapping functions. Many studies have been done to better understand their role during LR. First studies were based on mice lacking c-Met in the liver. On the one hand, inducible deletion of the c-Met gene in the liver caused a defective exit of hepatocytes from quiescence and a delay in cell cycle entry after PHx (Borowiak *et al.*, 2004). On the other hand, deletion of the c-Met gene in hepatocytes in a non-inducible manner showed the inability of these animals to survive the procedure showing severe necrosis (Huh *et al.*, 2004). Subsequent and complementary studies based on RNA interference (shRNA) against c-Met and HGF in rats supported the previous works (Paranjpe *et al.*, 2007). Moreover, a more recent work also showed a regenerative defect in hepatocyte-specific Met KO mice. Specifically, after PHx, Met-deficient hepatocytes showed a block in early/mid G_2 phase and consequently, a failure in G_2/M gene expression program. Similar alterations were found in primary hepatocytes from unchallenged Met KO mice, which could be partially reversed by treating the cells with EGF (Factor *et al.*, 2010). All these works confirmed the importance of c-Met in LR. Regarding EGFR pathway, to overcome the problem of redundancy among ligands, models lacking EGFR in hepatocytes were generated. The first study based on the genetic loss of EGFR in mice showed higher rates of mortality post-PHx. However, apoptosis was not increased, suggesting that activation of the EGFR pathway was not directly related with survival signals for hepatocytes. Moreover, delayed and reduced hepatocyte proliferation was observed in the remaining animals, although complete regeneration occurred, so EGFR would be important but not essential for LR (Natarajan *et al.*, 2007). Another study using interference RNA in rats confirmed the critical role of this pathway, observing decreased DNA replication after PHx, concomitant with an up-regulation of Met and other ErbB members and, finally, liver restoration occurred (Paranjpe *et al.*, 2010). In contrast to these two studies, animals treated with a neutralizing antibody against EGFR did not present altered LR (Van Buren *et al.*, 2008). More recently, a different study also using EGFR null mice showed delayed cell cycle progression and proliferation after PHx, but the overall effect on liver regeneration was relatively minimal. Additionally, after CCl_4-induced injury only the Met-KO, but not the EGFR-KO, displayed enhanced necrosis and delayed LR, effects that were more prominent in the EGFR-Met double KOs, suggesting that EGFR, and at a greater extent Met, may partially compensate each other (Scheving *et al.*, 2015). Regarding this issue, our group has recently developed a novel transgenic mouse model expressing a hepatocyte-specific truncated form of human EGFR, lacking the catalytic domain, thus acting as a negative dominant mutant (aEGFR) allowing definition of its tyrosine kinase-dependent functions. These animals displayed lower and delayed proliferation as well as lower activation of proliferative early signals after PHx, demonstrating a critical role during the initial phases of LR. Moreover, it correlated with an overactivation of the TGF-β pathway, and subsequent amplification of its cytostatic effects. Nevertheless, NEGFR were able to fully regenerate the liver by overactivating compensatory signals, such as c-Met pathway (López-Luque *et al.*, 2016). Regarding this issue, a very recent work has demonstrated that only the combined elimination of Met and the inhibition of the EGFR signaling pathway completely abolishes LR. They state that both pathways separately control many non-overlapping critical points, and inhibition of only one of them had distinct alterations in different signaling pathways, allowing for compensation when only one of the signals is blocked (Paranjpe *et al.*, 2016). All these works emphasize the requirement

of simultaneous co-activation of parallel signaling pathways for full mitogenic signaling and efficient LR.

It is important to mention that, after PHx, metabolic alterations occur in the remnant liver. The liver must continue regulating systemic energy levels while meeting its own demands for nucleotide and protein synthesis needed for cell division. Thus, translation is the control point that integrates nutrient levels with mitogenic signals and most proteins involved are downstream of mammalian target of rapamycin (mTOR). Almost a complete loss of hepatocyte DNA replication was observed in S6 KO mice after PHx (Volarevic *et al.*, 2000). Importantly, changes in lipid and glucose metabolism are observed. A regenerative transient steatosis is evident during LR after PHx at early stages, which is necessary for LR (Huang and Rudnick, 2014) and concomitant with upregulation of genes related to the adipogenic program (Shteyer *et al.*, 2004). Increased *de novo* hepatic fatty acid production and catabolism of systemic adipose tissue might be the main sources of the lipid that accumulates in the regenerating liver. It is required to meet the increased energy demand for rapid cell proliferation and essential for the enhanced biosynthesis of membrane phospholipids (Rudnick and Davidson, 2012). Disruption of hepatic adipogenesis and lipid accumulation is associated with impaired or inefficient LR following PHx (Shteyer *et al.*, 2004; Kohjima *et al.*, 2013). Interestingly, EGFR plays a role in the regulation of liver and plasma lipid levels in adult male mice (Scheving *et al.*, 2014). In addition, recently it has been shown that EGFR, but not the HGF/c-Met pathway, is required for fat accumulation and properly regulation of key enzymes related to the *de novo* lipid synthesis during LR, revealing a new function for EGFR kinase activity which is not overlapping with the HGF/c-Met pathway (López-Luque *et al.*, 2016; Paranjpe *et al.*, 2016). Regarding glucose metabolism, mice develop hypoglycemia soon after PHx, showing insulin decreased levels. To compensate this, many TFs are activated upregulating glucose metabolism. Studies postponing this hypoglycemic response delayed LR (Huang *et al.*, 2016). Finally, bile acid metabolism is also altered, increasing twice their amounts *per* liver mass without showing hepatotoxicity (Csanaky *et al.*, 2009).

Termination phase

The initial burst of hepatocyte proliferative activity is followed by secondary waves of mitosis until original liver mass is restored. However, growth must be finished to control liver size and homeostais, although the precise mechanisms of a proper termination are still very poorly understood, and even controversial. Generally, when the regenerating liver reaches certain size, several factors promote proliferation arrest. Surprisingly, many of them are also present in the first stages of LR. It has been proposed that these factors would act as a "brake", controlling the speed of proliferation and the terminal point of the process, and even ensuring the right direction, preventing oncogenesis. An extensive review focused in the termination phase has been recently published (Liu and Chen, 2017). For this reason, this phase is as important as the previous ones, and more studies should be performed to decipher its molecular mechanisms. It is known that it involves multiple factors, including TGF-β family (TGF-β1, activins), IL-1, and tumor suppressor genes (p53, p21) (Michalopoulos, 2010).

TGF-β1 is a known suppressor of hepatocyte proliferation and inducer of apoptosis. Then, it would be easy to think a role for it at the end of LR. Surprisingly, its expression increases rapidly after PHx, and levels are maintained until termination of the process (Braun *et al.*, 1988). In addition, TGF-β plasma levels rise together with HGF, suggesting that the cytokine is released after the remodeling of the ECM, where it is bound to decorin (Dudás *et al.*, 2001). However, responsiveness to this factor declines transiently in the regenerating liver. TGF-β inhibitory effects are surpassed by different mechanisms: TGF-β receptors downregulation (Chari *et al.*, 1995), increasing the transcriptional repressors *SnoN* and *Ski* (Macias-Silva *et al.*, 2002), as well as being removed and inactivated through binding α-2-macroglobulin in the circulation (LaMarre *et al.*, 1991). Moreover, regenerating hepatocytes showed a reduced response to TGF-β cytostatic and cytotoxic effects, through up-regulation of anti-apoptotic and anti-oxidant signals (Herrera *et al.*, 2004). Interestingly, mice lacking the type II TGF-β receptor in hepatocytes presented an increased proliferative response after PHx, but hyperproliferation was transient and normal regeneration was achieved (Romero-Gallo *et al.*, 2005). Thus, although initially it was supposed that TGF-β could play a relevant role in mediating termination of LR, more recent results indicate that it is not necessary during this stage. Higher and accelerated DNA synthesis peak after PHx was found in a *Tgfbr2* knockout animal model. Nevertheless, similarly to the previous mentioned work, a normal ending of LR occurred, associated with increased compensatory inhibitory signals, particularly Activin A (Oe *et al.*, 2004). Interestingly, TGF-β could play an essential role in the first stages of LR, as transgenic mice expressing EGFR form presented an overactivation of the TGF-β pathway leading to a delayed regeneration (López-Luque *et al.*, 2016). Thus, a perfect spatio-temporal orchestration of TGF-β signaling during the process is required, although it is still unclear. As the NADPH oxidase NOX4 is known to mediate TGF-β-induced apoptosis (Carmona-Cuenca *et al.*, 2008) and to inhibit hepatocyte proliferation (Crosas-Molist *et al.*, 2014), it would be a perfect candidate for mediating some of the TGF-β suppressor actions during LR. Interestingly, NOX4 has been shown to be also regulated during LR, decreasing its levels soon after PHx and recuperating them at the end of the process (Crosas-Molist *et al.*, 2014). This should be an additional mechanism to avoid TGF-β suppressor effects during the early liver regenerative process.

Among other players linked to the termination phase, the Integrin Linked Kinase (ILK) would play a role. ILK is under the plasma membrane associated with β1 integrin, suppressing hepatocyte growth. ILK hepatoespecific KO animals acquire hepatomegaly and enhanced hepatocyte proliferation, presenting an impaired termination of the process (Gkretsi *et al.*, 2008). On the other hand, Glypican 3, a GPI-linked protein on hepatocytes plasma membrane, has been shown to suppress proliferation after PHx when overexpressed in transgenic animals (Liu *et al.*, 2010). In addition, these animals also presented decreased expression of Yap (Yes-associated protein), controlled by the Hippo pathway, and which has been considered a central player controlling cell size and, finally, liver hepatostat.

Thus, a proper balance of all these signals during the different phases might be a good determinant of the efficacy of LR.

Second line of liver regeneration: the time for stem/progenitor cells

When the liver is damaged with specific chemicals, when injury is severe, or when mature hepatocytes are prevented from proliferation, the contribution of liver stem/progenitor cells (LS/PCs) is crucial for LR success (Fig. 3).

As it has been mentioned, during liver development, both hepatocytes and BECs originate from the common precursor named hepatoblast. It is suggested that combined hepatocyte loss concomitant with impaired hepatocyte proliferation is necessary to activate these LS/PCs during LR. This has been observed in models combining PHx with chemical inhibition of hepatocyte proliferation using 2-acetylaminofluorene (2-AAF) or retrorsine in rats, or a choline-deficient diet in mice (Liu *et al.*, 2016). LS/PCs reside in the transition region between the canaliculi and intrahepatic bile ducts, named Canal of Hering. It has been postulated that LS/PCs can be characterized by a positive staining for BEC markers, but also by the appearance of LS/PCs (named "oval cells" in rodents and "ductular structures" in humans) throughout the parenchyma when there is a liver repair process, often forming clusters of duct-like and/or cord-like structures (Itoh, 2016). Therefore, in an elegant review, Itoh propose a model for homeostatic maintenance and LR, where the scenario starts with hepatocyte proliferation upon acute or mild hepatocyte injury. However, if injury is severe or chronic, a novel epithelial cell population identified by Font-Burgada *et al.*, namely the periportal Hybrid hepatocytes (HybHPs), is activated, located in the portal triads of healthy livers and expressing low amounts of Sox9 and other bile-duct-enriched genes (Font-Burgada *et al.*, 2015). If they are selectively damaged, then a recently found population of "unipotent stem cell-like" hepatocytes with self-renewing abilities adjacent to the central vein (generally diploid and expressing *Axin2*, the canonical Wnt signaling target gene) can participate (Wang *et al.*, 2015). Finally, if all these cells fail, it is when LS/PCs in the biliary compartment become activated to re-establish the parenchyma (Itoh, 2016) (Fig. 3).

The response of LS/PCs includes activation, proliferation, migration and differentiation, finally leading to hepatocytes or cholangiocytes. Parenchymal and non-parenchymal cells in the liver, the ECM, GFs and signaling pathways participate contributing to LS/PCs niche during LR. Regarding the crosstalk with other cells, for instance, it is found that HSCs participate in the first stages of LR producing HGF, promoting proliferation in LS/PCs. However, at the end of the process, high levels of TGF-β produced also by HSCs suppressed DNA synthesis in the same cells, revealing a dual role for HSCs in LS/PCs-mediated proliferation (Chen *et al.*, 2012). Regarding the ECM, its remodeling by MMPs and the multiple factors found in it have been reported to stimulate LS/PCs proliferation. They comprise inflammatory cytokines (IL-6, TNF-α, Stromal Derived Factor (SDF-1), TNF-like weak inducer of apoptosis (TWEAK), Stem Cell Factor (SCF), etc.), regulatory proteins (such as MERLIN acting on EGFR), or other paracrine messengers from neighboring mesenchymal cells (HGF, FGF and TGF-α/β). Specifically, HGF, EGF, TGF-α and SCF stimulate LS/PCs proliferation. On the contrary, TWEAK/Fibronectin 14 would participate in the activation, and SDF-1/CXC receptor 4 (SDF-1/CXCR4) axis in the migration of these cells (Liu *et al.*, 2016). Among all these factors, as during LR from pre-existing hepatocytes, c-Met and EGFR pathways are essential for regeneration from LS/PCs. In this sense, Met signaling is essential for survival of oval cells *in vitro*, as its deletion increases sensitivity to TGF-β-induced apoptosis, through the activation of the PI3K signaling pathway (del Castillo *et al.*, 2008a; Martínez-Palacián *et al.*, 2013). Moreover, c-Met pathway promotes oval cell migration *in vitro*, activity that also involves PI3K signaling (Suárez-Causado *et al.*, 2015). *In vivo*, lack of c-Met has profound effects on the LS/PCs-driven regeneration, including impairment in cell proliferation, migration and differentiation into hepatocytes *(Ishikawa et al., 2012)*. Regarding EGFR, it also promotes oval cell proliferation and survival, although it is dispensable for c-Met-mediated proliferation and survival of these cells, functioning independently one from each other (Martínez-Palacián *et al.*, 2012). Interestingly, while c-Met is important for hepatocytic differentiation, EGFR promotes cholangiocyte specification concomitantly suppressing hepatocyte commitment inducing Notch1 (Kitade *et al.*, 2013). Therefore, it is very common to find that these factors play different, and sometime opposite, roles on hepatocytes and progenitors cells. Finally, all the above-mentioned GFs and cytokines trigger mul-
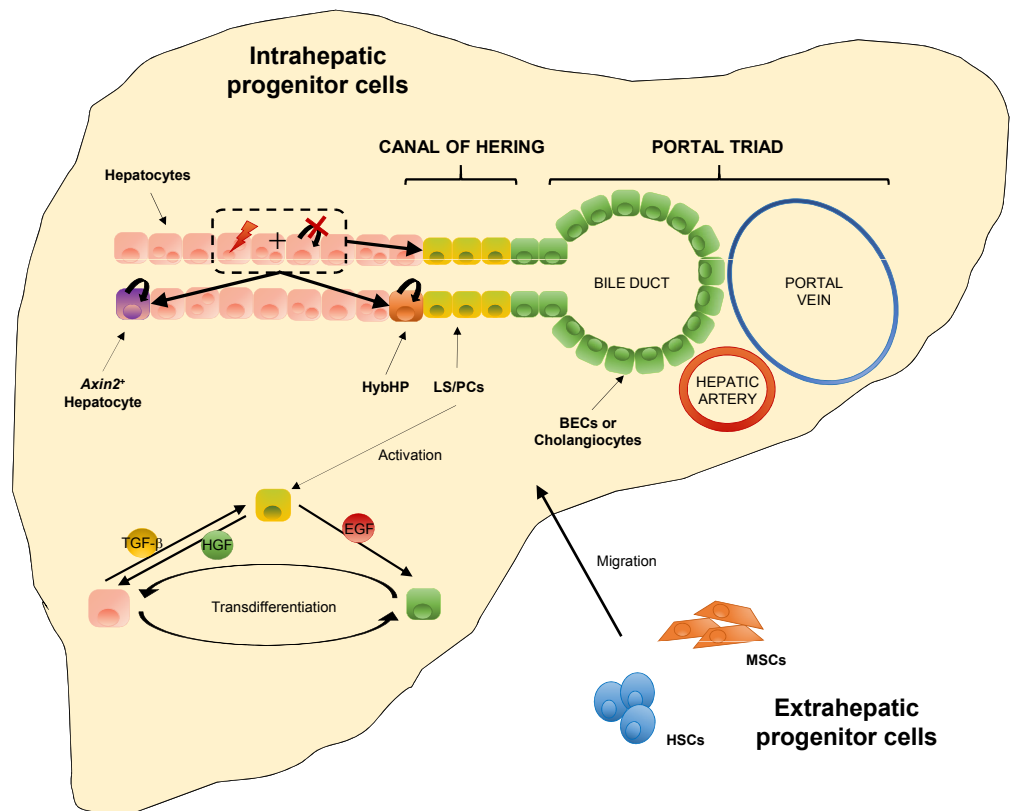

Fig. 3. Liver regeneration based on stem/progenitor cells. *Intrahepatic and extrahepatic liver progenitor cells participate in regenerating the liver when hepatocytes are prevented from proliferating or when injury is severe. See text for further details.*

tiple signaling pathways. For instance, activation of NF-κB and STAT3 in rat oval cells is required for their activation, expansion and differentiation (Sánchez *et al.*, 2004). Finally, Wnt and Notch pathways participate with opposite roles: while the former direct LS/PCs towards the hepatocytic fate, the second promotes their differentiation towards the biliary lineage (Boulter *et al.*, 2012). Of special relevance is also the implication of inflammatory signals and cells, known as "inflammatory niche", on the regeneration by progenitor cells. Although some of them have been mentioned just above, it is worthy to point out that many of them play contrary roles in the regeneration from mature hepatocytes and the one carried out by progenitor cells. For instance, while IFN-γ signaling appears to have a negative impact on LR by mature cells, the effects of this inflammatory cytokine on the progenitor cell compartment appear very different (Bisgaard *et al.*, 1999). However, what it is completely obvious is that the inflammatory microenvironment regulates liver progenitor cells expansion and fate, which has been extensively reviewed by others (Santoni-Rugiu *et al.*, 2005).

However, the huge controversial discussion comes up with the hypothesis that hepatocytes and cholangiocytes would be liver progenitors cells *per se* when undergoing transdifferentiation from one cell type to the other, acting as "facultative stem cells" (Michalopoulos and Khan, 2015). Many different studies have been focused on the possibility that cholangiocytes become LS/PCs to rescue hepatocytes. While most of them confirmed this hypothesis (Golding *et al.*, 1995; Huch *et al.*, 2015; Raven *et al.*, 2017), other groups did not (Schaub *et al.*, 2014; Yanger *et al.*, 2014). Almost all these studies prove that some cholangiocytes express hepatocyte-associated TFs, then proliferate as LS/PCs and, finally, become hepatocytes. Less controversy is found about the capacity of hepatocytes transdifferentiating directly to cholangiocytes. This has been observed in rats after biliary injury, without evidence of formation of LS/PCs (Michalopoulos *et al.*, 2005) (Fig. 3). However, various works point to the capacity of hepatocytes to acquire also stem cell properties. Thus, after TGF-β treatment, the subpopulation of rat fetal hepatocytes (FH) that survive to its apoptotic effects undergo epithelial-to-mesenchymal transition (EMT), expressing high levels of mesenchymal markers and lacking epithelial and differentiation ones, concomitant with expression of progenitor markers. Moreover, they acquire resistance to TGF-β-induced apoptosis (Valdés *et al.*, 2002; del Castillo *et al.*, 2006). Their isolation and characterization demonstrated that they behave as liver progenitors, supporting the hypothesis that hepatocytes can function as facultative liver stem cells and that TGF-β might play an essential role in this transdifferentiation process (del Castillo *et al.*, 2008b). Importantly, this response was observe in human hepatocytes (Caja *et al.*, 2011), reaffirming TGF-β as an inducer of EMT and promoting the acquisition of a stem-like phenotype. Interestingly, this was a reversible process, where mesenchymal stem-like cells were able to re-differentiate to hepatocytes, fact that opens feasible possibilities for human liver diseases.

Nevertheless, not only resident LS/PCs play a role in this second line of LR. Nowadays, there is a consensus that also stem cells from extrahepatic sites, in particular the bone marrow (BM), can participate and contribute to LR (Petersen *et al.*, 1999; Theise *et al.*, 2000) although with low efficiency. Hematopoietic Stem Cells (HSCs) and Mesenchymal Stem Cells (MSCs) are among the most

well defined ones, migrating to the injured liver through circulation to regenerate the organ (Fig. 3). The differentiation of MSCs into MSCs-derived hepatocytes (MDHs) arises with 3 important shifts: morphologically (changing from a fibroblast-morphology to a polygonal-epithelial shape), phenotipically (regarding several TFs) and functional (acquiring the ability for glycogen storage, detoxification and lipid metabolism, within others). Nevertheless, the mechanisms of MSCs and HSCs transdifferentiation to hepatocytes are controversial. In fact, cell fusion with hepatocytes is an alternative way by which both cell types can achieve the plasticity required to develop into hepatocytes (Vassilopoulos *et al.*, 2003; Wang *et al.*, 2003; Quintana-Bustamante *et al.*, 2006; Liu *et al.*, 2015).

Finally, considering both mechanisms of the liver to regenerate, it seems obvious that progenitor cells can be a very reasonable option and an attractive alternative to organ transplantation due to several benefits: they can be expanded in culture without losing bidirectional differentiation potential, and an autologous transplant after LS/PCs isolation from a patient would obviate the need for immunosuppression (Liu *et al.*, 2016). Several alternative candidates to liver transplants have been proposed based on the progenitor cells concept, including the above-mentioned MSCs and, more recently, induced pluripotent stem cells (iPSCs).

The attractive ability to derive pluripotent cells from adult human tissues opens new opportunities in research and, importantly, in therapy. This direct reprogramming towards producing pluripotent stem cells was first achieved and published in 2007 (Takahashi *et al.*, 2007; Yu *et al.*, 2007). With this in mind, several works have developed the technology in the liver field. Thus, iPSCs-derived hepatocytes proved to have functional and proliferative capabilities needed for LR *in vivo* in mice (Espejel *et al.*, 2010), and this could be induced modeling the embryonic liver development conditions (Sancho-Bru *et al.*, 2011). Later, a great achievement was carried out by Takebe *et al.,* with the formation of vascularized and functional human liver after transplantation of liver buds created *in vitro* derived form iPSCs (Takebe *et al.*, 2013). This represents a huge step forward in the field to cure human liver pathologies. Nevertheless, the liver is a very complex organ from a structural point of view and highly heterogeneous with different cell types participating to develop its function. For this reason, there is currently no technology available to grow a newly liver that accomplishes all the characteristics to the same extent as an original one. Liver engineering tries to solve these problems, through the repopulation of a decellularized liver stroma scaffold with hepatocytes and other liver cell types, or through 3D-bioprinting technology with biological materials (Kholodenko and Yarygin, 2017). However, these very promising approaches have not yet been completely successful as they are very challenging, although they clearly open a very encouraging landscape.

Future perspectives

LR is required to guarantee the liver function during processes of chronic liver diseases. Furthermore, liver resection is the most common approach used by clinicians in secondary/metastatic lesions in the liver. For this reason, it is indispensable to continue focusing on the molecular mechanisms that orchestrate LR to predict and even improve the response of each patient to the surgery. Deciphering the enigmas of LR might also contribute to

the development of artificial functional livers to be used in patients with liver failure.

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