Genes to Cells

REVIEW

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Liver regeneration: a unique and flexible reaction depending on the type of injury

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The liver can be thought of as a mysterious organ, because it has an elegant regenerative capability. This phenomenon has been well known since ancient times and is already applied to medical treatments for severe hepatic disorders by transplanting portions of liver received from living donors. However, it was not until quite recently that the mechanism underlying the principle of liver regeneration was investigated more deeply. Recent advances in the technologies for characterizing cell properties and examining the molecular nature of cells are enabling us to understand what occurs in the regenerating liver. After acute liver damage, hepatocytes actively proliferate in response to external stimulation by humoral factors. However, in the chronically injured liver, hepatocytes cannot proliferate well, but biliary cells appearing after chronic liver damage form primitive ductules around portal veins of the liver. These biliary cells may have a multiple origin, including hepatocytes, and contain progenitor cells giving rise to both hepatocytes and biliary cells, or represent cells that can be directly converted into hepatocytes. Although liver regeneration is more complicated than we had thought, unremitting efforts by researchers will certainly connect the numerous findings obtained in basic research with the development of new therapeutic strategies for liver diseases.

Introduction

As shown by the Greek myth of Prometheus and the liver, human beings have known that the liver can regenerate itself since before the Common Era, although the mechanism underlying liver regeneration has remained largely unknown (Chen & Chen 1994). In recent years, however, the mechanism has gradually become clearer, in accordance with the development of technologies for analytical methods.

It is generally known that the liver regenerates in two distinct ways, depending on the cellular compartments undergoing proliferation. After loss of liver mass or acute (mild) hepatic injury, the cells in the remaining liver tissue, especially hepatocytes, proliferate rapidly to restore the lost cells without any contribution from hepatic stem/progenitor cells. Although hepatocytes are fully differentiated, they can still actively proliferate by self-duplication in response to external stimulation by specific humoral factors, including cytokines, growth factors and bile acids (Michalopoulos & DeFrances 1997; Taub 2004; Huang *et al.* 2006). These humoral factor signals alter the gene expression pattern in hepatocytes by activating transcription factors and modulate the balance of protein quantity in hepatocytes, leading to induction of DNA synthesis in hepatocytes through regulation of the cell cycle machinery (Costa *et al.* 2003; Sekiya & Suzuki 2011). Then, to terminate liver regeneration, the external regenerative stimuli gradually decrease, and hepatocyte antiproliferative factors, including transforming growth factor (TGF) β and related TGFβ family members such as activin, effectively function (Michalopoulos & DeFrances 1997; Taub 2004).

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When the liver receives chronic injury, hepatocyte proliferation is impaired, and a ductular reaction, in which cells expressing biliary lineage markers appear and form primitive ductules, is often induced in periportal regions of the liver (Desmet *et al.* 1995; Libbrecht & Roskams 2002; Duncan *et al.* 2009). Prospective isolation and *in vitro* clonal analysis showed that hepatic progenitor cells capable of giving rise to both hepatocytes and biliary cells are contained among the cells with biliary properties within the chronically injured liver (Suzuki *et al.* 2008; Okabe *et al.* 2009; Dorrell *et al.* 2011; Shin *et al.* 2011). More recently, intensive studies using *in vivo* genetic lineage-tracing approaches have provided new insights into the nature of cellular identity and plasticity in the liver and suggested that adult liver cells can flexibly respond to injury by actively converting their own cell fate to other cell fates (Fan *et al.* 2012; Sekiya & Suzuki 2012, 2014; Michelotti *et al.* 2013; Yanger *et al.* 2013). In the current review, I place special focus on recent new findings in the study of liver regeneration, including those from our previous studies, and summarize them concisely.

Liver regeneration after loss of liver mass or acute hepatic injury

During liver development, hepatocytes that compose nearly 80% of the liver mass and have multiple functions involved in many aspects of metabolism arise from hepatic progenitor cells, hepatoblasts, together with biliary cells that line the intrahepatic biliary apparatus (Lemaigre 2009). Hepatocytes can proliferate to render the size of the liver suitable for the size of the whole body during a certain period in the postnatal stage, and then stop proliferation and reside in the liver for a prolonged period of time to exert multiple metabolic functions for the maintenance of homeostasis. This property of limited turnover of differentiated cells in the liver is similar to the cases of the heart and brain, but not to those of the intestine and skin, in which tissue-specific stem cells continuously supply differentiated cells. However, the liver can regenerate itself in response to hepatic damage. After partial hepatectomy (PH) or chemical-induced moderate liver injury, fully differentiated hepatocytes rapidly reenter the cell cycle and proliferate to restore the original liver mass without any contribution from hepatic stem/progenitor cells. At the onset of liver regeneration, DNA synthesis and subsequent hepatocyte cell divisions are initially activated by specific humoral factors, including interleukin-6 and hepatocyte growth factor, which are mainly produced by nonparenchymal cells in the liver (Michalopoulos & DeFrances 1997; Taub 2004). In addition, after the liver receives damage, the remnant hepatocytes can detect an increase in the amount of bile acids incorporated into hepatocytes, which flow into the liver from the gut. Subsequently, the hepatocytes can initiate proliferation through the bile acid signaling pathway depending on nuclear bile acid receptors (Huang *et al.* 2006).

As one of the intracellular targets activated by such humoral factors, we found that glycogen synthase kinase (GSK)-3β-dependent Snail degradation occurs in hepatocytes to initiate their proliferation (Sekiya & Suzuki 2011). Snail is one of the zinc-finger transcription factors and involved in various cellular functions, including cell motility, differentiation, proliferation and survival (Nieto 2002). GSK-3β can bind to and phosphorylate Snail in the nucleus and cytoplasm of cells and eventually lead to β-Trcp-mediated ubiquitination and subsequent proteasomal degradation of Snail (Zhou *et al.* 2004). As shown in (Fig. 1), the quantity and activity of GSK-3β are immediately increased in response to regenerative signals after a reduction of liver mass or hepatic

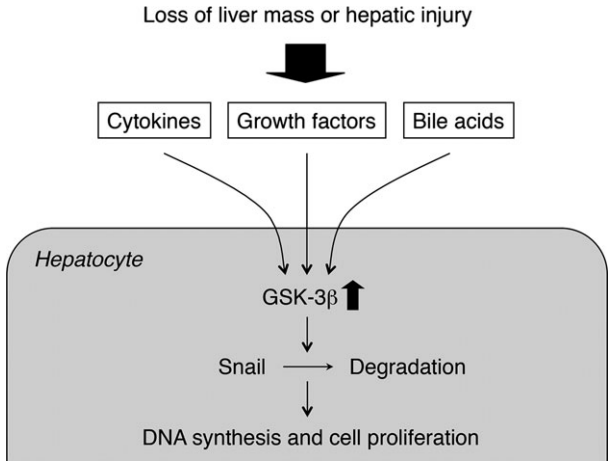

Figure 1 GSK-3β-dependent Snail degradation induces hepatocyte proliferation in liver regeneration after loss of liver mass or acute hepatic injury. The quantity and activity of GSK-3β are immediately increased as a result of cytokine, growth factor and bile acid signals that are known to drive liver regeneration. These changes lead to phosphorylation and subsequent degradation of Snail, and the decreased levels of Snail finally induce DNA synthesis in hepatocytes through up-regulation of cell cycle-related proteins.

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injury, although the mechanism underlying the rapid increase in GSK-3β is still unknown. Unbalanced amounts of GSK-3β and Snail in hepatocytes promote GSK-3β-mediated phosphorylation of Snail and lead to ubiquitination and proteasomal degradation of Snail. The decreased levels of Snail then induce DNA synthesis in hepatocytes through up-regulation of cell cycle-related proteins, including *cyclin D2*, which is directly regulated by Snail through binding to the E-box element in its promoter. As the external regenerative stimuli gradually decrease, the amounts of GSK-3β and Snail in hepatocytes become close to their basal levels, which may lead to the arrest of hepatocyte proliferation and terminate liver regeneration.

Similar to the case for mature hepatocytes, hepatoblasts in the developing liver cease proliferation after the introduction of a construct that highly expresses *Snail* (Sekiya & Suzuki 2011). Thus, in normal liver development, the amount of Snail is maintained at extremely low levels, which is probably mediated by the effect of abundant GSK-3β in the developing liver, to allow hepatoblast proliferation. In the adult liver, Snail is typically expressed in quiescent hepatocytes, in proportion to the decreased levels of GSK-3β, to block their proliferation. If the liver is injured and needs to regenerate itself, the quantitative balance of GSK-3β and Snail in hepatocytes is temporarily disrupted and reverts to a state in the developing liver, thereby allowing hepatocytes to proliferate like hepatoblasts. In other words, Snail acts as a gatekeeper or manacle to keep hepatocytes quiescent in the adult liver, and GSK-3β has a role to free hepatocytes from this restraint and induce their proliferation as a result of the initial regenerative responses to hepatic damage.

Normally, it is thought that hepatocytes are contained within the group of nonproliferative functionally differentiated cells, including neuronal cells and cardiomyocytes and that their proliferation is passively activated in response to hepatic damage, as if they suddenly awake from sleep. However, our findings provide a new theory for liver regeneration. Hepatocytes in the normal adult liver may be conventionally ready to proliferate, but this is usually blocked by Snail activity. Meanwhile, in the damaged liver, GSK-3β-dependent Snail degradation induces active proliferation of hepatocytes as a fundamental cue for the initiation of liver regeneration (Fig. 2). This hypothetical mechanism helps to explain why DNA synthesis in hepatocytes can be activated very quickly after hepatic damage.

Liver regeneration in the chronically injured liver

Under conditions of chronic liver injury, hepatocyte proliferation is blocked, and a ductular reaction occurs in portal areas of the hepatic lobule. Experimental rodent models of chronic liver injury can be induced by bile duct ligation and administration of potential carcinogens, including azo dyes, choline-deficient/ethionine-containing diet, D-galactosamine, 2-acetylaminofluorene, 3,5-diethoxycarbonyl-1,4-dihydrocollidine and Dipin (Farber 1956; Shinozuka *et al.* 1978; Tatematsu *et al.* 1984; Lemire *et al.* 1991; Factor & Radaeva 1993; Preisegger *et al.* 1999). Within biliary cells composing such newly generated primitive ductules and intrahepatic bile ducts, hepatic progenitor cells (often called oval cells) should be present. Although it is difficult to distinguish hepatic progenitor cells from other biliary cells owing to the lack of independent markers for each cell type, prospective isolation and *in vitro* clonal analysis of cells expressing common biliary antigens, including CD133/Prominin1, MIC1-1C3, epithelial cell adhesion molecule (EpCAM) and forkhead box L1 (Foxl1), showed that hepatic progenitor cells capable of giving rise to both hepatocytes and biliary cells are actually present in the chronically injured liver (Suzuki *et al.* 2008; Okabe *et al.* 2009; Dorrell *et al.* 2011; Shin *et al.* 2011).

As hepatic progenitor cells naturally possess phenotypic and morphological similarities to biliary cells, hepatocyte differentiation from progenitor cells could also be interpreted as direct conversion of biliary cells to hepatocytes. In our previous study, CD133/Prominin1-positive hepatic progenitor cells isolated from the chronically injured liver were originally identified as biliary cells without any expression of hepatocyte markers. However, these cells gradually became positive for the expression of hepatocyte markers during proliferation under appropriate culture conditions for hepatocyte differentiation and were eventually able to reconstitute hepatic tissues as mature hepatocytes after transplantation into the livers of fumarylacetoacetate hydrolase (*Fah*)-deficient ($Fah^{-/-}$) mice, as a mouse model of hereditary tyrosinemia type I (Suzuki *et al.* 2008). In addition, an *in vivo* genetic lineage-tracing analysis of sex-determining region Y-box 9 (Sox9)-positive or osteopontin-positive biliary cells showed that some of these cells give rise to hepatocytes after chronic liver injury (Furuyama *et al.* 2011; Español-Suñer *et al.* 2012). Taken together, these findings suggest that

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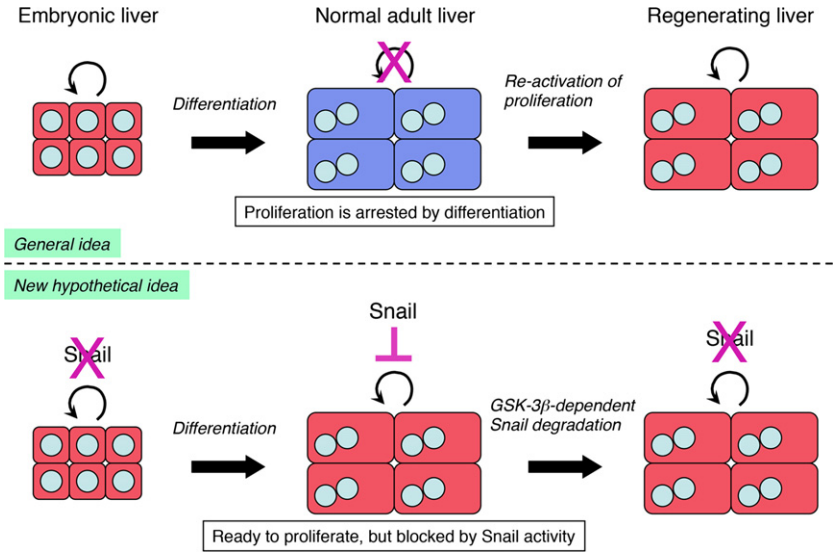

Figure 2 New hypothetical mechanism of liver regeneration. In the general idea, hepatocytes stop proliferation as a result of terminal differentiation, but their proliferation can be passively and quickly activated in response to hepatic damage. However, our previous findings provide a new theory for liver regeneration, in which hepatocytes in the adult liver may be conventionally ready to proliferate, but this is usually blocked by Snail activity. As the amount of Snail is maintained at an extremely low level in the developing liver, hepatoblasts are able to proliferate, unlike the case for hepatocytes. When hepatocyte proliferation is required for liver regeneration after loss of liver mass or acute hepatic injury, GSK-3β-dependent Snail degradation occurs in hepatocytes to initiate their proliferation.

biliary cells in the chronically injured liver contain immature progenitor cells with biliary properties, or represent cells that can be directly converted into hepatocytes.

The cellular origin of the biliary cells that specifically appear by the ductular reaction after chronic liver injury has been obscure. However, recent advances in the technologies available for cell-fate tracking using engineered mouse models allow approaches to not only their characterization, but also the mechanism underlying the emergence of these cells. Interestingly, the biliary cells that compose primitive ductules in the chronically injured liver may not be derived from a single type of cells, but appear to have a plural origin. The data obtained from *in vivo* genetic lineage-tracing analyses of albumin-positive or thyroid-binding globulin-positive hepatocytes and cytokeratin (CK) 19-positive, Sox9-positive or osteopontin-positive biliary cells within intrahepatic bile ducts have showed that biliary cells appearing in the chronically injured liver arise from both hepatocytes and intrahepatic biliary ductal cells (Furuyama *et al.* 2011; Español-Suñer *et al.* 2012; Yanger *et al.* 2013; Sekiya & Suzuki 2014). Moreover, Notch signal activation in hepatocytes is required for conversion of hepatocytes to biliary cells (Yanger *et al.* 2013; Sekiya & Suzuki 2014). However, Malato *et al.* (2011) reported the opposite data, showing that hepatocytes do not give rise to biliary cells in the chronically injured liver, based on experiments where hepatocytes were genetically marked by transthyretin expression. In contrast, a recent *in vivo* genetic lineage-tracing analysis showed that hepatic stellate cells (also known as Ito cells) residing in the space of Disse could be a source of the biliary cells found after chronic liver damage (Michelotti *et al.* 2013), whereas Mederacke *et al.* (2013) negated this conclusion. Although it is largely unknown why biliary cells need to be induced from many cell types in the chronically injured liver, the cell-fate conversion may be critically involved in the phenomenon of the ductular reaction. Similarly, it has been reported that a small number of hepatocytes in the adult liver can change their fate to that of biliary cells, even after PH and induction of acute hepatic injury by carbon tetrachloride (CCl_4) injection (Yanger *et al.* 2013; Sekiya & Suzuki 2014). Thus, the cellular interconversion between hepatocytes and biliary cells is not rare, but occurs spontaneously in response to liver damage.

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Cell turnover in hepatic homeostasis

In the adult liver, hepatocytes and biliary cells have essential, but clearly distinguishable, roles in hepatic functions, and thus both types of cells are required to maintain homeostasis throughout life. Indeed, recent *in vivo* genetic lineage-tracing analyses of albumin-positive, transthyretin-positive or thyroid-binding globulin-positive hepatocytes and CK19-positive or osteopontin-positive biliary cells in normal livers showed that both hepatocytes and biliary cells are maintained by self-duplication or without cell divisions for at least 6 months and never undergo mutual conversion (Malato *et al.* 2011; Español-Suñer *et al.* 2012; Sekiya & Suzuki 2012; Suzuki 2013; Yanger *et al.* 2013). Thus, the lineages of hepatocytes and biliary cells in the liver of adult organisms are completely distinct, and both cell types are present without interconversion under normal physiological conditions.

However, Furuyama *et al.* (2011) showed that Sox9-positive biliary cells composing intrahepatic bile ducts in the normal adult liver could continuously supply hepatocytes, suggesting that hepatic progenitor cells are contained within these cells, or spontaneous conversion of biliary cells to hepatocytes occurs even in the normal liver. In contrast, Carpentier *et al.* (2011) reported that biliary cells within intrahepatic bile ducts and a portion of periportal hepatocytes, both of which are progenies of Sox9-positive embry-

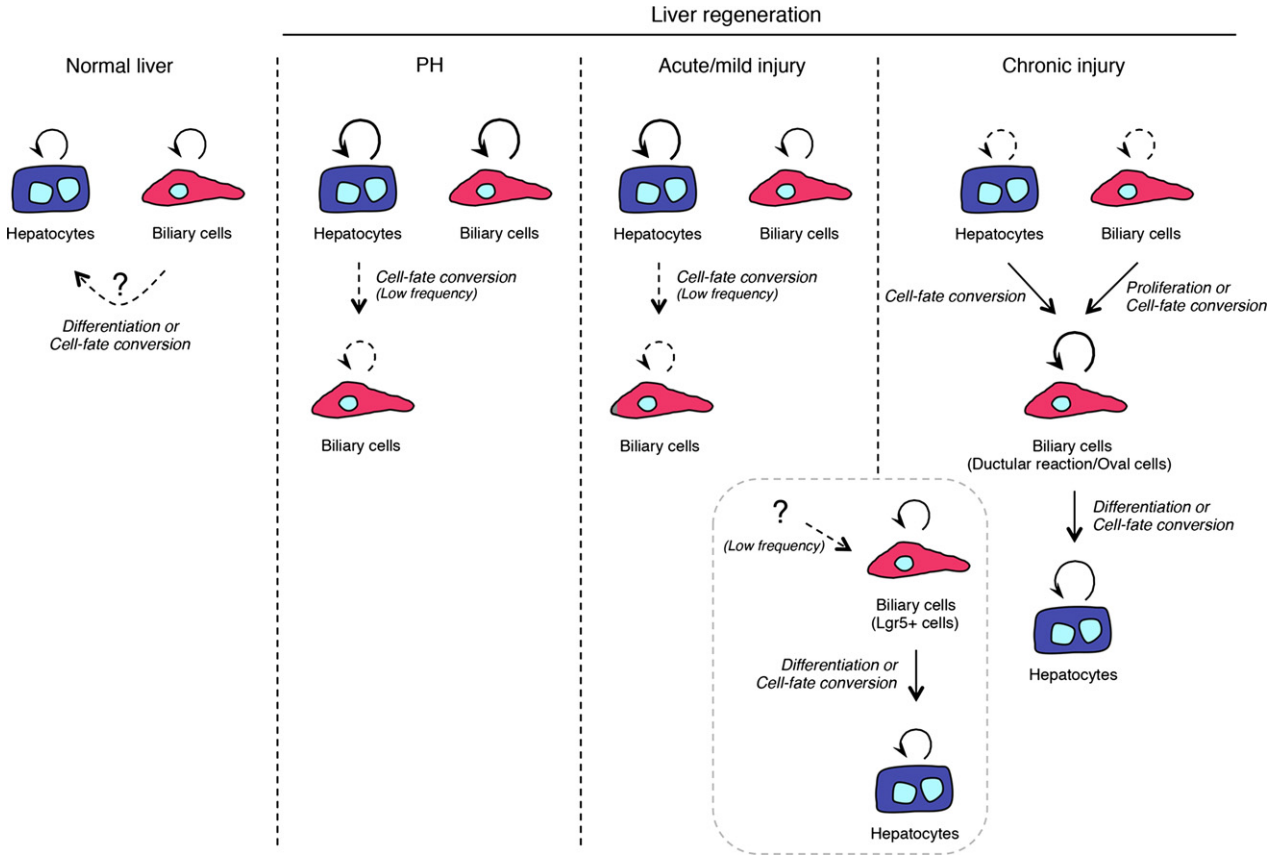

Figure 3 Summary of cellular identity and plasticity of hepatocytes and biliary cells in the normal and damaged liver. In the normal liver, hepatocytes and biliary cells that compose intrahepatic bile ducts are maintained by self-duplication without interconversion between these two types of cells. However, it is also suggested that Sox9-positive biliary cells within bile ducts can supply hepatocytes through differentiation or cell-fate conversion under normal physiological conditions. In the regenerating liver after PH or acute injury, hepatocytes and biliary cells proliferate by self-duplication in response to the regenerative signals, and to a lesser extent, hepatocytes can also be converted into biliary cells. In the chronically injured liver, biliary cells appearing through the ductular reaction arise from both hepatocytes and intrahepatic biliary ductal cells, and these newly generated biliary cells give rise to hepatocytes during recovery from chronic injury. Moreover, a small number of Lgr5-positive cells appear in both acutely and chronically injured livers and give rise to hepatocytes through differentiation or cell-fate conversion.

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onic ductal plate cells, could not colonize the liver by producing hepatocytes. In that study, however, Sox9-positive cells were only marked during liver development, and not in the adult liver. Thus, the fate of Sox9-positive biliary cells in the adult liver remains to be analyzed. In fact, biliary cells in the adult liver contain cells that are not derived from embryonic ductal plate cells (Carpentier *et al.* 2011). Thus, there is a possibility that Sox9-positive biliary cells in the adult liver are heterogeneous, and some of them have a potential to give rise to hepatocytes in hepatic homeostasis. However, Carpentier *et al.* (2011) also provided critical data showing that ectopic expression of Sox9 is induced in hepatocytes after injection of tamoxifen, suggesting that these Sox9-positive hepatocytes are also marked by the expression of reporter genes, similar to Sox9-positive biliary cells. Taken together, at least presently, the data obtained from genetic lineage-tracing analyses of Sox9-positive cells should be handled carefully, and more detailed examinations are required to clarify the role of Sox9-positive cells in the liver.

Identification of a new candidate for hepatic progenitor cells appearing in the injured liver

Recently, Huch *et al.* (2013) reported that leucine-rich repeat-containing G protein-coupled receptor (Lgr) 5-positive cells appear near intrahepatic bile ducts in both acutely and chronically injured livers, although they are not observed in normal livers. These Lgr5-positive cells are smaller than surrounding hepatocytes, co-express biliary lineage markers, but not hepatocyte and stellate cell markers, and give rise to not only hepatocytes in the recovery from the acute hepatic injury, but also both hepatocytes and biliary cells in the chronically injured liver, although the numbers of Lgr5-positive cell progenies in the liver are small. Interestingly, Lgr5-positive cells isolated from the injured livers form cyst-like structures consisting of biliary cells and expand under specific culture conditions. Upon induction of hepatocyte differentiation, cells within these cyst-like structures acquire the fate of hepatocytes *in vitro* and can reconstitute hepatic tissues after transplantation into $Fah^{-/-}$ mouse livers. As mentioned above, it is generally thought that hepatic progenitor cells do not contribute to liver regeneration after acute hepatic injury. However, the finding of Lgr5-positive cells that appear after induction of acute hepatic injury by CCl_4 injection and give rise to some periportal hepatocytes shows that hepatic progenitor cells are, at least in part, involved in liver regeneration after acute damage. Alternatively, these data may indicate direct cell-fate conversion of Lgr5-positive biliary cells into hepatocytes.

To date, however, it has remained unclear whether Lgr5-positive cells are actually required for liver regeneration, whether they also appear after PH as well as CCl_4-induced acute liver injury and whether they arise from biliary cells or other types of cells in the liver, including not only hepatocytes, but also mesenchymal and hematopoietic lineage cells via mesenchymal–epithelial transition. Nevertheless, the *in vitro* expansion of Lgr5-positive cells to form cyst-like structures and their differentiation into functional hepatocytes provide a possibility that these cells could contribute to the development of potential therapies for liver diseases.

Concluding remarks

As described above, the mechanism underlying liver regeneration is becoming clearer through the recent progress of new technologies, including advanced methods of molecular biology, development of cell separation techniques using flow cytometry and *in vivo* genetic lineage-tracing analyses. Unexpectedly, adult liver cells have the property of being extremely pliable for the repair of injured liver tissues (Fig. 3). This flexibility of liver cells raises new questions, such as why only the liver can respond to injury in a plastic manner and how liver cells are maintained against becoming abnormal under normal physiological conditions. Thus, liver regeneration is even more complicated than we had thought. However, researchers have definitely opened the door toward understanding the unique properties of the liver. This future challenge will lead to the development of distinctive approaches for the treatment of liver failure. The liver can be considered to be a strange organ, but that is precisely why we are fascinated and cannot stop studying this organ.

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