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Aberrant differentiation and proliferation of hepatocytes in chronic liver injury and liver tumors

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Abstract

Chronic liver injury induces liver cirrhosis and facilitates hepatocarcinogenesis. However, the effects of this condition on hepatocyte proliferation and differentiation are unclear. We showed that rodent hepatocytes display a ductular phenotype when they are cultured within a collagenous matrix. This process involves transdifferentiation without the emergence of hepatoblastic features and is at least partially reversible. During the ductular reaction in chronic liver diseases with progressive fibrosis, some hepatocytes, especially those adjacent to ectopic ductules, demonstrate ductular transdifferentiation, but the majority of increased ductules originate from the existing bile ductular system that undergoes extensive remodeling. In chronic injury, hepatocyte proliferation is weak but sustained, and most regenerative nodules in liver cirrhosis are composed of clonally proliferating hepatocytes, suggesting that a small fraction of hepatocytes maintain their proliferative capacity in chronic injury. In mouse hepatocarcinogenesis models, hepatocytes activate the expression of various fetal/neonatal genes, indicating that these cells undergo dedifferentiation. Hepatocyte-specific somatic integration of various oncogenes in mice demonstrated that hepatocytes may be the cells of origin for a broad spectrum of liver tumors through transdifferentiation and dedifferentiation. In conclusion, the phenotypic plasticity and heterogeneity of mature hepatocytes are important for understanding the pathogenesis of chronic liver diseases and liver tumors.

KEYWORDS

bile ducts/ductules, chronic liver injury, ductular reaction, fibrosis, hepatocellular carcinoma, hepatocytes, proliferation, regeneration, tissue remodeling, transdifferentiation

INTRODUCTION

Since the time of Greek mythology, the liver has been known to have a powerful regenerative capacity.[1,2] In fact, as shown in partial hepatectomy models in rodents, the liver is able to recover its original mass with full functions within two weeks following the removal of two-thirds of the parenchyma.[1] However, despite the seemingly perfect liver regeneration in the tragedy of *Prometheus Bound*, when the liver is repetitively excised or hepatocytes are continuously damaged, appropriate regeneration cannot be achieved due to insufficient

Abbreviations: AAV8, adeno-associated virus serotype 8; AFP, α-fetoprotein; CCA, cholangiocarcinoma; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; CK19, cytokeratin 19; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DEN, diethylnitrosamine; Dex, dexamethasone; DLK1, delta-like 1; EGF, epidermal growth factor; HCC, hepatocellular carcinoma; HNF-4α, hepatocyte nuclear factor-4α; IGF2, insulin-like growth factor-2; IL, interleukin; JNK, c-Jun N-terminal kinase; MKK7, mitogen-activated protein kinase kinase 7; NICD, Notch intracellular domain; OSM, oncostatin M; PRODH, proline dehydrogenase; TBG, thyroxine-binding globulin promoter; TNF-α, tumor necrosis factor-α.

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hepatocyte proliferation and progression of fibrosis, resulting in liver cirrhosis, which is characterized by regenerative nodules surrounded by interconnecting fibrous septa.[2,3] When the proliferative activity of hepatocytes is impaired in chronic liver injury, hepatic stem/progenitor cells, instead of hepatocytes, have been suggested to proliferate and contribute to liver regeneration.[4,5] Initially, liver stem/progenitor cells were thought to reside at the canals of Hering, the juncture of the hepatocyte canalicular system and the terminal branches of the biliary tree (bile ductules), but their exact nature, including their existence, is unclear.[6,7] Since regenerative nodules are definite factors in the development of liver tumors,[3,8] it is important to investigate and understand the mechanism of liver regeneration and hepatocarcinogenesis in chronic liver injury.

In various chronic liver diseases, an abnormal increase in bile ductules is frequently observed in association with liver fibrosis. This phenomenon is called the ductular reaction, and its cells of origin, mechanism, and importance have been the subjects of debate.[9–11] Many investigators have suggested that the increased bile ductules originate from putative hepatic stem/progenitor cells with bipotential differentiation, not from hepatocytes whose phenotype is considered to be fixed when they are terminally differentiated.[12] However, in the parenchyma in chronic liver injury, there are apparent transitions between increased ductules and neighboring hepatocytes, which often express bile duct-specific cytokeratins (keratins).[13,14] Thus, hepatocytes may change their phenotype to bile duct-like cells in an altered microenvironment.[9]

Understanding how hepatocytes might change their phenotype during hepatocarcinogenesis is important. The prototypes of primary liver cancers are hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA), and their cells of origin are presumed to be hepatocytes and bile ducts, respectively. However, there are also tumors with both of these distinct phenotypes, called combined hepatocellular-cholangiocarcinoma (cHCC-CCA).[15] Although the presentation of mixed phenotypes in such tumors suggests that they may be derived from putative hepatic stem/progenitor cells and there is experimental evidence in mice supporting the notion,[16] hepatocyte-derived tumors might demonstrate bile duct features upon transformation if the phenotype of hepatocytes is malleable.

We demonstrated the phenotypic plasticity of mature hepatocytes in rodents using in vitro and in vivo models. Hepatocytes can transdifferentiate into bile duct-like cells in three-dimensional cultures within a collagenous matrix,[17–20] as well as in the fibrotic liver environment following chronic injury, partly contributing to ductular reactions.[21] Moreover, we showed that during hepatocarcinogenesis, hepatocytes can transdifferentiate and/or dedifferentiate to varying extents, generating a broad spectrum of primary liver cancers, including HCC, CCA, cHCC-CCA, and hepatoblastoma-like tumors.[22–25] Here, I will review the experimental data demonstrating the aberrant differentiation and proliferation of adult hepatocytes in chronic liver diseases and primary liver cancers, thereby providing a comprehensive view of this highly debated research area with reference to implications for human pathology.

PHENOTYPIC PLASTICITY OF HEPATOCYTES: DUCTULAR TRANSDIFFERENTIATION OF MATURE HEPATOCYTES

For analysis of the phenotypic plasticity of hepatocytes, it would be helpful to review how the epithelial cells of the liver, for example, hepatocytes and bile ducts, differentiate from the hepatoblasts that develop in the hepatic diverticulum of the foregut.[26] The development and differentiation of epithelial cells in the liver can be followed by the expression of cell differentiation markers (Figure 1). Emerging hepatoblasts expressing delta-like 1 (DLK1), a specific marker for immature hepatoblasts, differentiate into those expressing hepatocyte nuclear factor-4α (HNF-4α) and albumin, and then, they differentiate into mature hepatocytes, as well as intrahepatic bile duct cells that are positive for cytokeratin 19 (CK19).[27–30] In contrast, the extrahepatic bile ducts (the common bile duct, cystic duct, and distal part of the hepatic ducts) are derived from immature hepatoblasts prior to the commitment of hepatocytic differentiation.[28] The development of portal connective tissues and the activation of the Notch signaling pathway are crucial for bile duct differentiation.[29,31–33]

Since intrahepatic bile ducts are derived from albumin-expressing hepatoblasts, hepatocytes may be converted to bile duct-like cells even after being terminally differentiated. In fact, hepatocyte-to-bile duct transition occurs in tissue culture experiments, in which aggregates of isolated rat hepatocytes are embedded within type I collagen gels that mimic the microenvironment of bile duct induction at the developing portal tract (Figure 2a).[17,18] In such a three-dimensional culture system, hepatocyte aggregates (spheroids) demonstrate branching morphogenesis in the presence of serum, insulin, and epidermal growth factor (EGF) (Figure 2b), as well as soluble factor(s) secreted by a fibroblast line (MRC-5), and the extending branching structures express CK19 (Figure 2c). The Notch signaling pathway involved in the development of bile ducts is activated during this process.[18] After culture for several weeks, the branching processes become ductular or microcystic structures surrounded by basement membranes.[18]

Although the expression of various markers for hepatocytes decreases and that for bile ducts

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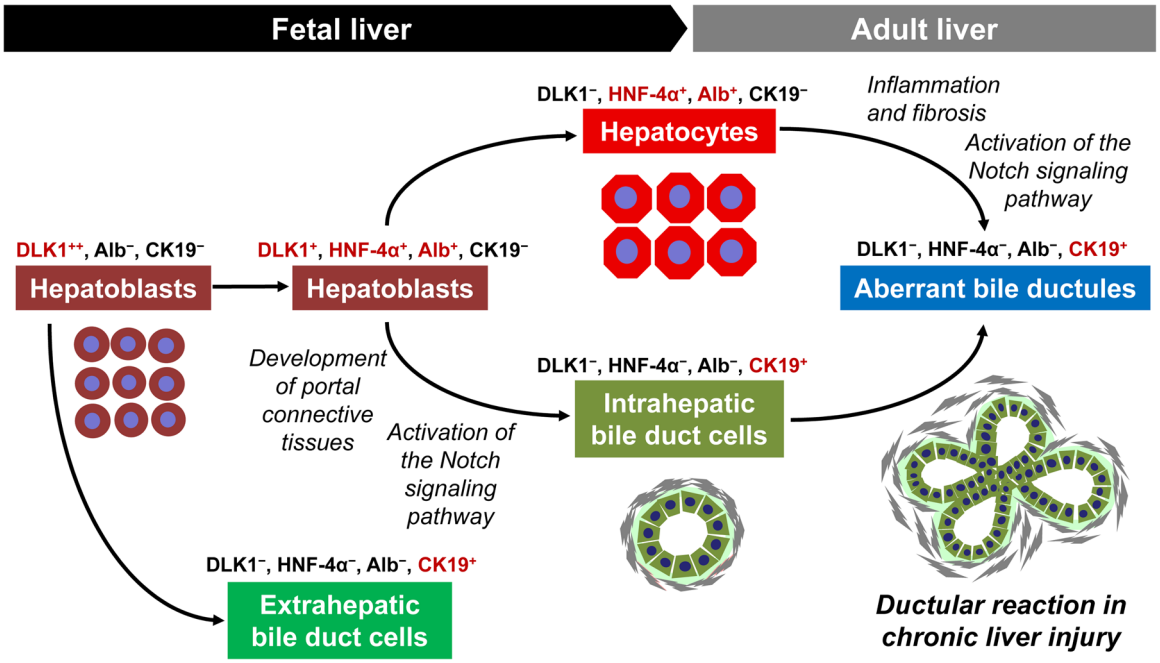

FIGURE 1 Development of hepatocytes and bile duct cells and possible cells of origin for the ductular reaction. Both hepatocytes and intrahepatic bile duct cells originate from Delta-like 1 (DLK1)-positive hepatoblasts, but differentiation of the latter occurs after the cells express albumin (Alb). The development of portal connective tissue and the activation of the Notch signaling pathway are important for the development of the intrahepatic bile ducts. Both hepatocytes and intrahepatic bile ducts contribute to ductular reactions in chronic liver diseases.

increases during branching ductular morphogenesis, the expression of markers for hepatoblasts, such as α-fetoprotein (AFP) and DLK1, is undetectable at any time point.[18] These results indicate that hepatocytes can transdifferentiate into bile duct-like cells without dedifferentiation into hepatoblasts. Similarly, bile ductular transdifferentiation of mature hepatocytes after culture between two type I collagen gel layers or in a roller bottle culture system has been reported.[34,35] Furthermore, we demonstrated that mouse hepatocytes undergo similar branching ductular morphogenesis without expressing DLK1 within collagen gels.[21]

The ductular morphogenesis of hepatocytes is increased by various inflammatory cytokines, particularly tumor necrosis factor-α (TNF-α).[19] In experiments using hepatocytes from Alb-DsRed2 transgenic rats, in which albumin-positive cells expressed an orange fluorescent protein, DsRed2, cultured hepatocytes were strongly fluorescent soon after isolation, but when their aggregates were embedded within the collagen gel matrix and cultured for 2 weeks with TNF-α, DsRed2 fluorescence was almost completely lost, as they demonstrated extensive branching tubular morphogenesis (Figure 2d).[20] However, these transdifferentiated hepatocytes recovered fluorescence after they were retrieved from gels and plated on Matrigel, a basement membrane-like matrix that has been shown to be suitable for the maintenance of hepatocyte differentiation,[34,36] and this recovery was strongly reinforced by the presence of dexamethasone (Dex) plus interleukin-6 (IL-6) or oncostatin M (OSM) (Figure 2d,e).[20] The combination of Dex and OSM has been reported to facilitate hepatocyte differentiation of cultured mouse hepatoblasts.[37]

Our experiments, as well as those performed by other investigators,[34,35] have helped elucidate the phenotypic plasticity of adult hepatocytes (Figure 3). During the development of the liver, hepatoblasts that have differentiated to express albumin form intrahepatic bile duct cells along the inchoate portal tract. Fully matured hepatocytes retain the capacity to transdifferentiate into bile duct cells in response to microenvironmental changes. In contrast, adult bile ducts exhibit limited phenotypic plasticity. In our preliminary experiments, bile duct/ductular cells from Alb-DsRed2 rats, which were cultured in spheroids on Matrigel in the presence of Dex and IL-6 (or OSM), became faintly fluorescent, but they never acquired a hepatocyte-like phenotype (Matsuo et al., unpublished data). This finding is consistent with the results of a previous report demonstrating that adult mouse bile duct cells lose the ability to differentiate into hepatocytes, which is evident in neonatal bile duct cells.[38] Cultured human, rat, and mouse hepatocytes have been reported to become highly proliferative, as well as bipotential, in several defined media.[39,40] Although these cells are designated progenitor cells, they do not express DLK1 or other hepatoblastic markers, therefore, their cellular behavior should be better categorized as transdifferentiation of hepatocytes.

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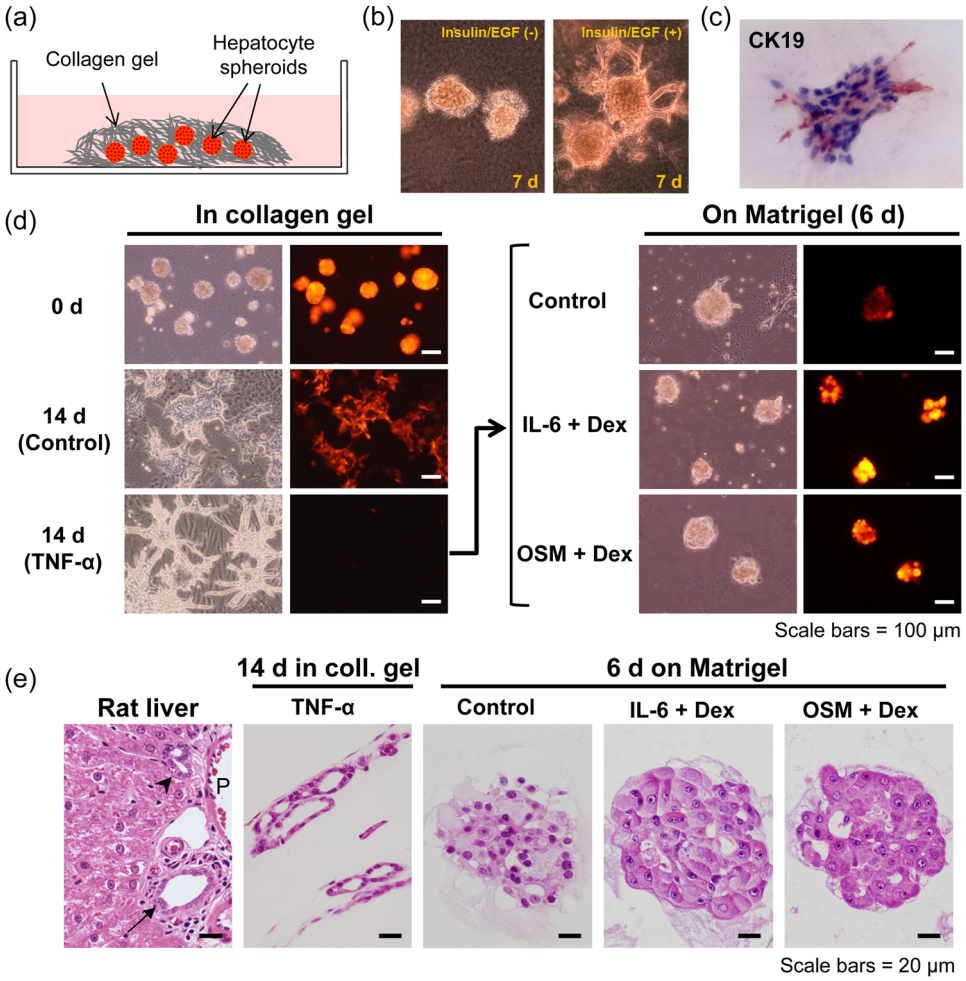

FIGURE 2 Bile ductular transdifferentiation of adult rat hepatocytes within a collagen gel matrix. (a) Schematic representation of collagen gel cultures of aggregates of isolated adult hepatocytes (spheroids). (b) Branching morphogenesis of rat hepatocyte spheroids in the presence of epidermal growth factor (EGF) and insulin (7 days). (c) Cytokeratin 19 (CK19) immunohistochemistry of rat hepatocyte spheroids cultured with MRC-5 fibroblast-conditioned medium (5 days). (d) Reversibility of the ductular differentiation of Alb-DsRed2 transgenic rat hepatocytes. Phase-contrast micrographs and DsRed2 fluorescence. The complete loss of DsRed2 fluorescence in hepatocyte spheroids within a collagen gel matrix after 14 days in the presence of TNF-α and the recovery of DsRed2 fluorescence after removal from the gel and transfer to Matrigel-coated surfaces in the presence of dexamethasone and interleukin-6 or oncostatin M. (e) Micrographs showing the ductular differentiation and redifferentiation of DsRed2 transgenic hepatocytes in vitro. Hematoxylin and eosin staining. The left panel shows intact rat liver tissue containing a portal vein (P), bile duct (arrow), and bile ductule (arrowhead). Panels (b), (c), (d), and (e) are reproduced from our previous publications[17,18,20] (Copyright Elsevier).

PATHOGENESIS OF THE DUCTULAR REACTION: DUCTULAR TRANSDIFFERENTIATION OF HEPATOCYTES AND REMODELING OF THE BILIARY SYSTEM

To further elucidate the pathogenesis of the ductular reaction, researchers must examine whether adult hepatocytes transdifferentiate into bile duct cells in vivo. Transdifferentiation of hepatocytes has been suggested to occur in rats following intrasplenic transplantation,[41] as well as following bile duct injury in the rat liver that is repopulated by dipeptidyl peptidase IV-positive hepatocytes.[42] Recently, the ROSA26 Cre-reporter system in mice, in which any types of cells are permanently labeled by constitutively expressing β-galactosidase or fluorescent proteins through Cre-mediated genetic recombination, has been applied to lineage tracing experiments in various organ systems, including the liver.[43] Adult hepatocytes can be effectively labeled in Alb-CreER^{TR}/ROSA26R (or ROSA26R-YFP) mice following tamoxifen treatment or in ROSA26R Cre-reporter mice that are infected with adeno-associated virus serotype 8 (AAV8) expressing Cre recombinase. However, the results of fate tracing of hepatocytes in ductular reactions induced by a biliary toxin (3,5-diethoxycarbonyl-1,4-dihydrocollidine [DDC]) or common bile duct ligation have been inconsistent; some investigators did not observe ductular transdifferentiation of hepatocytes,[44] whereas other investigators documented transdifferentiation to varying degrees.[45,46]

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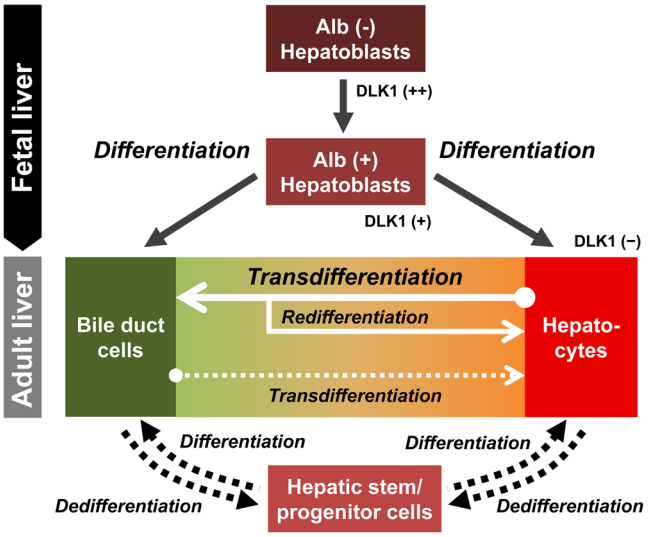

FIGURE 3 Phenotypic plasticity of mature hepatocytes in vitro in relation to the development of liver epithelial cells. Mature hepatocytes can transdifferentiate into bile duct cells, and this process is partly reversible. In contrast, mature bile duct cells possess only a limited capacity to transdifferentiate into hepatocytes. Dedifferentiation of hepatocytes toward hepatoblastic cells (putative hepatic stem/progenitor cells), as well as bile duct cells, is not evident in vitro.

In the adult liver of Alb-Cre/ROSA26R mice, both hepatocytes and intrahepatic bile ducts, but not extrahepatic bile ducts, are positive for X-gal histochemical staining due to β-galactosidase expression, consistent with observations in the developing liver.[21] We performed liver repopulation experiments in which selectively isolated X-gal-positive hepatocytes were transplanted into the livers of wild-type mice treated with retrorsine, an alkaloid that inhibits hepatocyte proliferation.[21] Ductular reactions induced by DDC or CCl_4 in the repopulated liver involve numerous bile ductules that are positive for both X-gal staining and CK19 immunohistochemistry, providing compelling evidence showing that mature hepatocytes retain the potential for ductular differentiation in vivo (Figure 4a).

In DDC-induced liver injury, the ductular reaction commences at the portal tract, where bile ducts/ductules are present, and extends into the hepatic lobule with the progression of periportal fibrosis. However, in chronic liver injury induced by CCl_4 or thioacetamide, a ductular reaction is observed in the damaged centrilobular region where activated hepatic stellate cells produce and deposit collagen. Interestingly, as the ductular reaction progresses in the centrilobular area, bile duct/ductular structures in the portal tract and periportal area gradually decrease and eventually disappear, indicating extensive remodeling of the bile duct system (Figure 4b).[21] The centrilobular ductular reaction called "reversed lobulation" has also been observed in human patients with congestive liver cirrhosis due to severe hepatic outflow obstruction, such as Budd-Chiari syndrome or veno-occlusive disease,[47] as well as primary pulmonary hypertension.[48] Similar ductular reaction with aberrant hepatic arteries has been documented in nonalcoholic steatohepatitis.[49] This centrilobular ductular reaction is also classified as a type IIB ductular reaction and has been suggested to be caused by transdifferentiation of hepatocytes in response to hypoxia,[10,50] but the mechanism and the cells of origin involved are unclear.

To determine whether the increased bile ducts/ductules are transdifferentiated hepatocytes or migrated bile duct/ductular cells, we used another lineage tracing system, Mx1-Cre/ROSA26R mice, which enables hepatocyte labeling in the intact adult liver.[21] The Mx1 promoter is activated by interferons induced by the injection of poly I:C, a synthetic double-stranded RNA, and Cre expression occurs in a hepatocyte-specific manner in the liver,[32,51,52] enabling the labeling of hepatocytes with β-galactosidase. In the centrilobular ductular reaction induced by CCl_4 or thioacetamide, approximately 10% of CK19-positive ductular cells were X-gal-positive and thus were considered transdifferentiated hepatocytes, but the majority of ductules were X-gal-negative, indicating that they are migrating and proliferating bile duct/ductules originally located in and around the portal tract. Interestingly, X-gal-negative ductular cells were significantly less proliferative than X-gal-negative cells. Importantly, communication between the common bile duct and the aberrant ductular structures, either the X-gal-positive or X-gal-negative ductules, was observed, demonstrating that the biliary system connection is maintained (Figure 4c). In the periportal ductular reaction, ductular transdifferentiation contributes relatively little (approximately 4%), and the majority of the increased ductules are derived from existing duct/ductular cells.[21,53] Recently, using AAV8-mediated hepatocyte lineage tracing, we examined chronic liver injury induced by a 0.1% methionine/choline-deficient L-amino acid-defined high-fat diet in a nonalcoholic steatohepatitis model and found that almost all of the ductular cells in the extensive ductular reaction are derived from existing duct/ductular cells that proliferate and migrate toward the centrilobular area.[54]

Our experiments in mice revealed that the ductular reaction in chronic liver injury is mainly due to proliferation and movement of the existing bile duct/ductular system, but there are also varying contributions of transdifferentiation of hepatocytes, especially in chronic injury following repeated tissue destruction (Figure 1). Although the contribution of ductular transdifferentiation of hepatocytes is relatively small, either in periportal or centrilobular ductular reactions, the presence of hepatocyte-derived ductular cells may be crucial for the establishment of bile flow between hepatocytes and remodeled bile ducts at their borders (Figure 4d).[21,55] Extensive remodeling may be mediated by S100-A4, a major tissue remodeling molecule that affects the expression of matrix

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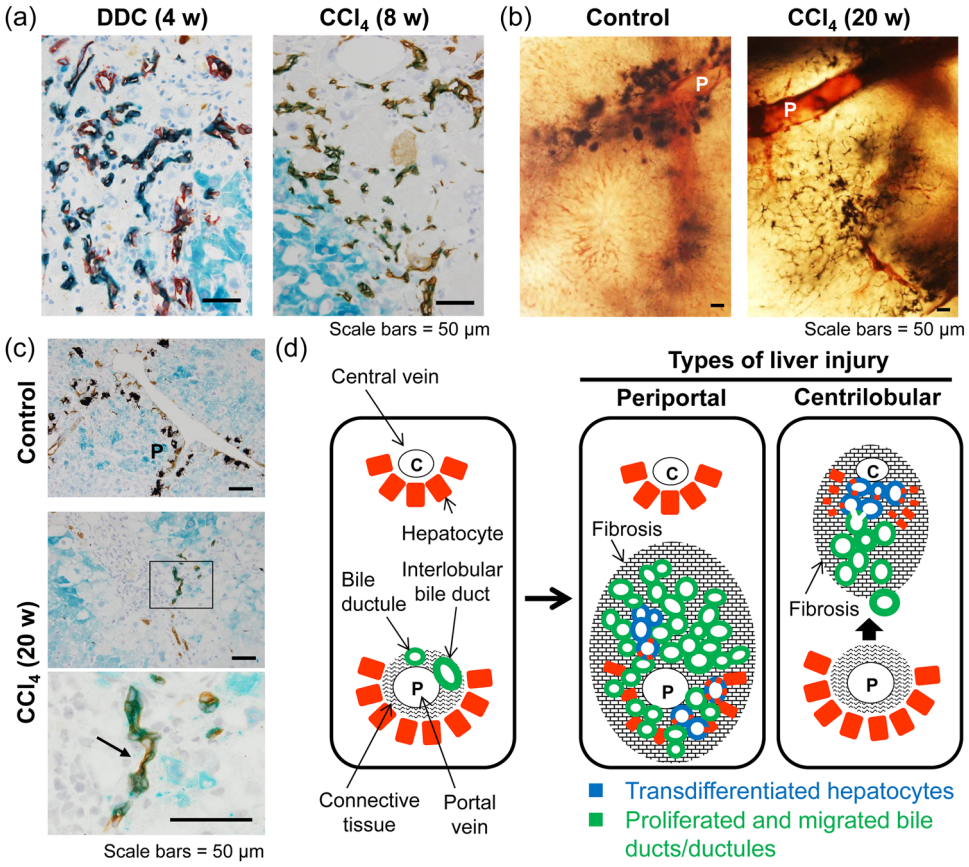

FIGURE 4 Ductular transdifferentiation of hepatocytes and extensive remodeling of the existing bile duct system in the ductular reaction. (a) Induction of the ductular reaction in the liver repopulated by β-gal-positive hepatocytes isolated from Alb-Cre × ROSA26R mice. Combined X-gal and CK19 immunohistochemistry. Diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet (4 weeks) and CCl_4 treatment (8 weeks). (b) Three-dimensional visualization of the bile ducts or ductules by retrograde injection of India ink and vermilion ink through the common bile duct and portal vein trunk, respectively. Intact (control) and CCl_4-treated (20 weeks) groups. P: portal vein. (c) Combined X-gal histochemistry and immunohistochemistry for CK19 of the liver in a hepatocyte lineage tracing system using Mx1-Cre × ROSA26R mice treated with poly I:C, in which India ink was injected through the common bile duct. Intact (control) and CCl_4-treated (20 weeks) groups. The boxed area in the middle panel is shown in detail in the bottom panel. Note the presence of India ink pigments (arrow) inside the lumen of the bile ductule composed of β-gal (+) and β-gal (–) cells. P: portal vein. (d) Schematic representation of two distinct origins of bile duct/ductular cells in the periportal and centrilobular ductular reactions. Hepatocytes transdifferentiate into biliary cells (blue), and existing bile duct/ductular cells actively proliferate and migrate (green). Panels (a), (b), and (c) are reproduced from our previous publication[21] (Copyright Elsevier).

metalloproteinases.[21,56] Furthermore, inflammatory cytokines, including TNF-α,[19] interleukin (IL)-13,[57] and IL-33,[58] which are released from damaged and inflamed sites, might play important roles in the proliferation and remodeling of bile ducts/ductules.

TWO DISTINCT MODES OF HEPATOCYTE PROLIFERATION: Myc-DEPENDENT ROBUST PROLIFERATION AND Myc-INDEPENDENT WEAK AND SUSTAINED PROLIFERATION

In acute liver injury, almost all hepatocytes that survive the damage proliferate robustly and synchronously.[1,2] In the partial hepatectomy model in rodents, the proliferation of hepatocytes in the remaining liver usually reaches its peak after 1 day (rat) or 2 days (mouse).[59] Although the mechanisms underlying hepatocyte proliferation following acute liver injury, including the roles of c-Jun and Myc, which are activated early in proliferative stimuli, have been extensively investigated, the factors that actually determine the robust proliferation of hepatocytes are unclear.

Mitogen-activated protein kinase kinase 7 (MKK7) activates c-Jun through the activation of c-Jun N-terminal kinase (JNK), and systemic knockout of this molecule is embryonically lethal due to defects in liver development.[60] We examined the effects of hepatocyte-specific knockout of MKK7 on the regenerative proliferation of hepatocytes following partial hepatectomy or acute CCl_4 injury and found that the loss of MKK7 does not affect hepatocyte proliferation, suggesting that the JNK-c-Jun signaling pathway is dispensable for hepatocyte proliferation itself.[61]

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However, since the loss of MKK7 delays the tissue repair process following CCl_4 injury, the JNK-c-Jun pathway is likely involved in the tissue repair process through the modulation of hepatocyte-extracellular matrix interactions.[61]

Myc suppression through siRNA-mediated RNA interference inhibits DNA synthesis in mouse hepatocytes in primary culture.[62] However, regarding the role of Myc in hepatocyte proliferation in vivo, Myc knockout experiments in mice performed by several investigators have led to inconsistent conclusions.[63–66] To elucidate the exact role of Myc in hepatocyte proliferation, we established the AAV8-mediated hepatocyte-specific expression of MadMyc, a chimeric protein composed of Mad and Myc, which strongly suppresses the transcriptional activity of Myc in a dominant-negative manner.[67] Using this system (Figure 5a), we examined the role of Myc in hepatocyte proliferation following partial hepatectomy or CCl_4 administration.[62] When MadMyc is expressed in hepatocytes, the recovery of liver weight is delayed, and the proliferation peak after 2 days is almost completely diminished, strongly suggesting that acute robust hepatocyte proliferation is dependent on Myc activation (Figure 5b). However, even when MadMyc was expressed, the liver weight recovered after 2 weeks. This delayed and weak proliferation was associated with the suppression of proline dehydrogenase (PRODH),[68] a proline-catabolizing enzyme that is involved in metabolic reprogramming (Figure 5c). Therefore, there are two distinct modes of hepatocyte proliferation: Myc-dependent robust and synchronized proliferation and Myc-independent weak and sustained proliferation.

In chronic liver injury induced by repetitive or continuous parenchymal damage, although robust proliferation of hepatocytes does not occur, the remaining hepatocytes continue to proliferate at lower rates, eventually forming nodular hepatocytic masses demarcated by fibrotic septa in liver cirrhosis. In the slowly proliferating hepatocytes in regenerative nodules, Myc is not activated, and the expression of PRODH is suppressed, suggesting that the regenerative proliferation of hepatocytes in chronic liver injury may be executed by a Myc-independent mechanism.[62]

CLONAL HEPATOCYTE PROLIFERATION IN CHRONIC LIVER INJURY: A POSSIBLE BASIS FOR HEPATOCARCINOGENESIS

It is unclear whether each hepatocyte is equally responsive to proliferation stimuli in chronic liver injury. To examine the features of gradual hepatocyte

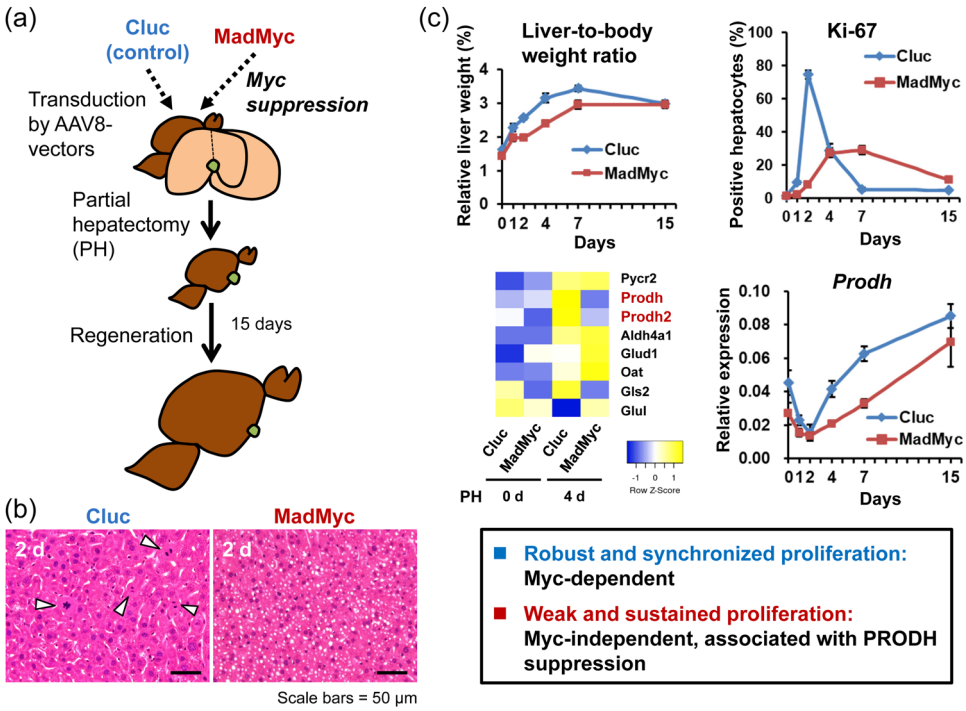

FIGURE 5 Myc-dependent and Myc-independent modes of hepatocyte proliferation. (a) Experimental procedures for examining the effect of in vivo Myc suppression on liver regeneration in mice. A two-thirds partial hepatectomy (PH) was performed following the infection of hepatocytes with AAV8 vectors with MadMyc, a competitive Myc inhibitor, or Cluc (control). (b) HE staining of liver tissue 2 days after PH. Arrowheads indicate mitotic figures. (c) Effects of Myc suppression by MadMyc on the liver-to-body weight ratio, Ki-67 labeling index, amino acid metabolism (microarray analysis), and mRNA expression of *Prodh* (quantitative reverse transcription-polymerase chain reaction). Panels (b) and (c) are reproduced from our previous publication[62] (Copyright Elsevier).

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accumulation over a substantial period of time, we evaluated the clonal-lineage relationships of regenerative nodules using a "rainbow mouse" model in collaboration with Dr. Hiroo Ueno, Kansai Medical University. In these mice, all cells in the body express GFP, but if cells express Cre recombinase, these cells lose GFP expression and instead express CFP, OFP, or RFP in a random manner.[69] When ROSA26R mice were infected with AAV8 expressing Cre recombinase under the control of a hepatocyte-specific thyroxine-binding globulin promoter (AAV8-TBG-Cre), the vast majority of hepatocytes in the normal liver randomly expressed a fluorescent protein other than GFP, whereas nonparenchymal cells, including bile ducts/ductules, expressed GFP (Figure 6a). In cirrhotic livers induced by chronic CCl_4 injury, most of the regenerative nodules were composed of hepatocytes expressing one of the fluorescent proteins, indicating clonal proliferation of hepatocytes (Figure 6a; Nishikawa et al., unpublished data). Consistent with these observations, several clonal analyses of regenerative nodules in human cirrhosis patients have also suggested that the majority of the nodules are monoclonal.[70–72] The preferential proliferation of particular fractions of hepatocytes suggests that there may be intrinsic or acquired heterogeneity of hepatocytes in terms of proliferative activity.[73–75] Notably, recent evidence has shown that regenerating hepatocytes in chronic liver injury increase their clonal fitness through somatic mutations of various genes, including those involved in metabolism.[76–78]

Consistent with our previous observations,[21] in the ROSA26R mice infected with AAV8-TBG-Cre, in which hepatocyte labeling with β-galactosidase is highly effective, most ductular cells in the ductular reaction induced by chronic administration of CCl_4 or thioacetamide were X-gal-negative and were thus derived from existing bile duct/ductular cells, although a small number of X-gal-positive ductules were present (Figure 6b). However, as lobular remodeling progressed, many clusters and nodules composed entirely of X-gal-negative hepatocytes appeared (Figure 6b). In the experiments using rainbow mice, regenerated nodules composed entirely of GFP-positive hepatocytes were also present (Figure 6a). Several investigators have reported similar observations and suggested that these nonrecombinant hepatocytes might be derived from nonhepatocytes, most likely bile duct/ductular cells.[44,79–81] However, there is no gradual transition between X-gal-negative hepatocytes and ductular cells in the ductular reaction or in the existing bile duct system. More critically, there are scattered HNF-4α-positive, X-gal-negative hepatocytes in the intact liver following AAV8-TBG-Cre infection, regardless of the location in the hepatic lobules (Figure 6b), and these hepatocytes gradually form larger clusters during tissue remodeling. These results suggest the presence of a small fraction of hepatocytes in the intact liver, which maintain proliferative capacity in chronic injury and propagate and form regenerative nodules in liver cirrhosis (Figure 6c).

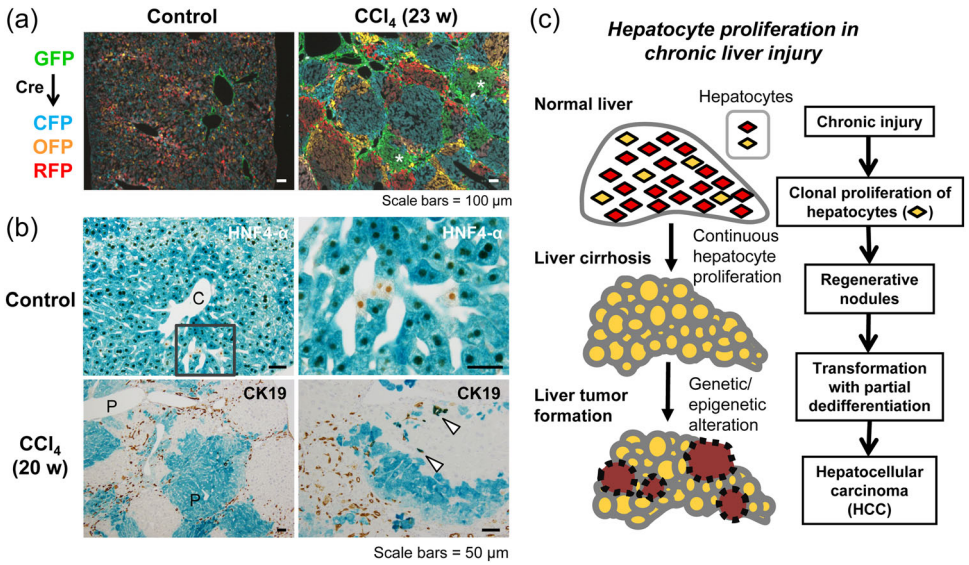

FIGURE 6 Skewed and clonal proliferation of hepatocytes in regenerative nodules in liver cirrhosis patients. (a) Evidence of clonal proliferation of subsets of hepatocytes in liver cirrhosis in rainbow mice infected with AAV8-Tbg-Cre. The intact (control) and CCl_4-treated (23 weeks) groups. Asterisks in the left panel indicate regenerative nodules with GFP fluorescence (nonrecombined). (b) Emergence of nonrecombined (X-gal-negative) regenerative nodules in ROSA26R mice infected with AAV8-Tbg-Cre following chronic CCl_4 treatment. The intact (control) and CCl_4-treated (20 weeks) groups. Combined X-gal histochemistry and immunohistochemistry for HNF-4α (control) and combined X-gal histochemistry and immunohistochemistry for CK19 (CCl_4). In the control liver, the boxed area in the left panels is magnified in the right panel. In CCl_4-treated livers, the arrowheads indicate β-gal-positive (hepatocyte-derived) ductules. (c) Clonal hepatocyte proliferation in chronic liver injury and liver cirrhosis as a possible background for the cirrhosis-carcinoma sequence.

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Many investigators have performed lineage tracing experiments using various systems and have demonstrated that putative hepatic stem/progenitor cells with biliary features do not or only partly contribute to liver regeneration in chronic injury,[44,81–88] although the activation of stem/progenitor-like cells appears to be triggered under particular conditions, such as the deletion of Mdm2, β1-integrin, or β-catenin in hepatocytes.[80,89,90] Several groups have proposed that hepatocyte-derived stem/progenitor-like cells might participate in liver regeneration in chronic injury.[91,92] Acutely injured hepatocytes were reported to express mRNA of the *Afp* gene and several genes that are active in the liver during the postnatal period,[93,94] and some embryonic morphogenesis-related genes are activated in the early phase of chronic CCl_4 injury.[95] Although hepatocyte-derived stem/progenitor-like cells may exhibit partially dedifferentiated features, AFP is constitutively expressed in adult hepatocytes, albeit at low levels,[96,97] and these bipotential hepatocytes do not express prototypical markers for fetal hepatoblasts, such as, most importantly, DLK1. Therefore, the emergence of such stem/progenitor-like cells in chronic liver injury should be considered transdifferentiation, rather than true dedifferentiation, of hepatocytes.

Thus, in chronic liver injury, hepatocytes are predominantly regenerated by self-reproduction. Hepatocytes in the adult liver are heterogeneous[98–101] and can demonstrate various proliferative activities following liver injury.[73–75] Although the subset of hepatocytes that retain a continuous proliferative capacity has not been characterized, such hepatocytes could be de facto liver stem/progenitor cells in the adult liver. Continuously proliferating hepatocytes in regenerative nodules might accumulate genetic or epigenetic alterations and eventually transform into tumor cells. Therefore, chronic clonal proliferation of hepatocytes may be the basis of hepatocarcinogenesis in chronically injured livers.

POSSIBLE MECHANISMS OF CHRONIC INJURY-MEDIATED HEPATOCARCINOGENESIS: DEDIFFERENTIATION OF HEPATOCYTES ASSOCIATED WITH EPIGENETIC ALTERATIONS

Elucidation of the intracellular events in hepatocarcinogenesis associated with chronic liver injury is important. However, at present, the molecular changes that occur during this process are still unclear. In mice, multiple very well-differentiated HCCs, which were originally designated hepatomas or hepatocellular adenomas,[102,103] can be induced in a cirrhotic background by repeated administration of CCl_4 or thioacetamide if the duration of the injury is more than 20 weeks, providing reliable and reproducible models for inflammatory hepatocarcinogenesis (Figure 7a).[97] In contrast, in the most popular mouse HCC model,[104] in which a necrotizing dose of a mutagen, diethylnitrosamine (DEN), is administered at 2 weeks after birth, multiple nodules of invasive HCC are induced in a noncirrhotic background (Figure 7a).[97]

We screened tumor-specific genes by oligonucleotide microarray analysis and identified 15 genes that were specifically expressed in CCl_4-induced liver tumors (*H19*, *Igf2*, *Cbr3*, and *Krt20*), DEN-induced liver tumors (*Tff3*, *Akr1c18*, *Gpc3*, *Afp*, and *Abcd2*), or both (*Ly6d*, *Slpi*, *Spink3*, *Scd2*, and *Cpe*) but not in nontumor liver tissues (intact liver or regenerative nodules).[97] All of these genes are also activated in fetal/neonatal livers, indicating that hepatocytes dedifferentiate into fetal hepatocytes or hepatoblasts to varying degrees during hepatocarcinogenesis. Unsupervised two-dimensional hierarchical cluster analysis revealed differences in the expression patterns of these fetal/neonatal genes between the cirrhotic liver and noncirrhotic liver tumors (Figure 7b). Importantly, lineage tracing experiments in mice have shown that hepatocytes, but not putative liver stem/progenitor cells, are the cells of origin for HCC induced by various hepatocarcinogenic protocols, including chronic CCl_4 injury and DEN administration.[105]

Although activating mutations in the *Hras* or *Braf* gene have been frequently identified in DEN-induced mouse liver tumors,[106,107] CCl_4-induced liver tumors lack any recurrent mutations in known driver genes (Tanaka et al., manuscript in preparation). However, a methylation analysis of the genome of CCl_4-induced liver tumors revealed that the major type of epigenetic alteration in these tumors is the demethylation of various genes, such as those involved in cell adhesion and iron transport, suggesting the importance of epigenetic gene alterations in the early step of inflammatory carcinogenesis associated with liver cirrhosis (Tanaka et al., manuscript in preparation).

In human hepatocarcinogenesis, alterations in DNA methylation have been shown to occur as early events and interact with genomic alterations.[108,109] As the importance of "nonmutational epigenetic reprogramming" in cancer is increasingly highlighted,[110] further investigation of epigenetic regulatory mechanisms that are influenced by microenvironmental changes during hepatocarcinogenesis, especially at earlier stages, is important.

ANALYSIS OF A BROAD SPECTRUM OF HEPATOCYTE-DERIVED TUMORS VIA THE INTERACTIONS OF DRIVER GENES

Comprehensive genomic studies of human and mouse HCC have identified the major intracellular pathways involved in hepatocarcinogenesis, including

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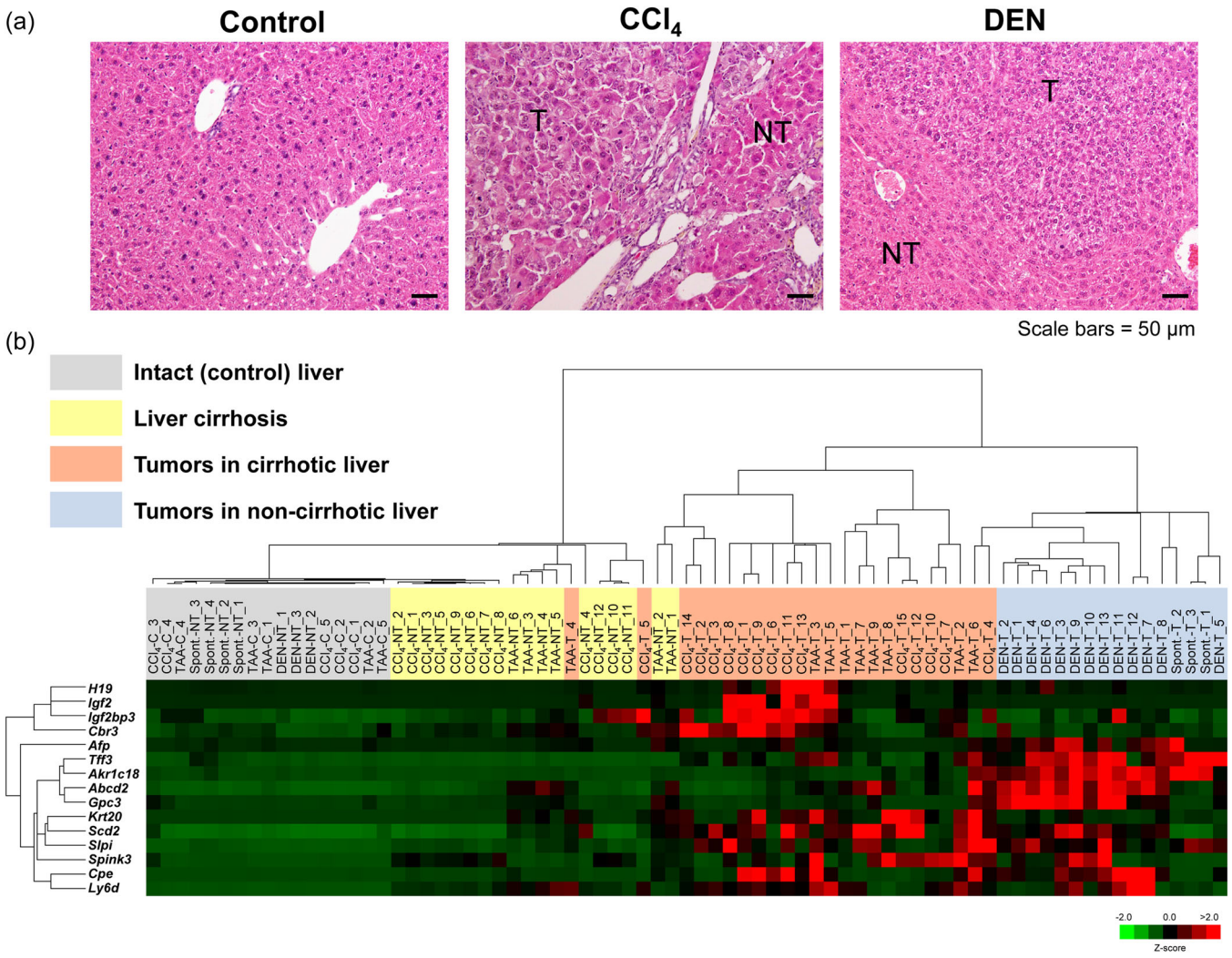

FIGURE 7 Fetal/neonatal gene expression in mouse liver tumors induced in cirrhotic and noncirrhotic hepatocarcinogenesis models. (a) Micrographs of intact (control), CCl_4-treated, and diethylnitrosamine (DEN)-treated liver tissues. HE staining. (b) Heatmap of unsupervised two-dimensional hierarchical clustering of mRNA expression of 15 tumor-associated genes in four different liver tumor models: CCl_4-induced, DEN-induced, thioacetamide (TAA)-induced, and spontaneous. NT, nontumor tissue; T, tumor.

the RTK/RAS, PI3K/AKT, p53, and Hippo/YAP pathways and those stimulated by the MYC family of transcription factors.[111,112] We studied how interactions between major oncogenes determine the phenotypes of tumors of adult hepatocyte origin using a combination of Sleeping Beauty transposon-mediated somatic gene transfer and hydrodynamic tail vein injection of various oncogenes.[22–25] This hepatocyte-specific gene transfer system enables us to induce multiple tumors in the mouse liver within several weeks and to examine the combined effects of oncogenes.[113,114]

The introduction of myristoylated AKT (AKT henceforth) or mutant HRAS ($HRAS^{V12}$; HRAS henceforth) alone induces multiple nodules of well-differentiated tumors after long incubation periods (20–28 weeks); however, when AKT and HRAS are simultaneously introduced, more aggressive, less differentiated HCC is formed within 4 weeks, and almost all the liver parenchyma is replaced with tumor tissues after 8 weeks (Figure 8a).[22] Consistent with previous observations with AKT/NRAS-induced HCC,[115] in the early stages of the development of AKT/HRAS-induced HCC, most tumor cells accumulate a large amount of lipids, but these cells show gradual loss of lipids and increased proliferative activity with the nuclear accumulation of the Myc protein,[22] probably due to Myc protein stabilization by the activated AKT and RAS pathways.[116]

Although transposon-mediated Myc overexpression alone was insufficient to induce tumors in our experimental system, Myc has been shown to induce mouse HCC[117] and is required to transform adult hepatocytes from mice and humans.[118,119] In fact, the spontaneous activation of Myc is critical for the development of AKT/HRAS-induced HCC, since tumorigenesis is almost completely abolished by cointroduction of MadMyc, and

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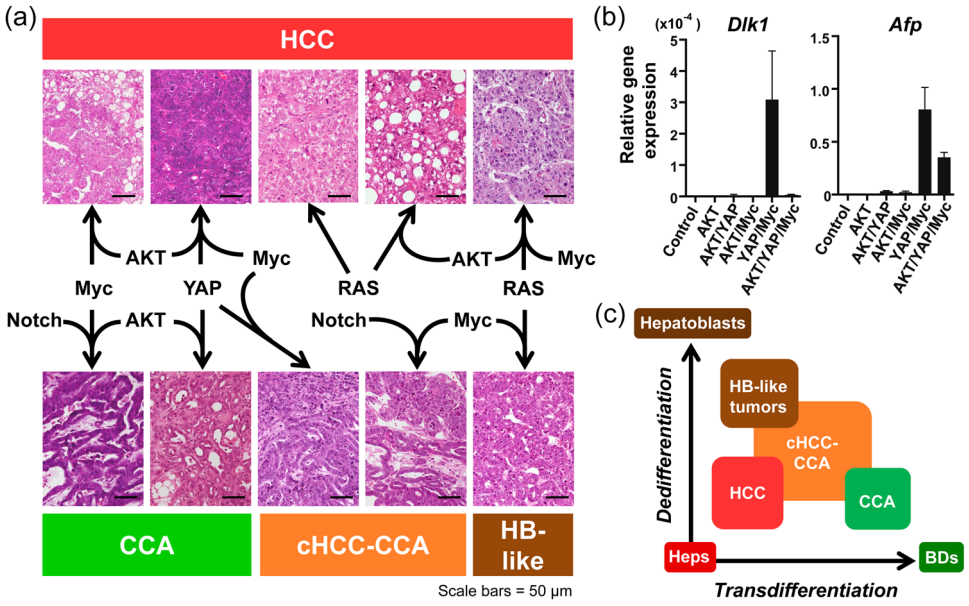

FIGURE 8 A broad spectrum of phenotypes of mouse hepatocyte-derived tumors induced by Sleeping Beauty transposon-mediated somatic integration of various oncogenes. (a) Generation of hepatocytic, cholangiocytic, and hepatoblastic tumors from hepatocytes through the interactions of activated AKT, HRAS, Myc, Notch (NICD), and YAP. HE staining. (b) The mRNA expression of *Dlk1* and *Afp* in tumors induced by various combinations of AKT, Myc, and YAP. (c) Hypothetical two-dimensional perspective of hepatocyte-derived tumors with respect to transdifferentiation and dedifferentiation. Panel (b) is reproduced from our previous publication[23] (Copyright Elsevier). BDs, bile ducts/ductules; CCA, cholangiocarcinoma; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; HB, hepatoblastoma; HCC, hepatocellular carcinoma; Heps, hepatocytes.

tumor progression is significantly inhibited by the Tet-On-mediated induction of MadMyc at the preneoplastic stage.[22] Interestingly, tumorigenesis induced by AKT/HRAS is effectively inhibited by coexpression of dual-specificity tyrosine-regulated kinase 2 (Dyrk2), whose low expression is associated with poor prognosis in liver cancer patients,[120] through proteasome-mediated degradation of the Myc and HRAS proteins.[121]

Myc overexpression strongly enhances tumorigenesis via AKT and/or HRAS, conferring increased proliferative activity.[22] The histology of AKT/Myc- and AKT/HRAS/Myc-induced tumors was similar to that of typical HCC, whereas HRAS/Myc-induced tumors were composed of cells with a high nuclear-cytoplasmic ratio, reminiscent of hepatoblasts in the fetal liver (Figure 8a).[22,24] Myc-overexpressing tumors are composed of tumor cells with prominent nucleoli and decreased (AKT/Myc) or no (HRAS/Myc, AKT/HRAS/Myc) lipid accumulation. Human HCC with distinct nuclear MYC expression is also characterized by prominent nucleoli, a cytoplasm devoid of lipid droplets, and increased proliferative activity.[22,122]

The activation of the Notch signaling pathway in adult mouse hepatocytes in vivo through the introduction of the Notch intracellular domain (NICD) is sufficient for the ductular transdifferentiation of hepatocytes.[46] Furthermore, cointroduction of NICD and AKT into mouse hepatocytes induces malignant tumors with features of CCA.[123] In our hands, simultaneous introduction of these genes generated cystic biliary tumors lacking definitive malignant morphological features.[23] However, cointroduction of Myc along with NICD and AKT induced highly proliferative CCA with marked invasive growth (Figure 8a). The Notch and YAP signaling pathways reciprocally interact with each other,[124] and the activation of the latter promotes bile duct proliferation in the ductular reaction,[125,126] as well as generating malignant tumors with biliary differentiation.[127] In fact, the cointroduction of AKT and mutant YAP (YAP^{S127A}) generates low-grade CCA with elevated mRNA expression levels of the genes encoding a Notch receptor (*Jag1*) and its effectors (*Hes1* and *Hes2*) (Figure 8a).[23] The introduction of mutant YAP and $PIK3CA^{H1047R}$ (a constitutively active mutant of PI3K) has been shown to induce not only CCA but also HCC and cHCC-CCA.[128] These results are consistent with the notion that mature hepatocytes can be transformed to generate tumors via cholangiocytic differentiation.[129,130]

Our immunohistochemical analyses of human HCC and CCA have validated some of the experimental data in mice.[23] YAP was detected in the nuclei of tumor cells in 33.3% of the HCC patients and in 94.1% of the CCA patients, suggesting that YAP plays a more important role in CCA than HCC. A substantial fraction (64.7%) of CCA patients were positive for phosphorylated glycogen synthase kinase-3 (GSK3β), a substrate for AKT, indicating an important correlation between the pathways involving these molecules. Phosphorylated AKT

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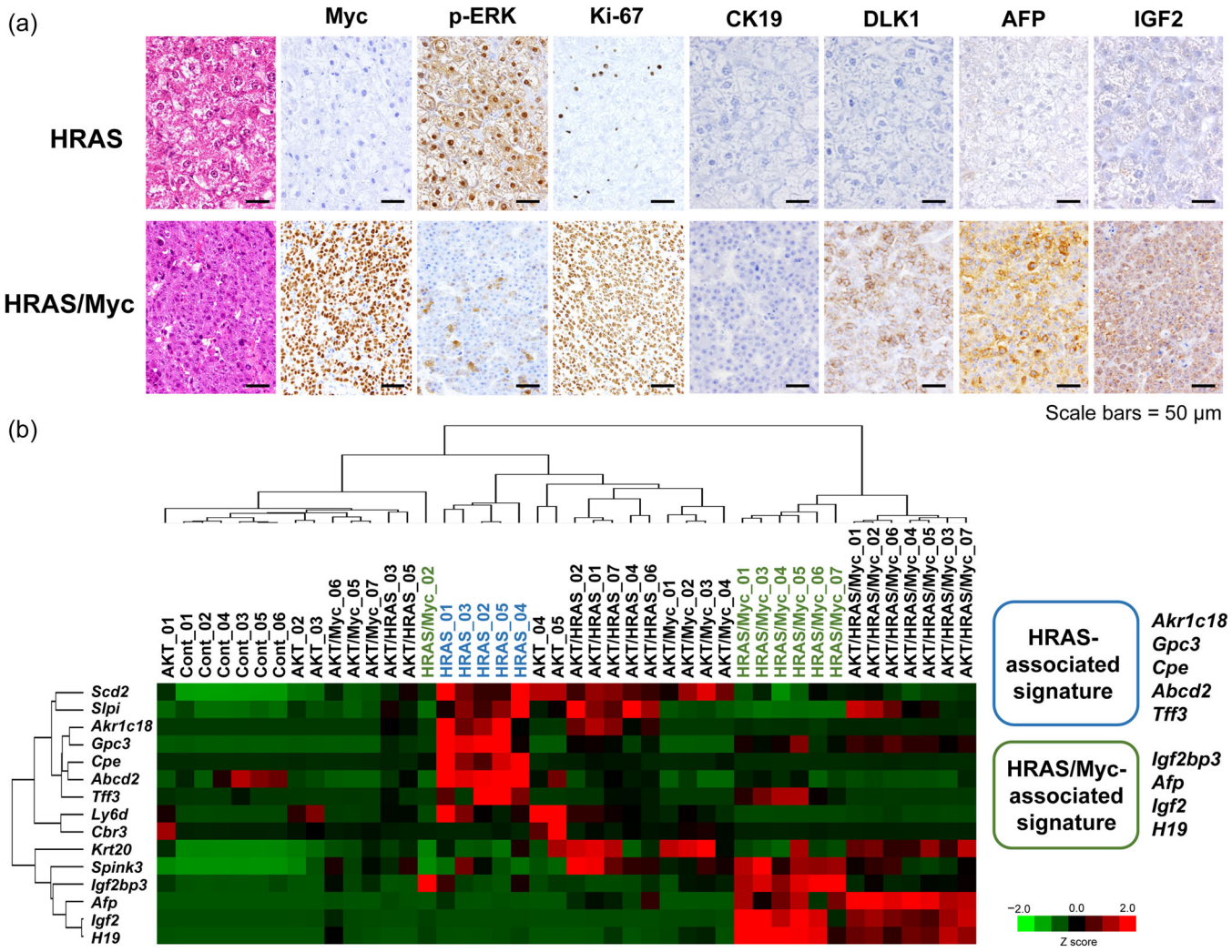

FIGURE 9 Dedifferentiated hepatoblastoma-like tumors induced by the combination of HRAS and Myc in mice. (a) Comparison of the phenotypes of HRAS- and HRAS/Myc-induced tumors. HE staining and immunohistochemistry for Myc, phosphorylated ERK (p-ERK), Ki-67, CK19, DLK1, AFP, and IGF2. (b) Heatmap of unsupervised two-dimensional hierarchical cluster analysis of the mRNA expression levels of the mouse tumor-associated fetal/neonatal genes that we previously identified[97] (see Figure 7) in the liver tumors induced by AKT, HRAS, AKT/HRAS, AKT/Myc, HRAS/Myc, and AKT/HRAS/Myc.

is often positive in HCC patients but not in CCA patients; phosphorylated S6 (another AKT substrate) is positive in most HCC patients but positive in approximately half of CCA patients.

The combination of Myc and YAP produced cHCC-CCA that were composed of tumor cells with elevated gene and protein expression of DLK1, as well as AFP, suggesting that these cells undergo dedifferentiation toward hepatoblasts (Figure 8a,b).[23] The expression of these dedifferentiation markers was significantly suppressed when AKT was concomitantly introduced (Figure 8b). Similarly, cHCC-CCA was also induced by the combination of Myc and NICD (Figure 8a). These results indicate that hepatocyte-derived tumor cells exhibit progenitor-like bipotential features as a result of the combination of dedifferentiation and transdifferentiation of hepatocytes (Figure 8c). The appearance of the biphenotypic histology may also be affected by the tumor microenvironment, since inflammatory backgrounds have been shown to affect the phenotype of mouse liver tumors.[131] In fact, HCC with abundant fibrous stroma (scirrhous HCC) may show CCA-like histology and gene expression patterns.[132] Interestingly, the genome-wide substitution patterns of human CCA and cHCC-CCA, which are associated with chronic hepatitis, may be similar to those of HCC.[133]

Compared with HRAS-induced tumors, HRAS/Myc-induced tumors, which are composed of immature hepatoblast-like cells, are extremely proliferative (Figure 9a). Both of these tumors do not show bile duct-like features and are negative for CK19, but HRAS/Myc-induced tumors are positive for dedifferentiation markers, including DLK1, AFP, and insulin-like growth factor-2 (IGF2) (Figure 9a). DLK1 has been reported to

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be a highly sensitive and specific marker for human hepatoblastoma.[134] Unsupervised two-dimensional hierarchical cluster analysis of the 15 liver tumor-associated fetal/neonatal genes we previously identified[97] revealed that the mRNA expression profiles of HRAS- and HRAS/Myc-induced tumors were clearly segregated (Figure 9b).[24] HRAS/Myc-induced tumors also express mRNAs for stem cell markers, such as *Nanog* and *Sox2*.[24] These results strongly suggest that Myc activation facilitates the dedifferentiation of transformed hepatocytes. As in the case of YAP/Myc-induced tumors,[23] when AKT is simultaneously activated with HRAS or HRAS/Myc, the expression of fetal/neonatal genes is suppressed, although the tumors become more proliferative and "less differentiated" (Figure 9b).

The genomic profile of human cHCC-CCA is more similar to that of HCC than that of CCA, and *TP53* is the most frequently mutated gene in cHCC-CCA.[135–137] Interestingly, mouse liver tumors with bidirectional differentiation have been generated upon liver-specific conditional knockout of *p53*, suggesting that the loss of p53 might affect the phenotype of primary liver cancer.[138] Transposon-mediated hydrodynamic somatic integration of activated NRAS in *p19Arf*-null mice, in which p53 is inactivated, has been shown to produce cHCC-CCA.[113] Furthermore, the loss of p53 has been demonstrated to confer bile ductular reprogramming to HCC cells induced by mutant KRAS.[139] Consistent with these reports, in our study, the phenotypes of the HRAS- and HRAS/Myc-induced tumors generated in p53-KO mice were consistent with those of cHCC-CCA.[25] However, the expression of fetal/neonatal liver proteins, including DLK1 and AFP, was detected in HRAS/Myc-induced but not in HRAS-induced cHCC-CCA tissues.[25] The dedifferentiation of HRAS/Myc-induced tumors is more notable in homozygous p53-KO mice than in heterozygous p53-KO mice and is associated with the activation of Myc and YAP and the suppression of ERK phosphorylation.[25] These results suggest that the loss of p53 promotes the ductular differentiation of hepatocyte-derived tumor cells through either transdifferentiation or Myc-mediated dedifferentiation.

MYC has been shown to be crucially involved in the pathogenesis of human hepatoblastoma.[140] In human HCC, MYC-positive tumors express AFP, IGF2, and DLK1 more frequently than MYC-negative tumors, indicating an important role in inducing the dedifferentiated phenotype.[24] Interestingly, some highly aggressive tumors with elevated levels of AKT phosphorylation do not express dedifferentiation markers even if the tumors are highly MYC positive, suggesting that activation of the PI3-AKT pathway suppresses dedifferentiation of hepatocytic tumors.[24] Therefore, the aggressiveness of liver tumors with higher cellular or structural atypia is separable from the degree of dedifferentiation, implying that the general notion that dedifferentiation correlates with higher tumor grades might not always be the case.

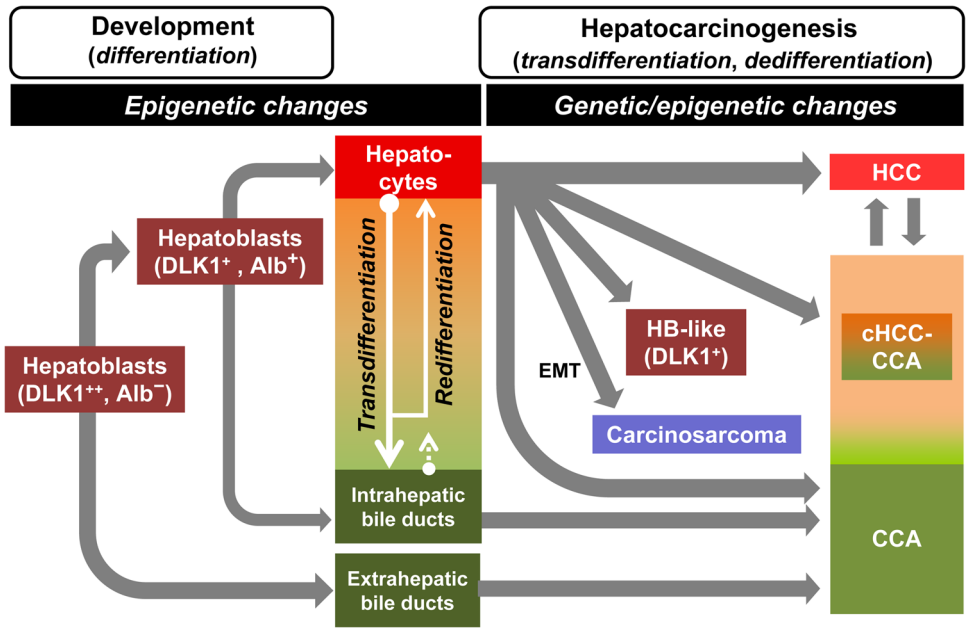

FIGURE 10 The development of the liver epithelial system and the plasticity of mature hepatocytes as frameworks for understanding the phenotypic diversity of liver tumors. Hepatocytes that are transformed by genetic and epigenetic alterations of various driver genes can be the source of HCC, but they may fully transdifferentiate to generate typical CCA. Furthermore, if these cells deeply dedifferentiate, for example, through Myc activation, hepatoblastoma (HB)-like immature tumors may develop. The biphenotypic features of cHCC-CCA can be the result of partial transdifferentiation or varying combinations of transdifferentiation and dedifferentiation. Evidence shows that even carcinosarcoma can develop if substantial epithelial–mesenchymal transition (EMT) occurs in transformed hepatocytes.

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We propose that it is possible to understand HCC, CCA, cHCC-CCA, and hepatoblastoma-like tumors from a two-dimensional perspective of transdifferentiation and dedifferentiation (Figure 8c).[25] Through the transformation of mature hepatocytes by genetic and epigenetic alterations of various driver genes, a broad spectrum of liver cancers can be generated (Figure 10). Furthermore, the combination of NICD and mutant HRAS induces sarcomatoid carcinoma composed of atypical spindle tumor cells with vimentin expression through epithelial–mesenchymal transition (Yamamoto et al., manuscript in preparation). The cells of origin for CCA can be hepatocytes, intrahepatic bile ducts, and extrahepatic bile ducts (including peribiliary glands), and regardless of the origin, carcinogenesis might be promoted by IL-33 released from hepatobiliary inflammation.[58,123,141–143]

CONCLUSIONS

In this review, the phenotypic plasticity and heterogeneity of adult hepatocytes are discussed. Whereas the capacity of hepatocytes to transdifferentiate to bile duct cells is restricted in chronic liver diseases, this process may be crucial to maintain connections with the existing bile ductular system that possesses extensive remodeling capacity. The transdifferentiation capacity is particularly relevant to the pathogenesis of hepatocyte-derived tumors with cholangiocytic differentiation. Hepatocytes can also dedifferentiate to varying degrees upon transformation, especially when Myc is activated, contributing to the phenotypic complexity of primary liver cancers. Although the debate on hepatic stem/progenitor cells has not yet been settled, the most relevant cells for parenchymal regeneration in acute and chronic injury appear to be hepatocytes. However, hepatocytes in the adult liver may be heterogeneous, including a fraction that can repeat cell divisions for a long period of time and form regenerative nodules in liver cirrhosis. Elucidation of the nature of such a hepatocyte fraction is critically important, and this issue warrants further investigation.

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CONFLICT OF INTEREST STATEMENT

None declared.

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