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Author manuscript

*Liver Res.* Author manuscript; available in PMC 2018 September 01.

Published in final edited form as:

*Liver Res.* 2017 September ; 1(2): 81–87. doi:10.1016/j.livres.2017.08.002.

## Hepatic progenitor cell activation in liver repair

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### Abstract

The liver possesses an extraordinary ability to regenerate after injury. Hepatocyte-driven liver regeneration is the default pathway in response to mild-to-moderate acute liver damage. When replication of mature hepatocytes is blocked, facultative hepatic progenitor cells (HPCs), also referred to as oval cells (OCs) in rodents, are activated. HPC/OCs have the ability to proliferate clonogenically and differentiate into several lineages including hepatocytes and bile ductal epithelia. This is a conserved liver injury response that has been studied in many species ranging from mammals (rat, mouse, and human) to fish. In addition, improper HPC/OC activation is closely associated with fibrotic responses, characterized by myofibroblast activation and extracellular matrix production, in many chronic liver diseases. Matrix remodeling and metalloprotease activities play an important role in the regulation of HPC/OC proliferation and fibrosis progression. Thus, understanding molecular mechanisms underlying HPC/OC activation has therapeutic implications for rational design of anti-fibrotic therapies.

### Keywords

Liver regeneration; Hepatic progenitor cells (HPCs); Oval cells (OCs); Liver injury; Hepatic fibrosis

## 1. Introduction

The liver is a vital organ within the body. A broad range of hepatic insults such as hepatitis virus, alcohol, fat accumulation, and drug toxicity can damage the liver to result in steatohepatitis, fibrosis, cirrhosis, and even development of hepatocellular carcinoma. Liver diseases have been a major health concern worldwide because of their high prevalence and

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### Author's contributions

AB and LP designed the concept of the review, prepared table, and wrote the review. JZ, QC, SW and BEP generated figures and provided critical comments on the paper.

### Conflict of interest

The authors declare that they have no conflict of interest.

poor long-term clinical outcome. This is particularly true in China, where approximately 300 million people suffer from liver diseases.<sup>1</sup> Liver transplantation is the only curative option for end-stage liver disease, but sources of donors are limited. Thus, there is an urgent need to develop alternative therapeutic strategies. Hepatic stem/progenitor cells (HPCs), also known as oval cells (OCs) in rodents, are one potential suitable source for liver cell replacement. These cells can be activated through orchestrated signaling networks mediated by a plethora of growth factors, cytokines, and enzymes in response to severe liver injury and/or compromised hepatocyte function. Abnormal amplification of HPC/OCs is also associated with pathological scarring processes and may contribute to the development of liver cancer. Crosstalk among damaged epithelial components, pro-fibrotic mesenchymal elements, and pro-inflammatory immune cells, as well as specialized extracellular matrix (ECM) in the HPC niche, is of fundamental importance in sustaining HPC activation and liver fibrosis in disease conditions. This review covers experimental and clinical studies of HPC activation and associated liver fibrosis, with emphasis on mediators of ECM regulation in the HPC niche.

## 2. HPC/OC characteristics and origins

Liver stem/progenitor cells include unique populations that are able to differentiate into hepatic parenchymal cells, hepatocytes, and/or bile ductular epithelial cells. During development, hepatoblasts appear in the foregut endoderm, where they give rise to both hepatocytes and cholangiocytes.<sup>2</sup> Similarly, adult livers contain so-called HPC/OCs, a heterogeneous population of transit-amplifying cells that expand during severe liver damage upon inhibition of hepatocyte proliferation.<sup>3</sup> HPC activation in the form of ductular reactions has been observed in many pathophysiological processes of human liver diseases.<sup>4</sup> In rodents, HPC/OCs represent small hepatobiliary reactive cells approximately 10  $\mu\text{m}$  in diameter with a large nuclear-to-cytoplasm ratio and oval-shaped nucleus (hence their name).<sup>5</sup> HPC/OCs represent dynamic cell populations that constantly change their phenotype depending on the injured cell type and consequent state of differentiation.<sup>6</sup> Despite lacking specific markers for HPC/OCs, a broad panel of surface antigens and intracellular proteins has been reported in these cells and their progeny (see Table 1).<sup>7-24</sup> Moreover, the bi-potential ability of HPC/OCs is evidenced by detection of markers for both bile ductular epithelial cells and hepatocytes in numerous experimental models and human studies.<sup>8,19</sup> HPC/OCs are thought to differentiate into hepatocytes to restore lost liver mass and function in response to hepatocyte damage, such as that occurring with severe viral hepatitis or massive hepatocyte loss in human patients.<sup>7,25,26</sup> In contrast, HPC/OCs mediate biliary regeneration in circumstances where bile ductular epithelium is the most damaged cell type, including diseases such as primary biliary cirrhosis or primary sclerosing cholangitis.<sup>27</sup> Recent studies using genetic lineage-tracing techniques in transgenic mice have challenged the contribution of HPC/OCs to liver regeneration.<sup>28,29</sup> Nevertheless, HPC/OC activation is often associated with myofibroblast cell activation and liver fibrosis during chronic liver disease.<sup>30-32</sup> In human studies, HPC/OCs accumulate as injuries become more severe, indicating HPC proliferation increases with progressively worsening liver injury and fibrosis.<sup>32</sup> Correlation between degree of HPC activation and the severity of liver disease has been shown in both acute and chronic human liver diseases.<sup>31</sup>

The origin of HPC/OCs is also a topic of contention with many different theories. Traditionally, they are considered to be small bile ductular epithelial cells located in the Canal of Hering, a transitional zone between the bile canaliculi and interlobular ductal systems in mammals.<sup>33</sup> An extrahepatic origin of rat OCs, such as bone marrow, has also been suggested.<sup>34</sup> In addition, it has been proposed that HPC/OCs may be derived from hepatic stellate cells (HSCs), as HSCs contain many of the same signaling pathways and express similar genes as undifferentiated cells in rats.<sup>35</sup> Other recent studies have demonstrated that conversion from hepatocyte or bile ductular epithelial cells to HPCs is a reversible process after recovery from injury, suggesting HPC/OCs may originate from dedifferentiation of mature hepatocytes or bile ductular epithelial cells in response to liver damage.<sup>36</sup> At least four distinct niches have been demonstrated: canal of Hering, intralobular bile ducts, peri-ductal cells, and peri-biliary hepatocytes.<sup>37</sup> The liver is believed to regenerate through a multi-tiered, flexible system rather than a single HPC/OC location in response to severe damage. This multi-tiered, flexible system determines phenotypic expression, proliferative capability, and differentiation properties of HPC/OCs under specific pathologic or pathophysiologic circumstances. Taken together, “location is everything”, as Petersen and Shupe stated.<sup>38</sup> This explains the diversity of HPC responses in different niches under distinct injury models.

### 3. Experimental models of HPC/OC activation

HPC/OCs can be activated in a number of animal models. In rats, surgical removal of two-thirds of the liver lobe by partial hepatectomy (PHx) has been combined with administration of hepatotoxins such as 2-acetylaminofluorene (2-AAF) to trigger an HPC/OC response.<sup>39</sup> In addition, bio-reactivation of chemicals can cause zonation-dependent regeneration. Allyl alcohol (AA) is metabolized to highly reactive aldehyde acrolein by alcohol dehydrogenase, which is principally localized in the periportal area.<sup>40</sup> Cytochrome P450 enzymes can convert CCl<sub>4</sub> into highly reactive metabolites that trigger lipid peroxidation, leading to hepatocyte death at centrilobular areas.<sup>41</sup> As shown in Fig. 1, activation of  $\alpha$ -smooth muscle actin (SMA)<sup>+</sup> myofibroblast cells and collagen deposition are common features of both types of liver damage. However, AA induces extensive periportal proliferation, whereas CCl<sub>4</sub> intoxication causes hepatocyte proliferation in central lobular parenchyma (Fig. 2). Combining 2-AAF implantation with either type of chronic liver injury triggers a robust HPC/OC response.<sup>42</sup>

There is remarkable heterogeneity of HPC/OCs in rat and mouse models. For instance, 2-AAF is unable to trigger an HPC/OC response in mice.<sup>43</sup> Instead, mouse HPC/OCs are activated using different dietary or toxin models, including the choline-deficient ethionine-supplemented diet, 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, and phenobarbital/cocaine.<sup>44,45</sup> The methionine-choline deficient (MCD) diet is also well known to induce fatty liver with HPC proliferation in mice.<sup>46</sup> These toxins can interfere with cellular and molecular mechanisms of liver regeneration through membrane damage, inflammatory reactions, or even activation of non-parenchymal cells such as Kupffer cells.<sup>47</sup> Recently, genetic murine models of HPC activation have been developed. For example, a system using  $\beta$ -naphthoflavone for inducible deletion of Mdm2, an E3 ubiquitin-protein ligase that degrades TRP53 (p53), was successful in over 98% of hepatocytes using an

*AhCre* system, and resulted in upregulation of p53, induction of p53-mediated hepatocyte death, and senescence of nearly all hepatocytes expressing p21.<sup>48</sup> These transgenic mice will be very valuable tools to model HPC activation, hepatocyte injury, and senescence in rats and human patients.

With regard to laboratory use, zebrafish are unique specimens for the study of liver regeneration for many reasons. First, they possess extensive regenerative capabilities. Second, as a result of the high genetic conservation of vertebrates, liver structure and signaling pathways are relatively conserved between mammals and zebrafish.<sup>49</sup> Third, each mating event results in hundreds of offspring that are easily genetically manipulated and transparent for observation of embryonic development. Finally, numerous methods for studying zebrafish liver regeneration exist, including 1/3 PHx, ethanol models, and ablation of hepatocytes in transgenic animals with nitroreductase, which converts metronidazole into a toxin that activates a fibrogenic response.<sup>49–51</sup>

#### 4. HPC/OC niche

The HPC niche consists of multiple types of stromal cells and surrounding ECM that forms a special microenvironment controlling the characteristics of HPC/OCs and balance of HPC/OC activation, proliferation, and differentiation (Fig. 3A). The significance of the microenvironment in control of cell behavior and fate has been demonstrated in elegant liver transplantation studies. Nonfunctional cells collected from cirrhotic rats can recover full metabolic and proliferative function after engraftment into non-cirrhotic animals.<sup>52</sup> Stromal cells, an important component of the HPC/OC compartment, include damaged epithelial cellular elements (bile ductular epithelial cells and hepatocytes), activated HSCs/myofibroblasts, and macrophages, as well as other inflammatory cells recruited after liver injury. Immune cells, such as macrophages and lymphocytes, have been shown to stimulate and initiate regenerative responses in experimental models.<sup>53</sup> In response to debris from damaged hepatocytes, macrophages can promote HPC/OCs towards hepatocyte differentiation through Wnt signaling.<sup>54</sup> This is in contrast to biliary injury in which HSCs are activated and promote HPC/OC differentiation into biliary duct epithelial cells through Jagged1/Notch pathways.<sup>54</sup> In addition, activated HSCs/myofibroblasts form intimate contact with HPC/OCs and produce ECM to support HPC cell adhesion and migration.<sup>5</sup> However, production of excessive matrix proteins, such as collagen, by activated HSCs/myofibroblasts causes disorganization of hepatic microarchitecture and interferes with blood/fluid exchange, ultimately contributing to the pathogenesis of liver fibrosis and cirrhosis.

#### 5. Signaling pathways involved in HPC activation

Many factors and signals produced by HPC/OCs and stromal cells regulate HPC proliferation, migration, and differentiation. These signaling pathways are outlined in the following sections.

### 5.1. Pathways for HPC/OC proliferation

Hepatocyte growth factor (HGF)/c-Met signaling is a critical regulator of liver regeneration, as it activates a variety of other signaling pathways involved in proliferation, motility, migration, and invasion.<sup>55</sup> Deletion of c-Met receptors in murine studies illustrates their importance in liver regeneration. In response to DDC-induced liver injury, c-Met-deficient mice exhibit a reduced HPC/OC response, impaired HPC/OC migration, and decreased hepatocytic differentiation *in vivo*.<sup>56</sup> Other important regulators are in the Hippo pathway, which controls the activity of the transcriptional co-activator Yes-associated protein (YAP). Phosphorylation of YAP by kinases in the Hippo signaling pathway can cause cytoplasmic retention of YAP, thus impeding its ability to access target gene promoters. YAP transcriptionally regulates connective tissue growth factor (CTGF, also termed CCN2) during cell adhesion and has emerging roles in controlling HSC activation.<sup>57</sup> Ectopic activation of YAP in differentiated hepatocytes can result in their dedifferentiation, which drives liver overgrowth and HPC/OC appearance.<sup>58,59</sup> Knocking out key components in the Hippo pathway, such as WW45, restrains HPC/OC proliferation, liver size, and the development of liver cancer.<sup>60</sup>

### 5.2. Pathways for HPC/OC and macrophage interaction

Tumor necrosis-like factor weak inducer of apoptosis (TWEAK), a member of the pro-inflammatory tumor necrosis factor family, is a regulator of HPC and macrophage interactions. It is strongly induced in monocytes, T lymphocytes and macrophages, and promotes HPC proliferation through its specific receptor, fibroblast growth factor-inducible 14 (Fn14).<sup>61</sup> This signal is powerful enough that injection of TWEAK can result in HPC/OC proliferation in unharmed mouse liver. In addition, chemokine stromal cell-derived factor 1 (SDF-1) has been shown to be upregulated in a wide range of human chronic liver diseases.<sup>62,63</sup> It binds to its receptor, CXCR4, and plays a variety of roles including recruitment of bone marrow stromal cells (BMSCs) to damaged liver. CXCR4 is expressed by many different cell types, including BMSCs and inflammatory cells. SDF-1 is produced by HPC/OCs and attracts CXCR4<sup>+</sup> inflammatory cells that also express TWEAK.<sup>63</sup> Finally, activation of the Wnt/beta-catenin signaling pathway plays a significant role in HPC/OC expansion during liver diseases, including hepatitis C and cirrhosis.<sup>54</sup> In hepatocytic diseases of humans and rodents, numerous macrophages infiltrate the periportal areas as part of the HPC/OC niche. As shown in Fig 3A, phagocytosis of hepatocyte debris by hepatic macrophages results in expression of Wnt3a. Wnt3a in close proximity to HPCs activates Wnt pathways leading to transcriptional activation of Numb (a Notch pathway inhibitor) and differentiation of progenitors into hepatocytes.<sup>54</sup>

### 5.3. Pathways for HPC/OC and myofibroblast interaction

Fibroblast growth factor 7 (FGF7) can regulate HPC/OC and myofibroblast interactions. The signal originating from activated HSCs is received by FGFR2-IIIb receptors located on hepatocytes.<sup>64</sup> *Fgf7*-deficient mice displayed significantly reduced OC proliferation and higher mortality when placed on a DDC diet or subjected to bile duct ligation.<sup>65</sup> Forced expression of FGF7 in knockout mice was able to reverse damage by decreasing levels of

hepatocyte injury and cholestasis markers weeks after liver injury, showing a potential role for FGF7 in liver treatment.

TGF- $\beta$  signaling stimulates several processes including cell differentiation, ECM synthesis, cell growth, cell death, and apoptosis. In liver regeneration, TGF- $\beta$  induces expression of ECM proteins, such as CTGF, in most types of fibrosis.<sup>66</sup> CTGF belongs to the Cyr61/CTGF/Nov (CCN) protein family and can potentiate the fibrogenic activity of TGF- $\beta$  through direct interaction.<sup>67,68</sup> We have found that CTGF is highly expressed in murine and rat HPC/OCs.<sup>69,70</sup> It binds to integrins on the surface of many cell types and is able to promote cell attachment and migration on provisional matrix.<sup>71</sup> Deletion of this molecule affects HPC/OC activation and biliary fibrosis during DD- induced liver injury in mice.<sup>70</sup> In addition, we have identified a disintegrin and metalloproteinase with thrombospondin type I repeat 7 (ADAMTS7) as a novel CTGF protease produced by myofibroblasts and hepatocytes in the HPC/OC niche.<sup>72</sup> Knocking out ADAMTS7 is associated with abnormal an HPC/OC response and biliary fibrosis in the DDC mouse model. As shown in Fig. 3B, this represents a new mechanism for regulation of CTGF activity during HPC/OC activation, bile ductular reaction, and biliary fibrosis. Given that CTGF is over expressed in many types of fibrotic disorders,<sup>67</sup> there is a clear clinical relevance of ADAMTS7 activity for targeting overexpressed CTGF in fibrotic disorders. Hedgehog (Hh) pathway activation leads to an increase in HPC/OCs, repair-related inflammation, vascular remodeling, and liver regeneration, while also contributing to fibrosis and carcinogenesis.<sup>73</sup> Deletion of an obligate intermediate of the Hh pathway, Smoothed (SMO), in cells expressing the myofibroblast-associated gene  $\alpha$ SMA can prevent induction of Hh-related transcription factors and inhibit expression of myofibroblast genes.<sup>74</sup> As SMO deletion prevents aggregation of myofibroblasts and liver epithelial progenitors, it is a demonstrated regulator of both fibrotic and regenerative responses.

In biliary diseases, the Notch pathway is implicated in differentiation of HPC/OCs towards biliary epithelial cells through interaction with myofibroblasts.<sup>54</sup> As shown in Fig 3A, myofibroblasts express Jagged1, which coordinates with Notch on the surface of HPC/OCs to activate Notch signaling, resulting in a biliary phenotype in response to biliary liver damage typically observed in primary biliary cirrhosis and primary sclerosing cholangitis.<sup>54,75</sup> Disruption of Notch signaling in male rats with a  $\gamma$ -secretase inhibitor after 2-AAF treatment followed by 70% PHx demonstrated that the Notch pathway is essential for producing properly functioning hepatocytes.<sup>76</sup> Deletion of the RBP-J effector of Notch signaling abolishes this YAP-mediated trans-differentiation between hepatocytes and HPC/OCs.<sup>59</sup> These phenomena demonstrate both the considerable degree of plasticity of multiple liver cell types and ambiguous function of HPC/OCs in liver regeneration.

## 6. Conclusion

HPC/OCs, a heterogeneous set of proliferating epithelial cells, exhibit various intermediate states and are activated when hepatocyte function is compromised during severe liver damage. The origin and contribution of HPC/OCs to damaged liver is controversial and may vary based on extent, type, and location of hepatic injury. Nevertheless, HPC activation is closely associated with fibrotic responses in many experimental animal models and chronic

liver diseases. New technologies, including genetic tools such as lineage tracing and knockout mouse models, as well as the development of various approaches to isolate cells of interest, have been developed to characterize molecular targets for HPC/OC activation. Understanding mechanisms underlying HPC/OC activation and liver fibrosis is critical for *in vivo* and *in vitro* manipulation of HPC/OC expansion and differentiation in large numbers. These studies also provide a fundamental basis for the development of therapeutic strategies against related chronic liver diseases.

## Acknowledgments

We would like to thank Ms. Alicia Brown for immunohistochemical analysis in Figs. 1 and 2. We thank Liwen Bianji, Edanz Group China ([www.liwenbianji.cn/ac](http://www.liwenbianji.cn/ac)), for editing the English text of a draft of this manuscript.

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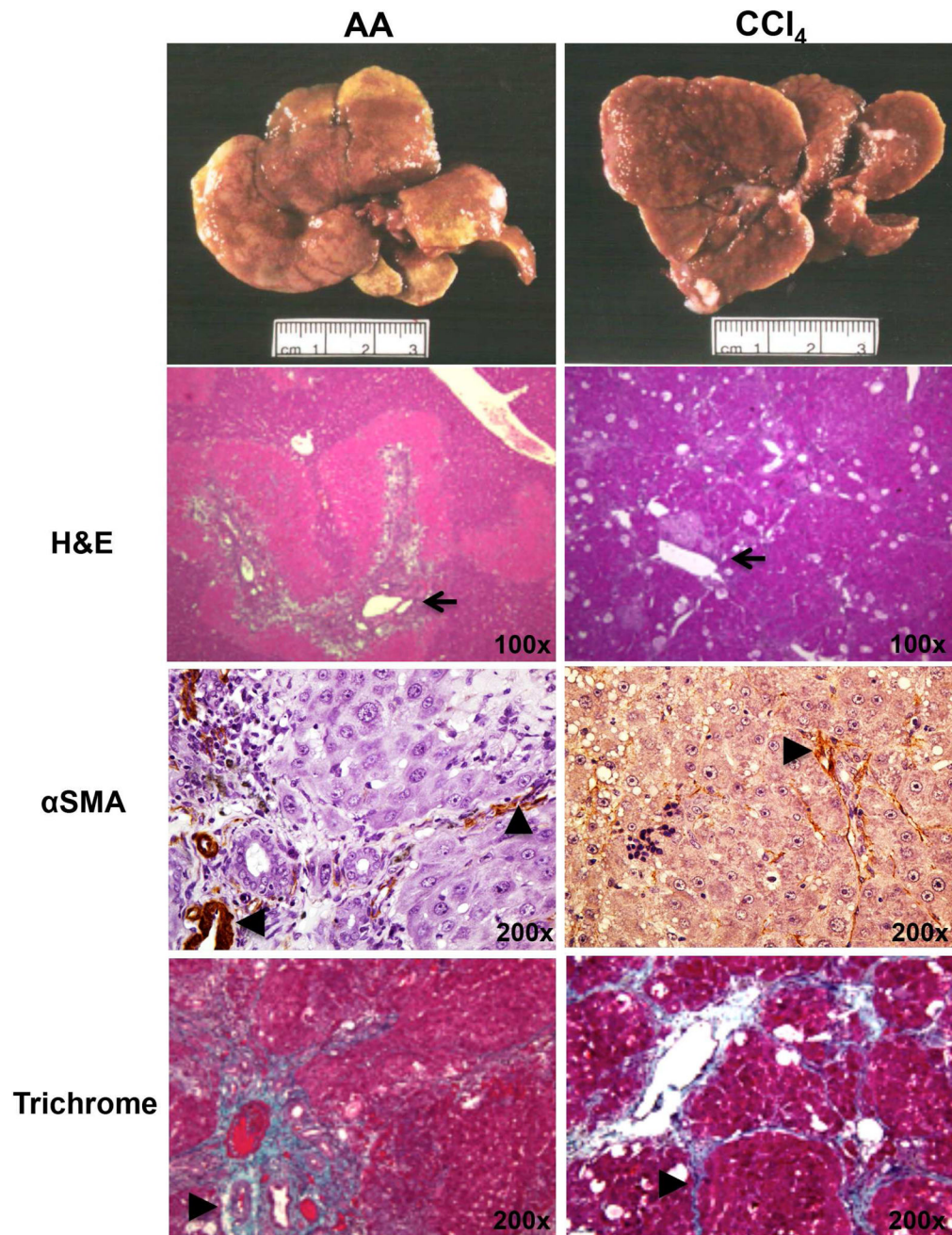
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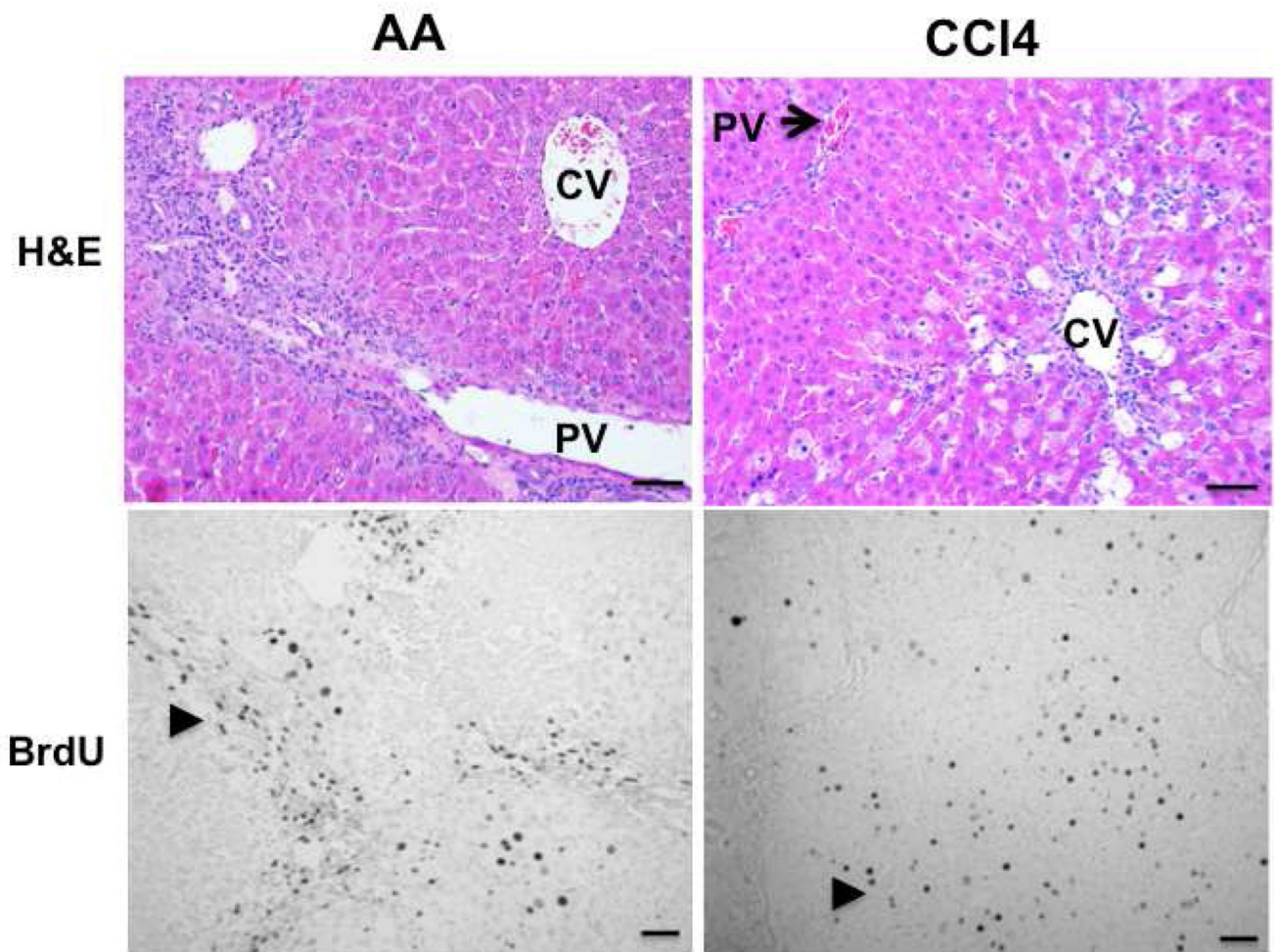


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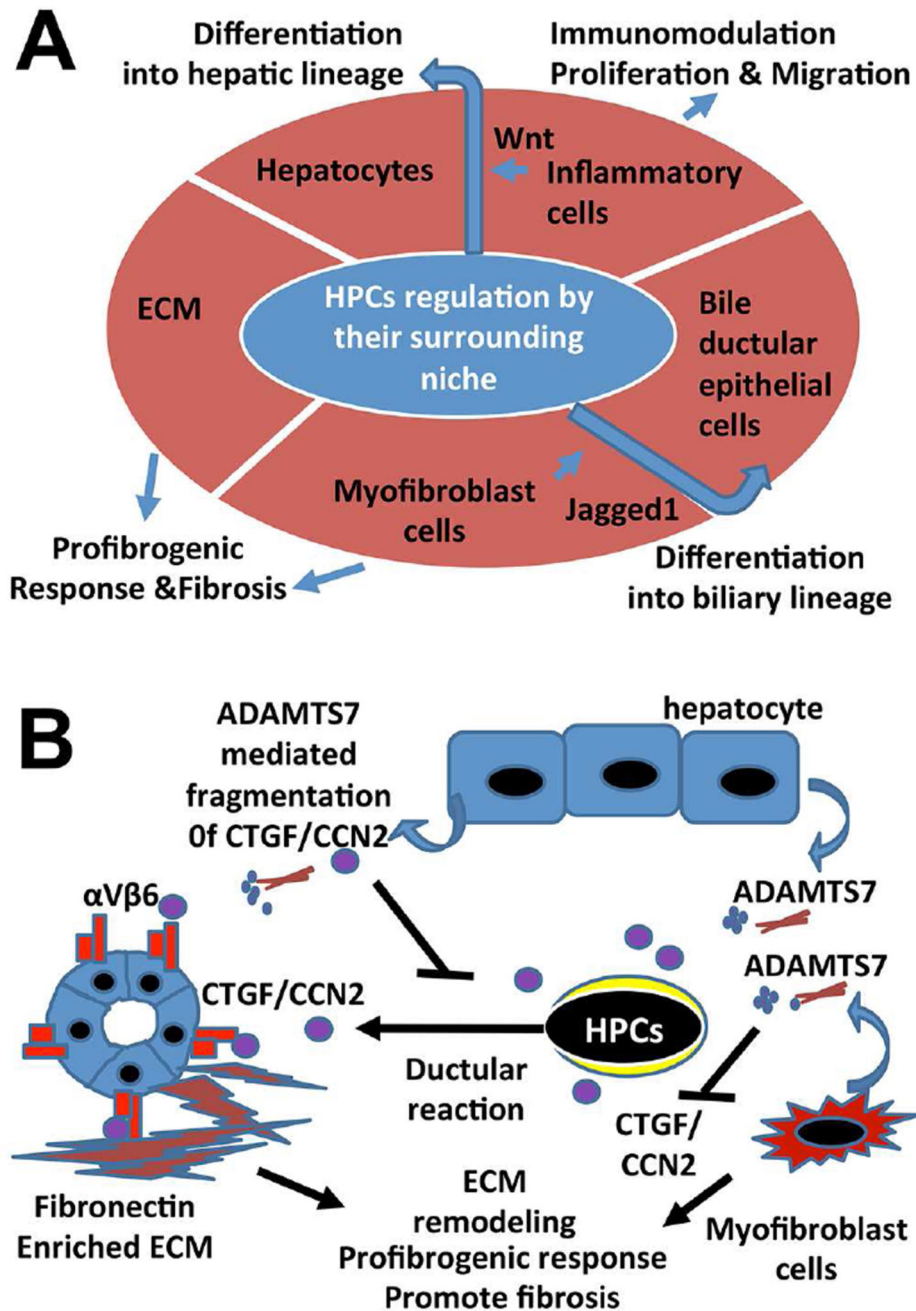
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**Fig.1.** Comparison of AA- or CCl<sub>4</sub>- induced liver fibrosis in rats. AA (7.4 mg/kg) and CCl<sub>4</sub> (300 mg/kg) were given to rats through IP injection for 90 days to induce chronic injury based on previous reports.<sup>40,41</sup> H&E, αSMA and Trichrome staining were carried out. Damaged and fibrotic areas (indicated by arrows or arrowheads) are shown.



**Fig. 2. AA or CCl<sub>4</sub> administration causes periportal or central lobular proliferation, respectively** AA (7.4 mg/kg) and CCl<sub>4</sub> (300 mg/kg) were administered to rats through IP injection for 45 days. BrdU (100 mg/kg body weight) was injected into animals 2 hours before sacrifice. H&E staining was performed to monitor histological changes in damaged livers. Proliferating cells were labeled with monoclonal mouse BrdU antibody followed by 3, 3'-diaminobenzidine (DAB) detection. Arrowheads indicate proliferating cells and damaged areas. Scale bar: 100  $\mu$ m. PV: portal vein; CV: central vein.



**Fig.3. HPC/OC niche and its damage responses**

(A) The HPC/OC niche consists of a specialized pro-fibrogenic extracellular matrix, epithelial elements (hepatocytes, cholangiocytes and their intermediate state), inflammatory cells, and myofibroblasts. Based on the type of injury, HPC/OCs can differentiate into either hepatocytes or bile ductular epithelial cells. Phagocytosis of hepatocyte debris by liver macrophages results in expression of Wnt3a. Wnt3a in close proximity to HPC/OCs activates Wnt pathways, leading to transcriptional activation of Numb (a Notch pathway inhibitor) and progenitor differentiation into hepatocytes.<sup>54</sup> In biliary injury, HPC/OC activation in the form of a ductular reaction is often associated with myofibroblast activation

and extensive collagen deposition, leading to liver fibrosis. Myofibroblasts express Jagged1 that activates Notch receptors in HPC/OCs leading to differentiation towards a biliary lineage.<sup>54</sup> (B) CTGF/CCN2 acts in an autocrine manner to promote HPC/OC activation, ductular reaction and biliary fibrosis by binding to the integrin  $\alpha v \beta 6$  receptor on HPC/OCs and cholangiocytes in DDC-induced murine liver injury.<sup>70</sup> ADAMTS7 protease can be secreted from hepatocytes and myofibroblasts in the HPC/OC niche to regulate HPC/OC activation and biliary fibrosis by CTGF/CCN2 fragmentation.<sup>72</sup>

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**Table 1**

Summary of markers immune-reactive for HPC/OC.

Marker name	Sources	Species	Author and references
Cytokeratin (K)7	Adult biliary marker	Human	Xiao et al. <sup>7</sup>
Cytokeratin (K)19	Adult biliary marker	Human	Lee et al. <sup>8</sup>
Cytokeratin (K)14	Adult biliary marker	Human	Xiao et al. <sup>7</sup>
Epithelial cell adhesion molecule (EPCAM)	Fetal hepatoblast and adult biliary marker	Mouse	Tanaka et al. <sup>9</sup>
		Human	Okabe et al. <sup>10</sup>
		Rat	Dan et al. <sup>11</sup>
			Schmelzer et al. <sup>12</sup> Yovchev et al. <sup>13,14</sup>
Sry-like HMG box protein 9 (Sox9)	Fetal hepatoblast and adult biliary marker	Mouse	Furuyama et al. <sup>15</sup>
Cytokeratin (K) 8	Adult hepatocyte marker	Human	Xiao et al. <sup>7</sup>
Cytokeratin (K) 18	Adult hepatocyte marker	Human	Xiao et al. <sup>7</sup>
c-Met	Adult hepatocyte marker	Human	Xiao et al. <sup>7</sup>
$\alpha$ -fetoprotein protein	Fetal hepatoblast marker	Mouse	Nierhoff et al. <sup>16</sup>
		Rat	Kuhlmann et al. <sup>17</sup>
		Human	Rao et al. <sup>18</sup>
albumin	Adult hepatocyte marker	Mouse	Fausto et al. <sup>19</sup>
		Rat	Kuijk et al. <sup>20</sup>
		Human	Xiao et al. <sup>7</sup>
c-Kit	Fetal hepatoblast and adult hematopoietic marker	Mouse	Petersen et al. <sup>21</sup>
Sca-1	Fetal hepatoblast and adult hematopoietic marker	Mouse	Nierhoff et al. <sup>16</sup>
			Petersen et al. <sup>21</sup>
Thy-1 (CD90)	Adult hematopoietic marker	Rat	Petersen et al. <sup>22</sup>
Prominin/CD133	Adult hematopoietic marker	Mouse	Suzuki et al. <sup>23</sup>
C-X-C chemokine receptor type 4 (CXCR4)	Adult hematopoietic marker	Mouse	Cardinale et al. <sup>24</sup>
Neural cell adhesion molecule (NCAM)	Adult neural cell marker	Human	Cardinale et al. <sup>24</sup>