Acknowledgment We thank Mrs. Mariko Yamada, Ms. Yoko Fukuzumi and Mrs. Isako Fujimoto for rearing the medaka used in this study.

References

Benson SC, Pershadsingh HA, Ho CI, Chittiboyina A, Desai P, Pravenec M, Qi N, Wang J, Avery MA, Kurtz TW (2004) Identification of telmisartan as a unique angiotensin II receptor antagonist with selective PPARgamma-modulating activity. Hypertension 43:993–1002

Brunt EM (2001) Nonalcoholic steatohepatitis: definition and pathology. Semin Liver Dis 21:3–16

Chu YC, Chiu HH (2008) A completely imbedded fish bone presenting as an esophageal tumor-like lesion: an unusual presentation. Gastrointest Endosc 68:1190–1191

Feldstein AE, Canbay A, Guicciardi ME, Higuchi H, Bronk SF, Gores GJ (2003) Diet associated hepatic steatosis sensitizes to Fas mediated liver injury in mice. J Hepatol 39:978–983

Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues.J Biol Chem 226:497–509

Fox ES, Brower JS, Bellezzo JM, Leingang KA (1997) N-acetylcysteine and alpha-tocopherol reverse the inflammatory response in activated rat Kupffer cells. J Immunol 158:5418–5423

Haukeland JW, Damas JK, Konopski Z, Loberg EM, Haaland T, Goverud I, Torjesen PA, Birkeland K, Bjoro K, Aukrust P (2006) Systemic inflammation in nonalcoholic fatty liver disease is characterized by elevated levels of CCL2. J Hepatol 44:1167–1174

Ishibashi M, Hiasa K, Zhao Q, Inoue S, Ohtani K, Kitamoto S, Tsuchihashi M, Sugaya T, Charo IF, Kura S, Tsuzuki T, Ishibashi T, Takeshita A, Egashira K (2004) Critical role of monocyte chemoattractant protein-1 receptor CCR2 on monocytes in hypertension-induced vascular inflammation and remodeling. Circ Res 94:1203–1210

Jin H, Yamamoto N, Uchida K, Terai S, Sakaida I (2007) Telmisartan prevents hepatic fibrosis and enzyme-altered lesions in liver cirrhosis rat induced by a choline-deficient L-amino acid-defined diet. Biochem Biophys Res Commun 364:801–807

Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin IT, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. Nature 447:714–719

Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ (2005) Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 41:1313–1321

Kolak M, Westerbacka J, Velagapudi VR, Wagsater D, Yetukuri L, Makkonen J, Rissanen A, Hakkinen AM, Lindell M, Bergholm R, Hamsten A, Eriksson P, Fisher RM, Oresic M, Yki-Jarvinen H (2007) Adipose tissue inflammation and increased ceramide content characterize subjects with high liver fat content independent of obesity. Diabetes 56:1960–1968

Kudo H, Yata Y, Takahara T, Kawai K, Nakayama Y, Kanayama M, Oya T, Morita S, Sasahara M, Mann DA, Sugiyama T (2009) Telmisartan attenuates progression of steatohepatitis in mice: role of hepatic macrophage infiltration and effects on adipose tissue. Liver Int 29:988–996

Masahito P, Aoki K, Egami N, Ishikawa T, Sugano H (1989) Life-span studies on spontaneous tumor development in the medaka (*Oryzias latipes*). Jpn J Cancer Res 80:1058–1065

Matsumoto T, Terai S, Oishi T, Kuwashiro S, Fujisawa K, Yamamoto N, Fujita Y, Hamamoto Y, Furutani-Seiki M, Nishina H, Sakaida I (2010) Medaka as a model for human nonalcoholic steatohepatitis. Dis Model Mech 3:431–440

Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ (1999) Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology 116:1413–1419

Powell EE, Cooksley WG, Hanson R, Searle J, Halliday JW, Powell LW (1990) The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. Hepatology 11:74–80

Sadler KC, Amsterdam A, Soroka C, Boyer J, Hopkins N (2005) A genetic screen in zebrafish identifies the mutants vps18, nf2 and foie gras as models of liver disease. Development 132:3561–3572

Sakaida I, Kubota M, Kayano K, Takenaka K, Mori K, Okita K (1994) Prevention of fibrosis reduces enzyme-altered lesions in the rat liver. Carcinogenesis 15:2201–2206

Sanyal AJ (2002) AGA technical review on nonalcoholic fatty liver disease. Gastroenterology 123:1705–1725

Schaffner F, Thaler H (1986) Nonalcoholic fatty liver disease. Prog Liver Dis 8:283–298

Schupp M, Clemenz M, Gineste R, Witt H, Janke J, Helleboid S, Hennuyer N, Ruiz P, Unger T, Staels B, Kintscher U (2005) Molecular characterization of new selective peroxisome proliferator-activated receptor gamma modulators with angiotensin receptor blocking activity. Diabetes 54:3442–3452

Tanaka T, Fukunaga Y, Itoh H, Doi K, Yamashita J, Chun TH, Inoue M, Masatsugu K, Saito T, Sawada N, Sakaguchi S, Arai H, Nakao K (2005) Therapeutic potential of thiazolidinediones in activation of peroxisome proliferator-activated receptor gamma for monocyte recruitment and endothelial regeneration. Eur J Pharmacol 508:255–265

Terai S (2010) Fish model leads to new findings in liver disease. Hepatol Res 40:111–113

Watanabe T, Asaka S, Kitagawa D, Saito K, Kurashige R, Sasado T, Morinaga C, Suwa H, Niwa K, Henrich T, Hirose Y, Yasuoka A, Yoda H, Deguchi T, Iwanami N, Kunimatsu S, Osakada M, Loosli F, Quiring R, Carl M, Grabher C, Winkler S, Del Bene F, Wittbrodt J, Abe K, Takahama Y, Takahashi K, Katada T, Nishina H, Kondoh H, Furutani-Seiki M (2004) Mutations affecting liver development and function in medaka, *Oryzias latipes*, screened by multiple criteria. Mech Dev 121:791–802

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr (2003) Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112:1796–1808

Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante AW Jr (2006) CCR2 modulates inflammatory and metabolic effects of high-fat feeding. J Clin Invest 116:115–124

Younossi ZM, Gramlich T, Liu YC, Matteoni C, Petrelli M, Goldblum J, Rybicki L, McCullough AJ (1998) Nonalcoholic fatty liver disease: assessment of variability in pathologic interpretations. Mod Pathol 11:560–565

REVIEW

Stem cell therapy in chronic liver disease

Taro Takami[a], *Shuji Terai*[b], *and Isao Sakaida*[b]

Purpose of review
To provide an overview of the current status of liver regeneration therapies for liver cirrhosis and future prospects.

Recent findings
Various clinical studies for liver disease have been reported, including hepatic administration of autologous CD34-positive cells induced by granulocyte colony-stimulating factor, portal vein administration of CD133-positive mononuclear cells, and administration of autologous bone marrow-derived mesenchymal stem cells. Effectiveness of these approaches has been shown in some patients. We have also reported improved liver fibrosis and function with infusion of autologous bone marrow cells in a basic study with mice, and on the basis of those results started autologous bone marrow cell infusion (ABM*i*) therapy for liver cirrhosis. The efficacy and safety of ABM*i* therapy has also been reported by other institutions.

Summary
Results of recent clinical studies strongly suggest that liver function-improving effects can be achieved using infusion of bone marrow (stem) cells for cirrhosis. New treatment methods using less-invasive bone marrow-derived cultured cells need to be developed.

Keywords
bone marrow, liver cirrhosis, liver regeneration, stem cell

INTRODUCTION

With the development of new antiviral medicines and advances in interferon therapy, viral elimination and cure of hepatitis can be expected even in some patients with chronic viral hepatitis, for which radical cure has been difficult. In many patients, however, liver cirrhosis has already occurred. In cases that have progressed to decompensated liver cirrhosis, the only radical treatment currently available is still liver transplantation. However, liver transplants are not widely performed worldwide due to problems such as chronic donor shortages, surgical invasiveness, risk of immunological rejection, and medical costs. To compensate for this, development of new regenerative therapies for liver cirrhosis is an urgent task. In 2000, Theise *et al.* [1] reported the existence of Y chromosome-positive cells in livers with chronic inflammation in autopsied women who had received therapeutic bone marrow transplantations from male donors, suggesting the existence of pluripotent stem cells among bone marrow cells. Since then, attention has been focused on bone marrow (stem) cells as a cell source for liver regenerative therapies [2–6,7▪▪]. Here, we present an overview of the current status of clinical trials and future prospects for liver regeneration therapies using stem cells, including autologous bone marrow-derived cells.

GRANULOCYTE COLONY-STIMULATING FACTOR

Various cell therapies for liver disease have been developed, including using CD34-positive cells induced with granulocyte colony-stimulating factor (G-CSF). Gordon *et al.* [8] collected CD34-positive cells from peripheral blood after induction with G-CSF, then administered these cells via a hepatic artery, and reported improved serum levels of bilirubin and albumin in some patients, despite a short

[a]Division of Laboratory, Yamaguchi University Hospital, Yamaguchi University School of Medicine and [b]Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan

Correspondence to Isao Sakaida, MD, PhD, Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Minami-kogushi 1-1-1, Ube, Yamaguchi 755-8505, Japan. Tel: +81 836 22 2238; fax: +81 836 22 2240; e-mail: sakaida@yamaguchi-u.ac.jp

Curr Opin Gastroenterol 2012, 28:203–208
DOI:10.1097/MOG.0b013e3283521d6a

KEY POINTS

- Previous clinical studies using autologous bone marrow cells suggest the efficacy and safety of this approach for advanced liver disease.
- Randomized controlled studies are needed to establish genuine efficacy because most of the published results have come from uncontrolled studies.
- New treatment methods using less-invasive bone marrow-derived cultured cell fractions showing treatment effects on liver fibrosis and functions need to be developed.

observation period of only 60 days. In addition, Spahr *et al.* [9] administered G-CSF to patients with alcoholic liver cirrhosis and reported increased proliferation of hepatic progenitor cells (HPCs), whereas Pai *et al.* [10] reported improvements in serum albumin, Child–Pugh score, and accumulation of ascites with in-vitro expansion of CD34-positive cells induced by G-CSF and administration via a hepatic artery in patients with alcoholic liver cirrhosis. A study by Han *et al.* [11] described G-CSF administration for hepatitis B virus (HBV)-related decompensated liver cirrhosis. In this study, administration of peripheral blood mononuclear cells induced with G-CSF showed significantly improved effects on serum albumin and Child–Pugh score at 6 months after treatment, compared with those in patients with only G-CSF mobilization for 4 days. However, rupture of the spleen during peripheral blood stem-cell mobilization by administration of G-CSF has been reported even in healthy individuals [12], and caution is needed in the administration of G-CSF to liver cirrhosis patients with splenomegaly.

NONCULTURED AUTOLOGOUS BONE MARROW CELLS

In our previous animal studies, we have developed an in-vivo mouse model [the green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model], and reported that GFP-positive bone marrow cells infused via a tail vein efficiently repopulated cirrhotic liver under conditions of persistent liver damage induced by CCl_4 [13]. In these processes, elevation in serum albumin levels [13], a significant increase in survival rate, and reduced liver fibrosis assessed by Sirius red staining were seen following infusion of GFP-positive bone marrow cells [14]. Infused bone marrow-derived GFP-positive cells were also confirmed to produce collagenases including matrix metalloproteinase (MMP) 2 and MMP-9 during these processes [14]. On the basis of the above basic study, infusion of autologous bone marrow cells via a peripheral vein in a chronic liver injury environment is thought to improve liver functions and reduce liver fibrosis, and to significantly improve vital prognosis in recipients. Our clinical study of autologous bone marrow cell infusion (ABM*i*) therapy for decompensated liver cirrhosis was therefore started in November 2003 [15]. In addition, a multicenter clinical trial of liver regeneration with cell transplantation was started in 2005. With that ABM*i* therapy, around 400 ml of autologous bone marrow cells was collected under general anesthesia, and the collected bone marrow fluid was concentrated and washed. Bone marrow mononuclear cells in that fluid were then purified and condensed according to standard operating procedures (SOP) at the regenerative and cell therapy center fully equipped with good manufacturing practice (GMP) grade facilities, and administered by drip infusion via a peripheral vein to the same patient. The course was observed for 6 months after ABM*i*, and efficacy and safety were evaluated using blood biochemistry tests, liver biopsy, abdominal ultrasonography, and abdominal computed tomography and so on. During the observation period, no changes in oral medications, antiviral drugs, or other agents were seen [15]. As a result, serum albumin levels, total protein levels, and Child–Pugh score at 6 months after ABM*i* were significantly improved in patients for whom the course could be observed for 6 months after ABM*i* [15]. Similar improvements were also seen in nine patients for whom the course could be observed for 15 months [16]. In 2011, Kim *et al.* [17▪] reported that ABM*i* improved serum albumin levels, Child–Pugh score, liver volume measured by MRI and accumulation of ascites in patients with HBV-related decompensated liver cirrhosis, and results from liver biopsies taken over time suggested the possibility of HPC activation as the underlying mechanism. All 10 patients who received ABM*i* also showed an improvement in quality of life, with no serious adverse events. Moreover, Saito *et al.* [18▪] recently reported the efficacy and safety of ABM*i* therapy for patients with alcoholic liver cirrhosis. Such results are gradually confirming the safety and efficacy of ABM*i* therapy. Therefore, we believe that ABM*i* therapy represents a promising treatment for advanced liver cirrhosis (Fig. 1).

Peng *et al.* [19▪▪] recruited 527 patients with HBV-related decompensated liver cirrhosis, divided into a group of 53 patients who received 120 ml of autologous bone marrow fluid via a hepatic artery and 105 patients who did not. Results of analysis showed no adverse effects from bone marrow administration.

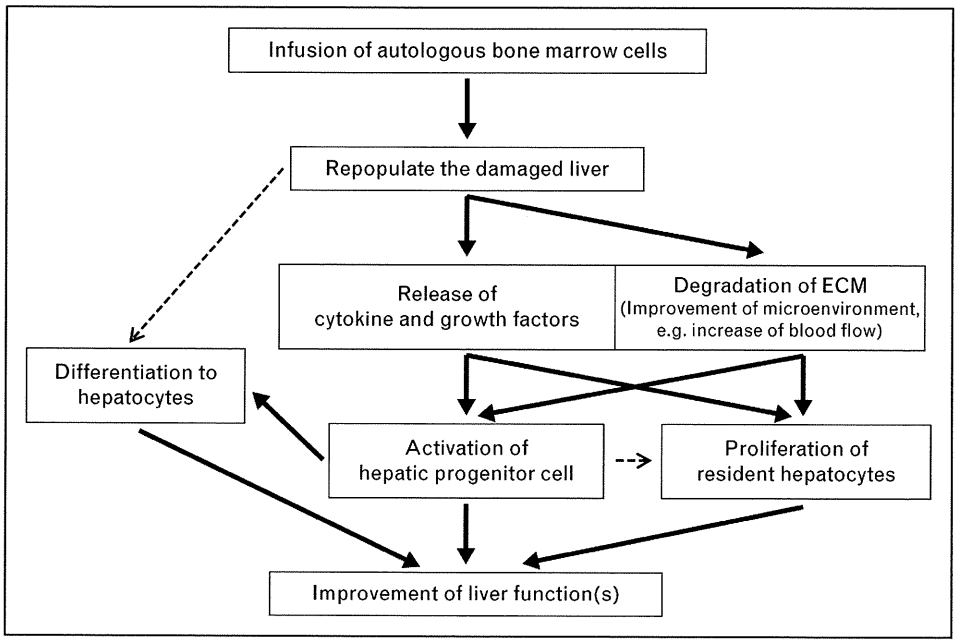

FIGURE 1. Possible mechanism(s) of autologous bone marrow cell infusion therapy. ECM, extracellular matrix.

Patients were divided into a short-term observation group (1–48 weeks) and a long-term observation group (until 192 weeks), and the results of analysis showed improved hepatic function in the early period. Long-term observation showed no change in the incidence of hepatocellular carcinoma (HCC) after the administration of bone marrow cells, suggesting the possibility of an improved survival rate [19▪▪]. Other studies to date on cell therapies using bone marrow (stem) cells for liver cirrhosis include not only those on our ABM*i* therapy [15,16] but also studies by Lyra *et al.* [20,21▪] on the effectiveness of infusion of bone marrow stem cells. Clinical studies by Lyra *et al.* in Brazil suggested the feasibility and safety of ABM*i* through a hepatic artery rather than a peripheral vein for chronic liver disease patients awaiting liver transplantation [20,21▪]. In other clinical studies, increased volumes of left lateral hepatic segments were reported by am Esch *et al.* [22,23] with intraportal administration of CD133-positive bone marrow cells after portal venous embolization of right liver segments. Conversely, death due to radiocontrast nephropathy has been reported as a result of infusion of concentrated CD34-positive cells from 200 ml of bone marrow fluid through a hepatic artery into patients with decompensated liver cirrhosis, and that clinical study was discontinued [24]. This indicates the need for clarification of the treatment indication criteria and full investigation of administration routes, cell concentrations and speed of drip infusion. The need for development of less-invasive liver regeneration therapies using cultured autologous bone marrow-derived cells is also suggested.

CULTURED AUTOLOGOUS BONE MARROW-DERIVED CELLS

Our ABM*i* therapy involves bone marrow aspiration under general anesthesia, and is not indicated for patients for whom general anesthesia is difficult. We therefore aimed to develop a new liver regeneration therapy in which cells having a curative effect on liver cirrhosis are isolated and cultured from a small amount of autologous bone marrow aspirated under local anesthesia and infused back into the same patient. The bone marrow contains two major sources of stem cells, hematopoietic stem cells (HSCs) and mesenchymal stem (stromal) cells (MSCs). Previous basic studies suggested the therapeutic effect on advanced liver diseases using these cell types in animal models. Therefore, issues that need to be investigated in the future will include identification of cells showing treatment effects from bone marrow cell fractions, and clarification of the mechanisms underpinning such actions. When cells with liver regeneration and restorative activity can be isolated from small amounts of bone marrow fluid, cultured, and then readministered, the indications would be able to be expanded, as collection of bone marrow fluid would no longer need to be performed under general anesthesia. However, safety evaluation guidelines for cultured cells are needed when autologous cultured bone marrow-derived cells

Table 1. Previous clinical trials in liver disease using autologous bone marrow (stem) cells

Type of infused cells	Number of infused cells	Improvement after the infusion	Number and etiology of patients	Reference
$CD34^+$ cells from G-CSF mobilized peripheral blood	1×10^6–2×10^8	Improved serum albumin and serum bilirubin	Five alcohol	[8]
Only G-CSF mobilization for 5 days	N/A	Elevated serum HGF. Increased hepatic $Ki67^+$/$cytokeratin7^+$ cells	24 alcohol, (11/24 ctrl.)	[9]
Cultured $CD34^+$ cells from G-CSF mobilized peripheral blood	2.3×10^8 (mean)	Improved serum bilirubin and CP	Nine alcohol	[10]
PBMCs from G-CSF mobilized peripheral blood	10^7–10^8/kg	Improved serum albumin and CP	40 HBV (20/40 ctrl., only G-CSF)	[11]
BMNCs from iliac crest (400 ml); ABM*i* therapy	$5.20 \pm 0.63 \times 10^9$	Improved serum albumin and CP	Five HCV, three HBV, one unknown	[15]
BMNCs from iliac crest (500–750 ml) ; ABM*i* therapy	0.48–1.48×10^8/kg	Activation of HPC ($cytokeratin7^+$cells). Increased liver volume	10 HBV	[17▪]
		Improved CP		
BMNCs from iliac crest (400 ml); ABM*i* therapy	$8.0 \pm 7.3 \times 10^9$	Improved serum albumin, PT, and CP	10 alcohol (5/10 ctrl.)	[18▪]
BMNCs (MSCs) from iliac crest (120 ml)	Not described	Improved serum albumin, bilirubin, PT, and MELD in the early period	158 HBV (105/158 ctrl.)	[19▪▪]
		No difference of HCC development through 48 weeks		
BMNCs from iliac crest (maximum 50 ml)	At least 1×10^8	Improved serum albumin and bilirubin	10	[20]
BMNCs from iliac crest (maximum 50 ml)	$3.78 \pm 2.69 \times 10^8$	Improved serum albumin, bilirubin, and CP for 60–90 days	30 (15/30 ctrl.)	[21▪]
Bone marrow-derived $CD133^+$ cells (60–220 ml)	2.4–12.3×10^6	Increased liver volume after liver resection	Six bearing liver tumors (3/6 ctrl.)	[22]
Bone marrow-derived $CD34^+$ cells from iliac crest (200 ml)	5.25×10^6 (mean) ($CD34^+$, 90.5%)	One case developed radiocontrast nephropathy and then died	One HBV, one PBC	[24]
			One AIH, one cryptogenic	
Cultured bone marrow-derived MSCs (80–100 ml)	31.7×10^6 (mean)	Improved MELD (two of four patients)	Three cryptogenic, one AIH	[28]
Cultured bone marrow-derived MSCs (20 ml)	3–5×10^7	Improved MELD, serum creatinine, prothrombin complex	Four HBV, two unknown	[29]
			One HCV	
Cultured bone marrow-derived MSCs stimulated to hepatic lineage using HGF-containing medium (95 ± 25 ml)	2×10^7 hepatic lineage-committed cells in a total of 2×10^8 cells	Improved MELD and CP	40 HCV (20/40 ctrl.)	[30▪]

ABM*i*, autologous bone marrow cell infusion; AIH, autoimmune hepatitis; BMNC, bone marrow mononuclear cell; CP, Child–Pugh score; ctrl., control; G-CSF, granulocyte colony-stimulating factor; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HGF; hepatocytes growth factor; HPC, hepatic progenitor cell; MELD, model for end stage liver disease; MSC, mesenchymal stem cell; N/A, not applicable; PBMC, peripheral blood mononuclear cell; PT, prothrombin time.

are used in liver cirrhotic patients, and a system conforming to SOP at a GMP-grade cell-processing center will be also essential.

Some clinical phase I trials using MSCs have been reported in patients with myocardial infarction [25], amyotrophic lateral sclerosis [26] and Crohn's disease [27]. We can check the current status of clinical trials in this database (*ClinicalTrials.gov*; http://www.clinicaltrials.gov/). About advanced liver disease, Mohamadnejad *et al.* [28] have shown improvements of the model for end-stage liver disease (MELD) score in some patients with peripheral vein administration of cultured MSCs in autologous bone marrow cells. Kharaziha *et al.* [29] also reported that liver function assessed by the MELD score decreased significantly from 17.9 ± 5.6 to 10.7 ± 6.3 after administration of cultured autologous MSCs in four hepatitis B, one hepatitis C, one alcoholic, and two cryptogenic cirrhotic patients. They aspirated around 20 ml of autologous bone marrow fluid from both posterior superior iliac spines under local anesthesia. The mononuclear cells were separated by the Ficoll separation method. Separated bone marrow mononuclear cells were cultured for 2 weeks, and then were collected. They infused about 3×10^7–5×10^7 cells expressing CD44, CD73, and CD105, consistent with MSCs characteristics to the same patient via a portal vein or peripheral vein. Moreover, recently, the clinical trial for patients with hepatitis C virus (HCV)-related liver failure has been reported by Amer *et al.* [30▪]. They also aspirated around 120 ml of autologous bone marrow fluid from the posterior superior iliac crest under local anesthesia, and then injected autologous cultured bone marrow-derived MSCs, which had been stimulated to hepatic lineage using hepatocytes growth factor (HGF) containing medium, into spleen or liver directly using a needle gauge 18 under abdominal ultrasonography guidance. In these patients, the MELD score and Child score were significantly lower than those in the control group from 2 weeks to 6 months after the injection. No difference between intrasplenic route and intrahepatic route was observed.

CONCLUSION

Previous clinical studies using autologous bone marrow cells for advanced liver disease suggested the efficacy and safety (Table 1). However, randomized controlled studies are needed to clearly show the efficacy and safety of liver regeneration therapies using autologous bone marrow (stem) cells or cultured bone marrow-derived cells. And new treatment methods using less-invasive bone marrow-derived cultured cells need to be developed.

Acknowledgements

This study was supported by Grants-in-Aid for scientific research from the Japan Society for the Promotion of Science (JSPS); Ministry of Health, Labour and Welfare, health and labour sciences research grants; and Japan Science and Technology Agency (JST), the project of realization of regenerative medicine to Sakaida I.

Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ▪ of special interest
- ▪▪ of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 291).

1. Theise ND, Nimmakayalu M, Gardner R, *et al.* Liver from bone marrow in humans. Hepatology 2000; 32:11–16.
2. Houlihan DD, Newsome PN. Critical review of clinical trials of bone marrow stem cells in liver disease. Gastroenterology 2008; 135:438–450.
3. Souza BS, Nogueira RC, de Oliveira SA, *et al.* Current status of stem cell therapy for liver diseases. Cell Transplant 2009; 18:1261–1279.
4. Bird TG, Lorenzini S, Forbes SJ. Activation of stem cells in hepatic diseases. Cell Tissue Res 2008; 331:283–300.
5. Almeida-Porada G, Zanjani ED, Porada CD. Bone marrow stem cells and liver regeneration. Exp Hematol 2010; 38:574–580.
6. Muraca M. Evolving concepts in cell therapy of liver disease and current clinical perspectives. Dig Liver Dis 2011; 43:180–187.
7. ▪▪ Stutchfield BM, Forbes SJ, Wigmore SJ. Prospects for stem cell transplantation in the treatment of hepatic disease. Liver Transpl 2010; 16:827–836.

This article gives a general overview about the clinical trials of liver regeneration therapy using autologous stem cells.

8. Gordon MY, Levicar N, Pai M, *et al.* Characterization and clinical application of human CD34+ stem/progenitor cell populations mobilized into the blood by granulocyte colony-stimulating factor. Stem Cells 2006; 24:1822–1830.
9. Spahr L, Lambert JF, Rubbia-Brandt L, *et al.* Granulocyte-colony stimulating factor induces proliferation of hepatic progenitors in alcoholic steatohepatitis: a randomized trial. Hepatology 2008; 48:221–229.
10. Pai M, Zacharoulis D, Milicevic MN, *et al.* Autologous infusion of expanded mobilized adult bone marrow-derived CD34+ cells into patients with alcoholic liver cirrhosis. Am J Gastroenterol 2008; 103:1952–1958.
11. Han Y, Yan L, Han G, *et al.* Controlled trials in hepatitis B virus-related decompensate liver cirrhosis: peripheral blood monocyte transplant versus granulocyte-colony-stimulating factor mobilization therapy. Cytotherapy 2008; 10:390–396.
12. Falzetti F, Aversa F, Minelli O, Tabilio A. Spontaneous rupture of spleen during peripheral blood stem-cell mobilisation in a healthy donor. Lancet 1999; 353:555.
13. Terai S, Sakaida I, Yamamoto N, *et al.* An in vivo model for monitoring the transdifferentiation of bone marrow cells into functional hepatocytes. J Biochem 2003; 134:551–558.
14. Sakaida I, Terai S, Yamamoto N, *et al.* Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice. Hepatology 2004; 40:1304–1311.
15. Terai S, Ishikawa T, Omori K, *et al.* Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. Stem Cells 2006; 24:2292–2298.
16. Terai S, Sakaida I. Current status of autologous bone marrow cell infusion therapy for liver cirrhosis patients. Hepatol Res 2008; 38:S72–S75.
17. ▪ Kim JK, Park YN, Kim JS, *et al.* Autologous bone marrow infusion activates the progenitor cell compartment in patients with advanced liver cirrhosis. Cell Transplant 2010; 19:1237–1246.

This clinical study strongly suggested the efficacy and safety of liver regeneration therapy by administration of autologous bone marrow cells for patients with HBV-related liver cirrhosis.

18. ▪ Saito T, Okumoto K, Haga H, *et al.* Potential therapeutic application of intravenous autologous bone marrow infusion in patients with alcoholic liver cirrhosis. Stem Cells Dev 2011; 20:1503–1510.

This clinical study also strongly suggested the efficacy and safety of liver regeneration therapy by administration of autologous bone marrow cells for patients with alcohol-related liver cirrhosis.

19. Peng L, Xie DY, Lin BL, *et al.* Autologous bone marrow mesenchymal stem cell
■■ transplantation in liver failure patients caused by hepatitis B: short-term and long-term outcomes. Hepatology 2011; 54:820–828.

In this clinical trial with long-term observation, 527 patients with HBV-related decompensated liver cirrhosis were recruited. And improved hepatic function in the early period and lower tendency of HCC-development in the long period were observed.

20. Lyra AC, Soares MB, da Silva LF, *et al.* Feasibility and safety of autologous bone marrow mononuclear cell transplantation in patients with advanced chronic liver disease. World J Gastroenterol 2007; 13:1067–1073.
21. Lyra AC, Soares MB, da Silva LF, *et al.* Infusion of autologous bone marrow
■ mononuclear cells through hepatic artery results in a short-term improvement of liver function in patients with chronic liver disease: a pilot randomized controlled study. Eur J Gastroenterol Hepatol 2010; 22:33–42.

This pilot clinical study also suggested the efficacy of liver regeneration therapy using noncultured autologous bone marrow cells for cirrhotic patients.

22. am Esch JS 2nd, Knoefel WT, Klein M, *et al.* Portal application of autologous CD133+ bone marrow cells to the liver: a novel concept to support hepatic regeneration. Stem Cells 2005; 23:463–470.
23. Furst G, Schulte am Esch J, Poll LW, *et al.* Portal vein embolization and autologous CD133+ bone marrow stem cells for liver regeneration: initial experience. Radiology 2007; 243:171–179.
24. Mohamadnejad M, Namiri M, Bagheri M, *et al.* Phase 1 human trial of autologous bone marrow-hematopoietic stem cell transplantation in patients with decompensated cirrhosis. World J Gastroenterol 2007; 13:3359–3363.
25. Lasala GP, Silva JA, Kusnick BA, *et al.* Combination stem cell therapy for the treatment of medically refractory coronary ischemia: a phase I study. Cardiovasc Revasc Med 2011; 12:29–34.
26. Mazzini L, Ferrero I, Luparello V, *et al.* Mesenchymal stem cell transplantation in amyotrophic lateral sclerosis: a phase I clinical trial. Exp Neurol 2010; 223:229–237.
27. Duijvestein M, Vos AC, Roelofs H, *et al.* Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut 2010; 59:1662–1669.
28. Mohamadnejad M, Alimoghaddam K, Mohyeddin-Bonab M, *et al.* Phase 1 trial of autologous bone marrow mesenchymal stem cell transplantation in patients with decompensated liver cirrhosis. Arch Iran Med 2007; 10:459–466.
29. Kharaziha P, Hellstrom PM, Noorinayer B, *et al.* Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I-II clinical trial. Eur J Gastroenterol Hepatol 2009; 21:1199–1205.
30. Amer ME, El-Sayed SZ, El-Kheir WA, *et al.* Clinical and laboratory evaluation
■ of patients with end-stage liver cell failure injected with bone marrow-derived hepatocyte-like cells. Eur J Gastroenterol Hepatol 2011; 23:936–941.

In this randomized controlled study, authors injected autologous cultured bone marrow-derived cells (stimulated to hepatic lineage using HGF) into spleen or liver under ultrasonography guidance for liver regeneration in HCV-related liver cirrhotic patients.

J. Biochem. 2011;149(3):231–239 doi:10.1093/jb/mvr001

JB THE JOURNAL OF BIOCHEMISTRY

JB Review

Liver stem/progenitor cells: their characteristics and regulatory mechanisms

Received November 16, 2010; accepted December 13, 2010; published online January 8, 2011

Minoru Tanaka[1], Tohru Itoh[1], Naoki Tanimizu[2] and Atsushi Miyajima[1,*]

[1]Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032 and [2]Cancer Research Institute, Sapporo Medical University School of Medicine, S-1, W-17, Chuo-ku, Sapporo 060-8556, Japan

*Atsushi Miyajima, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. Tel: +81 3 5841 7884, Fax: +81 3 5841 8475, email: miyajima@iam.u-tokyo.ac.jp

Liver stem cells give rise to both hepatocytes and bile duct epithelial cells also known as cholangiocytes. During liver development hepatoblasts emerge from the foregut endoderm and give rise to both cell types. Colony-forming cells are present in the liver primordium and clonally expanded cells differentiate into either hepatocytes or cholangiocytes depending on culture conditions, showing stem cell characteristics. The growth and differentiation of hepatoblasts are regulated by various extrinsic signals. For example, periportal mesenchymal cells provide a cue for bipotential hepatoblasts to become cholangiocytes, and mesothelial cells covering the parenchyma support the expansion of foetal hepatocytes by producing growth factors. The adult liver has an extraordinary capacity to regenerate, and after 70% hepatectomy the liver recovers its original mass by replication of the remaining hepatocytes without the activation of liver stem cells. However, in certain types of liver injury models, liver stem/progenitor-like cells, known as oval cells in rodents, proliferate around the portal vein, while the roles of such cells in liver regeneration remain a matter of debate. Clonogenic and bipotential cells are also present in the normal adult liver. In this minireview we describe recent studies on liver stem/progenitor cells by focusing on extracellular signals.

Keywords: cytokine/development/differentiation/hepatocyte/regeneration.

Abbreviations: 2-AAF, 2-acetylaminofluoren; AFP, alpha-fetoprotein; ALB, albumin; BMEL, bipotential mouse embryonic liver cell; CDE, choline-deficient, ethionine-supplemented; DDC, 3,5-diethoxycarbonyl-1,4-dihydro-collidine; Dlk, Delta-like protein 1; DPPIV, dipeptidyl peptidase IV; EpCAM, epithelial cell adhesion molecule; FGF, fibroblast growth factor; H-CFU-C, hepatic colony-forming unit in culture; MC, mesothelial cell; OSM, oncostatin M; PH, partial hepatectomy; STM, septum transversum mesenchyme; TNF, tumour necrosis factor; Wt1, Wilms' tumour 1.

The liver is a central organ for homeostasis owing to its numerous functions, including carbohydrate metabolism, glycogen storage, biosynthesis of various biochemical components including amino acids and nucleotides, lipid metabolism, urea synthesis, drug detoxification, production of plasma proteins and hormones, and destruction of erythrocytes. Because the liver is such an essential organ, liver diseases are often fatal. Liver insults such as hepatitis viruses, drugs, alcohol and genetic, metabolic and immune disorders can lead to steatosis, hepatitis, fibrosis, cirrhosis and cancer and liver disease is a major cause of death. The liver is also known as a unique organ that can regenerate, making it possible to transplant the liver from a living donor. However, the molecular mechanisms underlying organogenesis, maintenance, pathogenesis and regeneration of the liver are not well understood. As the liver is a large organ with a variety of functions, it has been used for many decades as a source to purify numerous enzymes for biochemical studies. By contrast, much progress has been made relatively recently in the characterization of each type of liver cell and analysis of their interactions. Those studies have been facilitated by new technologies such as genomics, mouse mutants and the development of various tools to isolate the cells of interest. In this review, we describe recent studies on liver stem/progenitor cells together with the environments that support their proliferation and differentiation during development and pathogenesis.

Liver architecture and liver stem cells

The liver is divided into lobules and each lobule consists of plates of hepatocytes lined by sinusoidal capillaries that radiate towards a central efferent vein (Fig. 1). Liver lobules are hexagonal and at each of six corners there is a portal triad of vessels consisting of a portal vein, hepatic artery and bile duct. Sinusoids are composed of liver-specific capillaries with fenestrated endothelial cells, hepatic stellate cells (Ito cells), liver-resident macrophages (Kupffer cells) and large granular lymphocytes (pit cells). The liver has a dual blood supply, namely, via the portal vein and the hepatic artery. The portal vein delivers the venous blood flowing from the intestines, pancreas and spleen. The hepatic artery supplies oxygen to the liver. The blood flows from a portal triad through a sinusoidal capillary to a central efferent vein. Hepatocytes are major parenchymal cells carrying out most of the metabolic functions and account for ~60% of the total liver cell population and 80% of the volume of the organ. Hepatocytes are highly polarized epithelial cells and

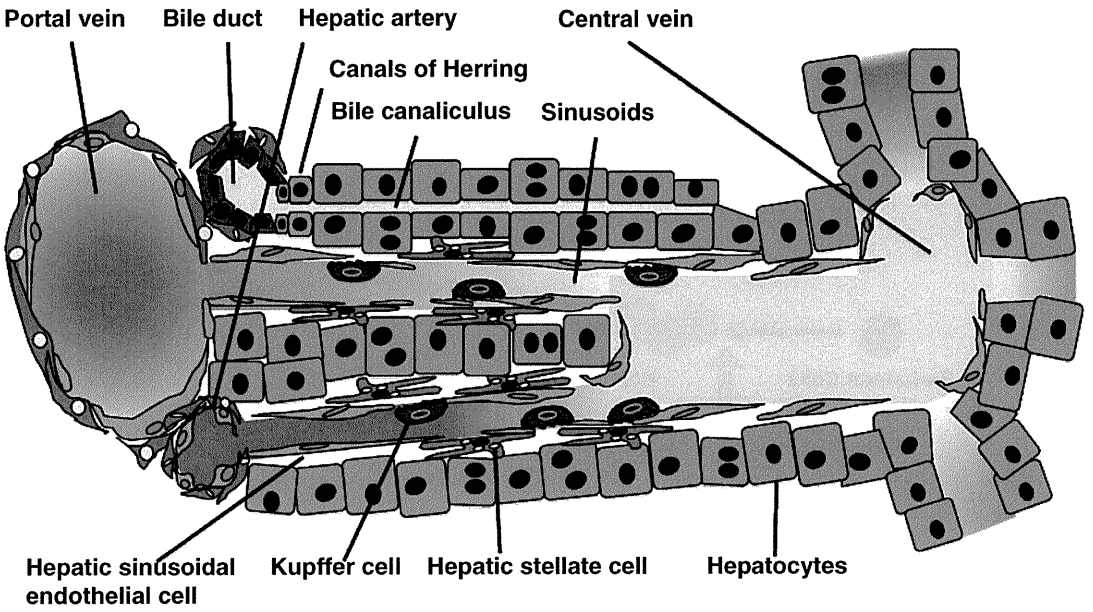

Fig. 1 Liver architecture. In the liver, blood flows from portal blood vessels through sinusoids to central efferent veins. Sinusoids are liver-specific capillaries with fenestrated endothelial cells, hepatic stellate cells (Ito cells) and blood cells such as liver-resident macrophages (Kupffer cells). Hepatocytes are highly polarized epithelial cells forming cords, and plates of hepatocytes are lined by sinusoidal capillaries that radiate towards a central efferent vein. Tight junctions formed between hepatocytes create a canaliculus that surrounds each hepatocyte. Bile salts produced in hepatocytes are excreted into canaliculi that are linked to bile ducts. The region that connects the bile canaliculus and the biliary tree is called 'canals of Hering'.

form cords. Their basolateral surfaces face fenestrated sinusoid endothelial cells, facilitating the transfer of materials between hepatocytes and blood flows. Tight junctions formed between hepatocytes create a canaliculus that surrounds each hepatocyte. Bile salts produced in hepatocytes are excreted into canaliculi that are linked to bile ducts at the portal triad. Bile ducts are formed by a specialized type of epithelial cell called a biliary epithelial cell or a cholangiocyte.

In general, stem cells are characterized by their ability to self-renew and differentiate to multiple lineages. As hepatocytes and cholangiocytes, the two types of liver epithelial cells, are derived from a common origin during organogenesis, those cells with the potential to proliferate and give rise to both types of liver epithelial cells are considered to be liver stem cells. Although there are many reports describing liver stem cells, the definitions of stem cells are rather vague in many of them. As it is not an easy task to distinguish stem cells from progenitors because of the difficulty of proving the unlimited self-renewal activity of stem cells in many situations, we use the term stem/progenitor cells to describe such cells in this review article.

The onset of liver development

Liver organogenesis begins at embryonic day (E) 8.5 in the mouse from the foregut endoderm. The ventral wall of the foregut endoderm faces the developing heart by approximately E8 and receives inductive signals for hepatic fate, such as fibroblast growth factor (FGF) from the heart (*1–3*) and bone morphogenetic protein from the septum transversum mesenchyme (STM) (*4*). Wnt2b is expressed in the lateral plate mesoderm adjacent to the endoderm destined to be the liver and is essential for the onset of liver development in zebrafish (*5*). By these signals, hepatoblasts emerge from the foregut endoderm and migrate as cords into the surrounding STM (*6*, *7*). Analysis of Flk1-deficient mouse embryos revealed that $Flk1^+$ endothelial cells are required for proliferation of hepatoblasts (*8*). Because hepatoblasts proliferate and give rise to both hepatocytes and cholangiocytes as described below, they are considered to be embryonic liver stem/progenitor cells.

Identification and characterization of hepatoblasts

As cell sorting using antibodies is a powerful means to isolate and characterize a specific cell type, efforts have been made to search for specific cell surface antigens on hepatoblasts (Fig. 2). Kubota and Reid (*9*) showed that the $RT1A1^-$ $OX18^{low}$ $ICAM\text{-}1^+$ fraction of E13 rat foetal liver contained hepatoblasts. Suzuki *et al.* (*10*) developed a single cell-based assay designated the hepatic colony-forming unit in culture (H-CFU-C) and showed that the $CD45^-$ $TER119^-$ $c\text{-}Kit^-$ $CD29^+$ $CD49f^+$ and $CD45^-$ $TER119^-$ $c\text{-}Kit^-$ $c\text{-}Met^+$ $CD49f^{+/low}$ fraction of E13.5 mouse liver contained hepatic progenitor/stem cells. They also showed that $CD45^-$ $TER119^-$ $c\text{-}Kit^-$ $c\text{-}Met^+$ $CD49f^{+/low}$ cells of E11.5 mouse liver had high H-CFU-C potential and that clonally expanding cells reconstituted the liver, pancreas and intestine *in vivo*. On the other hand, Minguet *et al.* (*11*) reported that $CD45^-$ $TER119^-$ $c\text{-}Kit^{low}$ cells in E11 mouse liver contained the earliest hepatic progenitors, also displaying features of

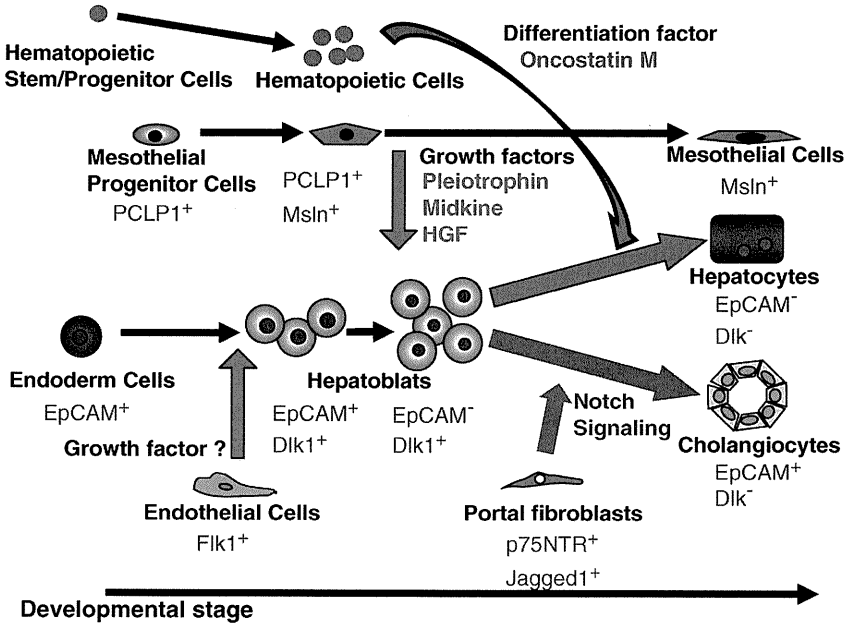

Fig. 2 Development of liver cells and expression of cell surface markers. The EpCAM$^+$DLK1$^+$ hepatoblasts emerge from EpCAM$^+$ foregut endoderm cells and form liver primordium. Then, the hepatoblasts dramatically reduce the expression of EpCAM. EpCAM is upregulated again in biliary epithelial cell precursor cells around the portal vein, where p75NTR$^+$ Jagged1$^+$ portal fibroblasts interacted with hepatoblasts. PCLP1$^+$ mesothelial progenitor cells produce growth factors for hepatoblasts to proliferate. OSM secreted by haematopoietic cells induces hepatocytic differentiation of hepatoblasts.

liver-repopulating stem cells. Delta-like protein 1 (Dlk1), also known as Pref-1, was strongly expressed in liver buds as early as E10.5 in mice. Dlk1$^+$ cells isolated from E14.5 livers expressed albumin (ALB) and formed colonies composed of the hepatocyte and cholangiocyte lineages in the presence of hepatocyte growth factor and epidermal growth factor, indicating that liver stem cell activity is present in this population (*12*). As in mouse foetal liver, Dlk1 is also expressed strongly in human foetal liver (*13*, *14*). Nierhoff *et al.* showed that murine foetal liver alpha-fetoprotein (AFP)$^+$/ALB$^+$ cells were positive for Dlk1 and E-cadherin and that purified E-cadherin$^+$ epithelial cells formed clusters in cell culture and differentiated along the hepatocytic lineage. Interestingly, AFP$^+$/E-cadherin$^+$ epithelial cells were Sca-1$^+$, but showed no expression of c-Kit. In order to examine their *in vivo* capacity, wild-type E12.5 mouse liver epithelial cells were transplanted into adult dipeptidyl peptidase IV (DPPIV) knockout mice, and DPPIV expression was used as a marker to discriminate the donor from recipient cells. This resulted in incorporation of the DPPIV$^+$ donor-derived cells into the hepatic parenchymal cords of the recipient liver, showing a repopulation and differentiation capacity of the E12.5 E-cadherin$^+$ cells (*15*).

Epithelial cell adhesion molecule (EpCAM) is expressed in HNF4α^+ hepatoblasts of liver buds as early as E9.5 in mice (Fig. 2). Colony-forming assays using sorted E11.5 liver cells revealed that the EpCAM$^+$ Dlk1$^+$ cell population contained *in vitro* colony-forming cells, indicating that liver stem cell activity is present in this population. EpCAM expression declined by E13.5 in mouse liver, while Dlk1 expression was sustained by E16.5 (*16*). In humans, Dan *et al.* (*17*) reported that multipotent progenitor cells derived from human foetal liver expressed EpCAM,and Schmelzer *et al.* (*18*) reported that pluripotent precursors of hepatoblasts expressed EpCAM and were located in ductal plates in human foetal liver. CD13 (aminopeptidase N) was detected on the cells of the Dlk1$^+$ hepatic stem/progenitor fraction. Colony formation assays revealed that hepatic stem/progenitor cells were enriched in the CD13$^+$ fraction, compared with the Dlk1$^+$ fraction, of non-haematopoietic cells in foetal liver (*19*).

Characteristics of foetal liver stem/progenitor cells

Dlk1$^+$ cells contain some clonogenic cells named hepatic progenitor proliferating on laminin that continuously proliferate on laminin-coated plates and differentiate to both hepatocytes and cholangiocytes depending on culture conditions, suggesting that they are liver stem cells (*20*). Bipotential cell lines, referred to as bipotential mouse embryonic liver cell (BMEL), were also obtained after a long latency in culture of foetal liver cells and they were shown to give rise to both hepatocytes and cholangiocytes in recipient mice, although the origin of BMEL was unknown (*21*). These cell lines are used to study the mechanisms of hepatocytic and/or cholangiocytic differentiation from liver stem cells.

In the past decade, a number of cell surface markers for foetal liver cells have been found and used to prospectively isolate and to localize them in the liver. While some studies used transplantation assays to

investigate the repopulation capacity, the ability to form a colony and differentiate to both lineages *in vitro* is a practical criterion to evaluate hepatoblasts in most of these studies. In the case of haematopoietic stem cells, a single purified stem cell can be shown to propagate and give rise to all kinds of haematopoietic cells for the long term in an irradiated recipient mouse, providing clear evidence for stemness *in vivo*, that is self-renewal ability and multi-lineage differentiation. By contrast, as liver repopulation assays require a large number of cells to be transplanted to demonstrate engraftment capacity, rigorous proof of stemness *in vivo* is difficult. Nonetheless, there is little doubt that hepatoblasts possess capacities of liver stem cells on the basis of numerous previous works as described above (*9–12*, *15–19*).

Differentiation of hepatoblasts to cholangiocytes

Bile ducts are formed only around the portal vein, suggesting that regionally specific signals induce cholangiocytes from hepatoblasts. Indeed, two signalling pathways, TGFβ/Activin and Notch, are specifically activated in hepatoblasts near the portal vein. TGFβ2 and TGFβ3 are predominantly expressed in the portal region (*22*), and the Onecut family of transcription factors, HNF6 (OC-1) and OC-2, promote expression of α2-macroglobulin and follistatin, inhibitors of the TGFβ/Activin pathway, in the parenchymal region (*23*). $Dlk1^+$ hepatoblasts express Notch2, whereas $p75NTR^+$ periportal fibroblasts express Jagged-1 (*24*). Forced expression of Notch intracellular domain in $Dlk1^+$ hepatoblasts resulted in differentiation to cholangiocytes (*25*). These results strongly suggest that cholangiocyte differentiation is induced by Notch signalling in the periportal region. Although differentiation of hepatoblasts to cholangiocytes by TGFβ and Notch signalling occurs in mid-gestation, surprisingly, hepatocytes turned to cholangiocytes and formed ectopic duct structures in the parenchyma by Notch activation after birth (*26*). These results indicate that not only hepatoblasts but also hepatocytes are competent to differentiate to cholangiocytes at least by the neonatal period (Fig. 2).

Immature cholangiocytes form a ductal plate, a single cell layer, around the portal vein. Tubular morphogenesis of bile ducts proceeds through the rearrangement of a single layer of the ductal plate. Recent studies on mice lacking Sox9, a transcription factor, or Notch 2 in the liver indicated the second wave of cholangiocyte differentiation adjacent to the initial single layer of the ductal plate, which was regulated by TGFβ and Notch pathways and involved in tubular morphogenesis. In a model proposed on the basis of those studies, after the initial induction of cholangiocytes near the portal vein, cholangiocyte differentiation and tubular morphogenesis progress in parallel (*22*, *26–30*). However, the precise mechanisms of bile duct morphogenesis have not been completely understood.

In addition, studies using mutant mice have implicated transcription factors including HES1, HNF6, HNF1β, Tbx3, FoxA2 and A3, FoxM1b, Hex and Sall4 in bile duct differentiation and/or morphogenesis (*31–38*) (Fig. 3). Although a network of these transcription factors and a link between transcription factors and Notch/TGFβ pathways are being uncovered (*39*), studies on gene expression and histology of mutant mice are insufficient to understand how these factors regulate complicated processes of tubulogenesis. As an alternative approach, *in vitro* culture systems allowing hepatoblasts to form bile duct structures are helpful to understand the lineage commitment of hepatoblasts and tubular morphogenesis (*22*, *25*, *40*, *41*).

Proliferation and differentiation of hepatocytes in foetal liver

At an early stage of hepatogenesis, endothelial cells contribute to the proliferation of hepatoblasts (*8*) and the vast majority of hepatoblasts become parenchymal hepatocytes at a later stage. The liver parenchyma is covered with the mesothelium consisting of the surface mesothelial cell (MC) layer, $ALCAM^+$ submesothelial cells and fibroblasts (*42*). At a later stage of hepatogenesis, MCs seem to contribute to the expansion of hepatoblasts (*43*). Foetal liver MCs are characterized by the expression of a sialomucin, PCLP1, and become adult liver MCs expressing mesothelin. Comparison of the gene expression profiles between foetal and adult MCs revealed that foetal $PCLP1^+$ MCs express various growth factors for hepatocytes such as Midkine and Pleiotrophin, and co-culture of $Dlk1^+$ foetal hepatocytes with $PCLP1^+$ foetal MCs in a transwell enhanced hepatocyte proliferation. Wilms' tumour 1 (Wt1) knockout mice were embryonic lethal, exhibiting impaired liver development. Cytokine production by Wt1 knockout MCs was reduced, while proliferation of $Dlk1^+$ cells from Wt1 knockout embryos was normal in a co-culture with wild-type MCs, indicating that defects in liver development of Wt1

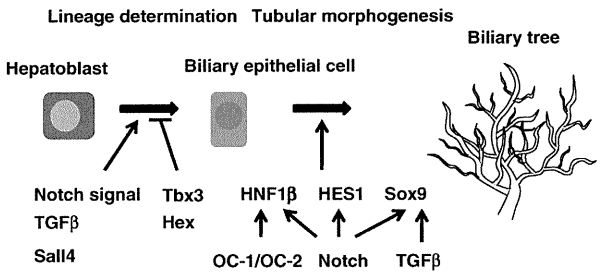

Fig. 3 Bile duct development. There are two steps in bile duct development. First, hepatoblasts are induced to differentiate to biliary epithelial cells around the portal vein. This step is promoted by TGFβ and Notch signals as well as a transcription factor, Sall4, whereas it is inhibited by two transcription factors, Tbx3 and Hex. Biliary epithelial cells then undergo tubular morphogenesis and form the biliary tree. Three transcription factors, HNF1β, HES-1 and Sox9, are involved in tubular morphogenesis. The Notch signal is upstream of all the three transcription factors, whereas OC-1 and OC-2, and the TGFβ signal are upstream of HNF1β and Sox9, respectively.

knockout mouse are due to MCs. MCs were also shown to delaminate and give rise to mesenchymal cells in the liver (*44*). These results indicate that the mesothelium is not only a protective sheet covering the liver parenchyma but also actively involved in liver organogenesis (Fig. 2).

Foetal liver is a major tissue for haematopoiesis, and hepatocytes acquire various metabolic functions at perinatal and postnatal stages. Mice lacking gp130, the common receptor subunit of the IL-6 family cytokines, develop liver with impaired functions, indicating that some of the IL-6 family cytokines are required for functional maturation of the liver (*45*). Oncostatin M (OSM), a member of the IL-6 family, strongly enhanced differentiation of foetal hepatocytes, while liver development is normal in OSM-deficient mice, suggesting that another member of the family may play a similar role. In the foetal liver, immigrating haematopoietic stem cells proliferate and produce numerous blood cells with the help of liver cells including hepatocytes and endothelial cells. Haematopoietic activity in foetal liver declines with hepatocyte differentiation (Fig. 2). As OSM is secreted from haematopoietic cells proliferating in the foetal liver and induces differentiation of hepatocytes, it is likely that OSM plays a role for coordination of liver development and haematopoiesis (*46*).

Adult liver stem/progenitor cells

Adult liver has a potential to regenerate under conditions of severe parenchymal loss, although hepatocytes and cholangiocytes are mitotically dormant under normal conditions. Hepatocytes themselves have a remarkable ability to self-replicate to restore liver mass (*47*) and are capable of at least 80 doublings by serial transplantation (*48*), allowing the liver to regenerate. Thus, the contribution of liver stem cells to regeneration after partial hepatectomy (PH) seems to be minimal if any. However, in liver injury that limits this pathway there is an accompanying expansion of a potential stem cell compartment in the periportal area, which is known as ductular reaction (*49–51*) (Fig. 4). These proliferating epithelial cells are often referred to as oval cells in rodents because of their oval nucleus (*52*). Upon activation of oval cells, they expand into liver parenchyma from the portal area, and selective damage of the periportal zone reduces oval cell proliferation, supporting the notion that oval cells are derived from the periportal region, in particular canals of Hering that connect the bile canaliculus and the biliary tree (*53*). In addition, an extrahepatic origin of oval cells such as bone marrow was also suggested (*54*); however, the exact origin of oval cells still remains to be established. While oval cells have been most extensively studied in rodents, similar cells have been found in various human liver diseases, such as chronic viral hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease and fulminant hepatitis, and also implicated in tumourigenesis (*55*, *56*). Oval cells express both ALB and cytokeratin 19, which are hepatocytic and cholangiocytic markers, respectively, and are believed to differentiate to hepatocytic and biliary lineages, similar to hepatoblasts in the embryonic liver.

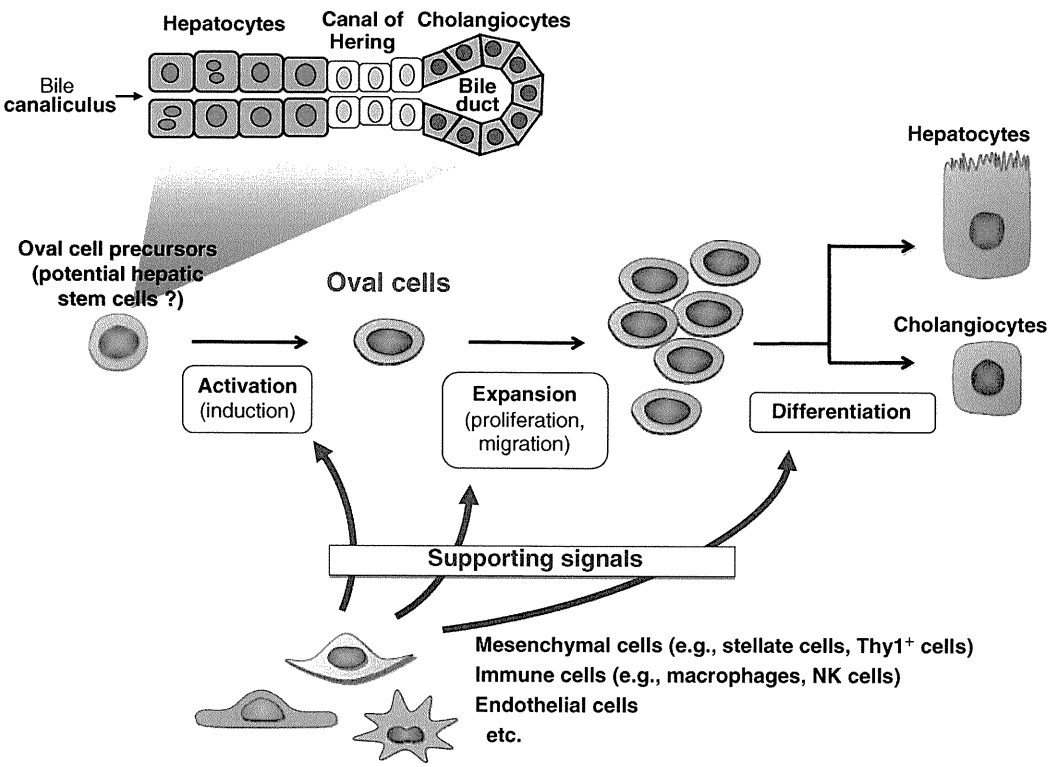

Fig. 4 Induction of oval cells. Oval cells and Thy1$^+$ cells are simultaneously induced in severe liver damage conditions. The origin of oval cells is still under debate. FGF7 is produced by Thy1$^+$ cells in response to liver damage conditions, and oval cells receive its signal.

Thus they are thought to be facultative stem/progenitor cells in the adult liver (Fig. 4).

The nature of oval cells as liver stem cells was debated in numerous reports of studies using various rodent models. The 2-acetylaminofluoren (2-AAF)/PH model, *i.e.* blocking hepatocyte proliferation by 2-AAF prior to PH, has been extensively used to characterize oval cells in rat (*57*, *58*). However, the same procedure does not induce oval cells in mice, and alternative protocols such as a choline-deficient, ethionine-supplemented (CDE) diet and 3,5-diethoxycarbonyl-1,4-dihydro-collidine (DDC) diet have been developed to induce oval cells in mice (*59–61*). Although the proliferating epithelial cells in the periportal region upon injury by various insults are collectively referred to as oval cells, it remains unclear whether or not the oval cells in different species by different protocols have common characteristics. A major problem in characterizing oval cells was the lack of appropriate cell surface markers to identify and isolate the oval cell compartment.

In the rat 2-AAF/PH model, Dabeva *et al.* reported that $EpCAM^+$ oval cells are bipotential adult hepatic progenitors (*62*, *63*). Suzuki *et al.* (*64*) reported that $CD133^+$ cells isolated from DDC-treated mouse liver could form large colonies in culture. These large colony-forming cells gave rise to both hepatocytes and cholangiocytes, while maintaining undifferentiated cells by self-renewing cell divisions. In order to isolate and characterize mouse oval cells, Okabe *et al.* (*65*) searched for cell surface molecules expressed on oval cells in mouse fed DDC diet. EpCAM was expressed in both mouse normal cholangiocytes and oval cells, and its related protein TROP2 was expressed exclusively in oval cells, establishing TROP2 as a novel marker to distinguish oval cells from normal cholangiocytes (*65*). Some of the $EpCAM^+$ cells isolated from injured liver proliferate to form colonies *in vitro*, and the clonally expanded cells differentiate into hepatocytes and cholangiocytes, suggesting that the oval cell fraction contains potential liver stem cells.

Interestingly, such cells with liver stem cell characteristics are also found in $EpCAM^+$ cells of the normal liver. Intriguingly, comparison of the colony formation of $EpCAM^+$ cells between normal and injured livers revealed little difference in the frequency of potential liver stem cell activity between them, strongly suggesting that most of the proliferating mouse oval cells represent transit-amplifying cells rather than stem cells. Bipotential clonal cell lines can be obtained from the healthy liver of adult mice and participate in liver regeneration in severe combined immune-deficient mice expressing urokinase-type plasminogen by the ALB promoter, where they differentiate in clusters of hepatocytes and occasionally bile ducts (*66*). Kamiya *et al.* (*67*) found progenitor cells in the $CD13^+CD49f^+CD133^+$ subpopulation of non-haematopoietic cells derived from postnatal livers. These results demonstrate the existence, in normal adult mouse liver, of a pool of clonogenic cells that are (or can become) bipotential.

As mentioned above, oval cells are induced in liver with severe or chronic damage. Chronic injury conditions in the liver are usually associated with inflammation, and the roles of lymphocytes and inflammatory responses in oval cell regulation have also been suggested (*68*, *69*). In accordance with this notion, several kinds of inflammatory cytokines, such as tumour necrosis factor (TNF)-α, lymphotoxin-β, interferon-γ and IL-6, have been shown to modulate oval cell response (*70*, *71*). Perhaps the best established inflammatory cytokine to be involved in oval cell response is a TNF family member ligand, TNF-like weak inducer of apoptosis (Tweak). Thus, transgenic mice overexpressing this cytokine in the liver exhibit periportal oval cell hyperplasia, while administration of a blocking anti-Tweak monoclonal antibody significantly reduced oval cell response in mice fed DDC diet (*72*). Furthermore, in mice lacking Fn14, the cognate receptor for Tweak, induction of oval cells was attenuated in both DDC diet and CDE diet models (*72*, *73*). These inflammatory cytokines are considered to function as part of the innate immune system sensing damage to the tissue and serve as the earliest signals for triggering the process of liver regeneration (Fig. 4).

Mesenchymal cells such as stellate cells have long been suggested to physically interact with oval cells and thus considered to induce some signals in them (*53*). Recent studies using several rat and mouse models have demonstrated that a population of mesenchymal cells expressing thymus cell antigen-1 (Thy-1; also known as CD90) resides in close proximity to and expands in parallel with oval cells (*74*) (H. Takase, T. Itoh and A. Miyajima, unpublished observation). Furthermore, these $Thy1^+$ cells were found to express FGF7, and its cognate receptor FGFR2b was detected in oval cells. FGF7 knockout mice showed a defect in oval cell response, while overexpression of FGF7 *in vivo* in normal mouse liver led to induction and proliferation of cells with markers of oval cells in the periportal area. Together, these results strongly suggest that FGF7 plays a key role in adult liver stem/progenitor cell response as well as that the $Thy1^+$ cells may serve as the niche for oval cells by providing this cytokine (Takase, H., Itoh, T. and Miyajima A., unpublished observations). As a signal related to oval cell response, several recent studies have implicated the canonical Wnt/β-catenin pathway in oval cell regulation (*75–78*) (Fig. 4). The Wnt/β-catenin pathway is well known to play important roles in stem cell regulation including self-renewal in various other organs and tissues, and also in carcinogenesis including liver tumours. In both rat and mouse models, expression of some Wnt ligands in damaged liver and concomitant activation of the β-catenin pathway in oval cells were observed. In conditional knockout mice lacking β-catenin in both hepatocytes and cholangiocytes, DDC diet-induced oval cell response in the liver was significantly reduced, although not completely abrogated. While several factors have been shown to be involved in oval cell response, the precise modes of their actions and their relationship are currently unclear and should be determined.

Concluding remarks

Traditionally, research on liver biology mostly relied on relatively crude cell separation methods based on cell density and centrifugation. In the last decade, identification of specific cell surface markers for each of the liver cell types, production of corresponding monoclonal antibodies and cell sorting techniques have together revolutionized the field and enabled us to perform much more detailed characterization of liver cells, particularly non-parenchymal cells including the stem/progenitor cells. It has also become possible to analyse the modes of interaction among different types of these cells *in vivo* by means of combinatorial use of specific markers/antibodies as well as *in vitro* with co-culture systems using the isolated viable cell populations. Elucidation of the molecular basis for the signals that regulate development, proliferation and differentiation of liver stem/progenitor cells should not only advance our understanding of the basic pathophysiology of the liver but also help to establish better protocols to generate mature hepatocytes and other liver cells *in vitro* for cell-based therapy, transplantation and drug discovery.

Funding
This work was supported by research grants from the Ministry of Education, Sports, Science, and Technology, and the Ministry of Health, Labour and Welfare, Japan, and a research grant from the Takeda Science Foundation.

Conflict of interest
None declared.

References

1. Douarin, N.M. (1975) An experimental analysis of liver development. *Med. Biol.* **53**, 427–455
2. Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J.R., and Zaret, K.S. (1996) Hepatic specification of the gut endoderm *in vitro*: cell signaling and transcriptional control. *Genes Dev.* **10**, 1670–1682
3. Jung, J., Zheng, M., Goldfarb, M., and Zaret, K.S. (1999) Initiation of mammalian liver development from endoderm by fibroblast growth factors. *Science* **284**, 1998–2003
4. Rossi, J.M., Dunn, N.R., Hogan, B.L., and Zaret, K.S. (2001) Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes Dev.* **15**, 1998–2009
5. Ober, E.A., Verkade, H., Field, H.A., and Stainier, D.Y. (2006) Mesodermal Wnt2b signalling positively regulates liver specification. *Nature* **442**, 688–691
6. Sosa-Pineda, B., Wigle, J.T., and Oliver, G. (2000) Hepatocyte migration during liver development requires Prox1. *Nat. Genet.* **25**, 254–255
7. Zaret, K. (1998) Early liver differentiation: genetic potentiation and multilevel growth control. *Curr. Opin. Genet. Dev.* **8**, 526–531
8. Matsumoto, K., Yoshitomi, H., Rossant, J., and Zaret, K.S. (2001) Liver organogenesis promoted by endothelial cells prior to vascular function. *Science* **294**, 559–563
9. Kubota, H. and Reid, L.M. (2000) Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. *Proc. Natl. Acad. Sci. USA* **97**, 12132–12137
10. Suzuki, A., Zheng, Y., Kondo, R., Kusakabe, M., Takada, Y., Fukao, K., Nakauchi, H., and Taniguchi, H. (2000) Flow-cytometric separation and enrichment of hepatic progenitor cells in the developing mouse liver. *Hepatology* **32**, 1230–1239
11. Minguet, S., Cortegano, I., Gonzalo, P., Martinez-Marin, J.A., de Andres, B., Salas, C., Melero, D., Gaspar, M.L., and Marcos, M.A. (2003) A population of c-Kit(low)(CD45/TER119)- hepatic cell progenitors of 11-day postcoitus mouse embryo liver reconstitutes cell-depleted liver organoids. *J. Clin. Invest.* **112**, 1152–1163
12. Tanimizu, N., Nishikawa, M., Saito, H., Tsujimura, T., and Miyajima, A. (2003) Isolation of hepatoblasts based on the expression of Dlk/Pref-1. *J. Cell. Sci.* **116**, 1775–1786
13. Floridon, C., Jensen, C.H., Thorsen, P., Nielsen, O., Sunde, L., Westergaard, J.G., Thomsen, S.G., and Teisner, B. (2000) Does fetal antigen 1 (FA1) identify cells with regenerative, endocrine and neuroendocrine potentials? A study of FA1 in embryonic, fetal, and placental tissue and in maternal circulation. *Differentiation* **66**, 49–59
14. Yanai, H., Nakamura, K., Hijioka, S., Kamei, A., Ikari, T., Ishikawa, Y., Shinozaki, E., Mizunuma, N., Hatake, K., and Miyajima, A. (2010) Dlk-1, a cell surface antigen on foetal hepatic stem/progenitor cells, is expressed in hepatocellular, colon, pancreas and breast carcinomas at a high frequency. *J. Biochem.* **148**, 85–92
15. Nierhoff, D., Ogawa, A., Oertel, M., Chen, Y.Q., and Shafritz, D.A. (2005) Purification and characterization of mouse fetal liver epithelial cells with high in vivo repopulation capacity. *Hepatology* **42**, 130–139
16. Tanaka, M., Okabe, M., Suzuki, K., Kamiya, Y., Tsukahara, Y., Saito, S., and Miyajima, A. (2009) Mouse hepatoblasts at distinct developmental stages are characterized by expression of EpCAM and DLK1: drastic change of EpCAM expression during liver development. *Mech. Dev.* **126**, 665–676
17. Dan, Y.Y., Riehle, K.J., Lazaro, C., Teoh, N., Haque, J., Campbell, J.S., and Fausto, N. (2006) Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages. *Proc. Natl. Acad. Sci. USA* **103**, 9912–9917
18. Schmelzer, E., Zhang, L., Bruce, A., Wauthier, E., Ludlow, J., Yao, H.L., Moss, N., Melhem, A., McClelland, R., Turner, W., Kulik, M., Sherwood, S., Tallheden, T., Cheng, N., Furth, M.E., and Reid, L.M. (2007) Human hepatic stem cells from fetal and postnatal donors. *J. Exp. Med.* **204**, 1973–1987
19. Kakinuma, S., Ohta, H., Kamiya, A., Yamazaki, Y., Oikawa, T., Okada, K., and Nakauchi, H. (2009) Analyses of cell surface molecules on hepatic stem/progenitor cells in mouse fetal liver. *J. Hepatol.* **51**, 127–138
20. Tanimizu, N., Saito, H., Mostov, K., and Miyajima, A. (2004) Long-term culture of hepatic progenitors derived from mouse Dlk+ hepatoblasts. *J. Cell. Sci.* **117**, 6425–6434
21. Strick-Marchand, H., Morosan, S., Charneau, P., Kremsdorf, D., and Weiss, M.C. (2004) Bipotential mouse embryonic liver stem cell lines contribute to liver regeneration and differentiate as bile ducts and hepatocytes. *Proc. Natl. Acad. Sci. USA* **101**, 8360–8365
22. Antoniou, A., Raynaud, P., Cordi, S., Zong, Y., Tronche, F., Stanger, B.Z., Jacquemin, P., Pierreux, C.E., Clotman, F., and Lemaigre, F.P. (2009) Intrahepatic bile

ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. *Gastroenterology* **136**, 2325–2333
23. Clotman, F., Jacquemin, P., Plumb-Rudewiez, N., Pierreux, C.E., Van der Smissen, P., Dietz, H.C., Courtoy, P.J., Rousseau, G.G., and Lemaigre, F.P. (2005) Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. *Genes Dev.* **19**, 1849–1854
24. Suzuki, K., Tanaka, M., Watanabe, N., Saito, S., Nonaka, H., and Miyajima, A. (2008) p75 Neurotrophin receptor is a marker for precursors of stellate cells and portal fibroblasts in mouse fetal liver. *Gastroenterology* **135**, 270–281 e273
25. Tanimizu, N. and Miyajima, A. (2004) Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. *J. Cell. Sci.* **117**, 3165–3174
26. Zong, Y., Panikkar, A., Xu, J., Antoniou, A., Raynaud, P., Lemaigre, F., and Stanger, B.Z. (2009) Notch signaling controls liver development by regulating biliary differentiation. *Development* **136**, 1727–1739
27. Geisler, F., Nagl, F., Mazur, P.K., Lee, M., Zimber-Strobl, U., Strobl, L.J., Radtke, F., Schmid, R.M., and Siveke, J.T. (2008) Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. *Hepatology* **48**, 607–616
28. Lozier, J., McCright, B., and Gridley, T. (2008) Notch signaling regulates bile duct morphogenesis in mice. *PLoS One* **3**, e1851
29. Sparks, E.E., Huppert, K.A., Brown, M.A., Washington, M.K., and Huppert, S.S. (2010) Notch signaling regulates formation of the three-dimensional architecture of intrahepatic bile ducts in mice. *Hepatology* **51**, 1391–1400
30. Tchorz, J.S., Kinter, J., Muller, M., Tornillo, L., Heim, M.H., and Bettler, B. (2009) Notch2 signaling promotes biliary epithelial cell fate specification and tubulogenesis during bile duct development in mice. *Hepatology* **50**, 871–879
31. Clotman, F., Lannoy, V.J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G.G., and Lemaigre, F.P. (2002) The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* **129**, 1819–1828
32. Coffinier, C., Gresh, L., Fiette, L., Tronche, F., Schutz, G., Babinet, C., Pontoglio, M., Yaniv, M., and Barra, J. (2002) Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. *Development* **129**, 1829–1838
33. Hunter, M.P., Wilson, C.M., Jiang, X., Cong, R., Vasavada, H., Kaestner, K.H., and Bogue, C.W. (2007) The homeobox gene Hhex is essential for proper hepatoblast differentiation and bile duct morphogenesis. *Dev. Biol.* **308**, 355–367
34. Kodama, Y., Hijikata, M., Kageyama, R., Shimotohno, K., and Chiba, T. (2004) The role of notch signaling in the development of intrahepatic bile ducts. *Gastroenterology* **127**, 1775–1786
35. Krupczak-Hollis, K., Wang, X., Kalinichenko, V.V., Gusarova, G.A., Wang, I.C., Dennewitz, M.B., Yoder, H.M., Kiyokawa, H., Kaestner, K.H., and Costa, R.H. (2004) The mouse Forkhead Box m1 transcription factor is essential for hepatoblast mitosis and development of intrahepatic bile ducts and vessels during liver morphogenesis. *Dev. Biol.* **276**, 74–88
36. Li, Z., White, P., Tuteja, G., Rubins, N., Sackett, S., and Kaestner, K.H. (2009) Foxa1 and Foxa2 regulate bile duct development in mice. *J. Clin. Invest.* **119**, 1537–1545
37. Oikawa, T., Kamiya, A., Kakinuma, S., Zeniya, M., Nishinakamura, R., Tajiri, H., and Nakauchi, H. (2009) Sall4 regulates cell fate decision in fetal hepatic stem/progenitor cells. *Gastroenterology* **136**, 1000–1011
38. Suzuki, A., Sekiya, S., Buscher, D., Izpisua Belmonte, J.C., and Taniguchi, H. (2008) Tbx3 controls the fate of hepatic progenitor cells in liver development by suppressing p19ARF expression. *Development* **135**, 1589–1595
39. Raynaud, P., Carpentier, R., Antoniou, A., and Lemaigre, F.P. (2011) Biliary differentiation and bile duct morphogenesis in development and disease. *Int. J. Biochem. Cell Biol.* **43**, 245–256
40. Tanimizu, N., Miyajima, A., and Mostov, K.E. (2007) Liver progenitor cells develop cholangiocyte-type epithelial polarity in three-dimensional culture. *Mol. Biol. Cell* **18**, 1472–1479
41. Tanimizu, N., Miyajima, A., and Mostov, K.E. (2009) Liver progenitor cells fold up a cell monolayer into a double-layered structure during tubular morphogenesis. *Mol. Biol. Cell* **20**, 2486–2494
42. Asahina, K., Tsai, S.Y., Li, P., Ishii, M., Maxson, R.E. Jr, Sucov, H.M., and Tsukamoto, H. (2009) Mesenchymal origin of hepatic stellate cells, submesothelial cells, and perivascular mesenchymal cells during mouse liver development. *Hepatology* **49**, 998–1011
43. Onitsuka, I., Tanaka, M., and Miyajima, A. (2010) Characterization and functional analyses of hepatic mesothelial cells in mouse liver development. *Gastroenterology* **138**, 1525–1535, 1535 e1521–1526
44. Ijpenberg, A., Perez-Pomares, J.M., Guadix, J.A., Carmona, R., Portillo-Sanchez, V., Macias, D., Hohenstein, P., Miles, C.M., Hastie, N.D., and Munoz-Chapuli, R. (2007) Wt1 and retinoic acid signaling are essential for stellate cell development and liver morphogenesis. *Dev. Biol.* **312**, 157–170
45. Kamiya, A., Kinoshita, T., Ito, Y., Matsui, T., Morikawa, Y., Senba, E., Nakashima, K., Taga, T., Yoshida, K., Kishimoto, T., and Miyajima, A. (1999) Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. *EMBO J.* **18**, 2127–2136
46. Kinoshita, T., Sekiguchi, T., Xu, M.J., Ito, Y., Kamiya, A., Tsuji, K., Nakahata, T., and Miyajima, A. (1999) Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. *Proc. Natl. Acad. Sci. USA* **96**, 7265–7270
47. Michalopoulos, G.K. (2010) Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *Am. J. Pathol.* **176**, 2–13
48. Overturf, K., al-Dhalimy, M., Ou, C.N., Finegold, M., and Grompe, M. (1997) Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes. *Am. J. Pathol.* **151**, 1273–1280
49. Alison, M.R., Golding, M.H., and Sarraf, C.E. (1996) Pluripotential liver stem cells: facultative stem cells located in the biliary tree. *Cell Prolif.* **29**, 373–402
50. Roskams, T.A., Theise, N.D., Balabaud, C., Bhagat, G., Bhathal, P.S., Bioulac-Sage, P., Brunt, E.M., Crawford, J.M., Crosby, H.A., Desmet, V., Finegold, M.J., Geller, S.A., Gouw, A.S., Hytiroglou, P., Knisely, A.S., Kojiro, M., Lefkowitch, J.H., Nakanuma, Y., Olynyk, J.K., Park, Y.N., Portmann, B., Saxena, R., Scheuer, P.J., Strain, A.J., Thung, S.N., Wanless, I.R., and West, A.B. (2004) Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. *Hepatology* **39**, 1739–1745
51. Theise, N.D., Saxena, R., Portmann, B.C., Thung, S.N., Yee, H., Chiriboga, L., Kumar, A., and Crawford, J.M.

(1999) The canals of Hering and hepatic stem cells in humans. *Hepatology* **30**, 1425–1433
52. Farber, E. (1956) Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylamino-fluorene, and 3′-methyl-4-dimethylaminoazobenzene. *Cancer Res.* **16**, 142–148
53. Paku, S., Schnur, J., Nagy, P., and Thorgeirsson, S.S. (2001) Origin and structural evolution of the early proliferating oval cells in rat liver. *Am. J. Pathol.* **158**, 1313–1323
54. Petersen, B.E., Bowen, W.C., Patrene, K.D., Mars, W.M., Sullivan, A.K., Murase, N., Boggs, S.S., Greenberger, J.S., and Goff, J.P. (1999) Bone marrow as a potential source of hepatic oval cells. *Science* **284**, 1168–1170
55. Fausto, N. (2004) Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. *Hepatology* **39**, 1477–1487
56. Lee, J.S., Heo, J., Libbrecht, L., Chu, I.S., Kaposi-Novak, P., Calvisi, D.F., Mikaelyan, A., Roberts, L.R., Demetris, A.J., Sun, Z., Nevens, F., Roskams, T., and Thorgeirsson, S.S. (2006) A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat. Med.* **12**, 410–416
57. Evarts, R.P., Nagy, P., Marsden, E., and Thorgeirsson, S.S. (1987) A precursor-product relationship exists between oval cells and hepatocytes in rat liver. *Carcinogenesis* **8**, 1737–1740
58. Laishes, B.A. and Rolfe, P.B. (1981) Search for endogenous liver colony-forming units in F344 rats given a two-thirds hepatectomy during short-term feeding of 2-acetylaminofluorene. *Cancer Res.* **41**, 1731–1741
59. Akhurst, B., Croager, E.J., Farley-Roche, C.A., Ong, J.K., Dumble, M.L., Knight, B., and Yeoh, G.C. (2001) A modified choline-deficient, ethionine-supplemented diet protocol effectively induces oval cells in mouse liver. *Hepatology* **34**, 519–522
60. Preisegger, K.H., Factor, V.M., Fuchsbichler, A., Stumptner, C., Denk, H., and Thorgeirsson, S.S. (1999) Atypical ductular proliferation and its inhibition by transforming growth factor beta1 in the 3,5-diethoxycarbonyl-1,4-dihydrocollidine mouse model for chronic alcoholic liver disease. *Lab. Invest.* **79**, 103–109
61. Wang, X., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., and Grompe, M. (2003) The origin and liver repopulating capacity of murine oval cells. *Proc. Natl. Acad. Sci. USA* **100 (Suppl. 1)**, 11881–11888
62. Yovchev, M.I., Grozdanov, P.N., Joseph, B., Gupta, S., and Dabeva, M.D. (2007) Novel hepatic progenitor cell surface markers in the adult rat liver. *Hepatology* **45**, 139–149
63. Yovchev, M.I., Grozdanov, P.N., Zhou, H., Racherla, H., Guha, C., and Dabeva, M.D. (2008) Identification of adult hepatic progenitor cells capable of repopulating injured rat liver. *Hepatology* **47**, 636–647
64. Suzuki, A., Sekiya, S., Onishi, M., Oshima, N., Kiyonari, H., Nakauchi, H., and Taniguchi, H. (2008) Flow cytometric isolation and clonal identification of self-renewing bipotent hepatic progenitor cells in adult mouse liver. *Hepatology* **48**, 1964–1978
65. Okabe, M., Tsukahara, Y., Tanaka, M., Suzuki, K., Saito, S., Kamiya, Y., Tsujimura, T., Nakamura, K., and Miyajima, A. (2009) Potential hepatic stem cells reside in EpCAM+ cells of normal and injured mouse liver. *Development* **136**, 1951–1960
66. Fougere-Deschatrette, C., Imaizumi-Scherrer, T., Strick-Marchand, H., Morosan, S., Charneau, P., Kremsdorf, D., Faust, D.M., and Weiss, M.C. (2006) Plasticity of hepatic cell differentiation: bipotential adult mouse liver clonal cell lines competent to differentiate *in vitro* and *in vivo*. *Stem Cells* **24**, 2098–2109
67. Kamiya, A., Kakinuma, S., Yamazaki, Y., and Nakauchi, H. (2009) Enrichment and clonal culture of progenitor cells during mouse postnatal liver development in mice. *Gastroenterology* **137**, 1114–1126, 1126 e1111–1114
68. Knight, B., Akhurst, B., Matthews, V.B., Ruddell, R.G., Ramm, G.A., Abraham, L.J., Olynyk, J.K., and Yeoh, G.C. (2007) Attenuated liver progenitor (oval) cell and fibrogenic responses to the choline deficient, ethionine supplemented diet in the BALB/c inbred strain of mice. *J. Hepatol.* **46**, 134–141
69. Strick-Marchand, H., Masse, G.X., Weiss, M.C., and Di Santo, J.P. (2008) Lymphocytes support oval cell-dependent liver regeneration. *J. Immunol.* **181**, 2764–2771
70. Akhurst, B., Matthews, V., Husk, K., Smyth, M.J., Abraham, L.J., and Yeoh, G.C. (2005) Differential lymphotoxin-beta and interferon gamma signaling during mouse liver regeneration induced by chronic and acute injury. *Hepatology* **41**, 327–335
71. Knight, B., Yeoh, G.C., Husk, K.L., Ly, T., Abraham, L.J., Yu, C., Rhim, J.A., and Fausto, N. (2000) Impaired preneoplastic changes and liver tumor formation in tumor necrosis factor receptor type 1 knockout mice. *J. Exp. Med.* **192**, 1809–1818
72. Jakubowski, A., Ambrose, C., Parr, M., Lincecum, J.M., Wang, M.Z., Zheng, T.S., Browning, B., Michaelson, J.S., Baetscher, M., Wang, B., Bissell, D.M., and Burkly, L.C. (2005) TWEAK induces liver progenitor cell proliferation. *J. Clin. Invest.* **115**, 2330–2340
73. Tirnitz-Parker, J.E., Viebahn, C.S., Jakubowski, A., Klopcic, B.R., Olynyk, J.K., Yeoh, G.C., and Knight, B. (2010) Tumor necrosis factor-like weak inducer of apoptosis is a mitogen for liver progenitor cells. *Hepatology* **52**, 291–302
74. Yovchev, M.I., Zhang, J., Neufeld, D.S., Grozdanov, P.N., and Dabeva, M.D. (2009) Thymus cell antigen-1-expressing cells in the oval cell compartment. *Hepatology* **50**, 601–611
75. Apte, U., Thompson, M.D., Cui, S., Liu, B., Cieply, B., and Monga, S.P. (2008) Wnt/beta-catenin signaling mediates oval cell response in rodents. *Hepatology* **47**, 288–295
76. Hu, M., Kurobe, M., Jeong, Y.J., Fuerer, C., Ghole, S., Nusse, R., and Sylvester, K.G. (2007) Wnt/beta-catenin signaling in murine hepatic transit amplifying progenitor cells. *Gastroenterology* **133**, 1579–1591
77. Itoh, T., Kamiya, Y., Okabe, M., Tanaka, M., and Miyajima, A. (2009) Inducible expression of Wnt genes during adult hepatic stem/progenitor cell response. *FEBS Lett.* **583**, 777–781
78. Yang, W., Yan, H.X., Chen, L., Liu, Q., He, Y.Q., Yu, L.X., Zhang, S.H., Huang, D.D., Tang, L., Kong, X.N., Chen, C., Liu, S.Q., Wu, M.C., and Wang, H.Y. (2008) Wnt/beta-catenin signaling contributes to activation of normal and tumorigenic liver progenitor cells. *Cancer Res.* **68**, 4287–4295

Chapter 14
Liver Stem Cells

Tohru Itoh, Minoru Tanaka, and Atsushi Miyajima

Abstract The liver is an essential organ for life, serving as the center for metabolism and playing various critical functions in controling systemic homeostasis. Among multiple types of cells comprising the liver, hepatocytes and cholangiocytes are the two epithelial cell lineages in the organ and commonly originate from hepatoblasts during organogenesis in the developing embryos. Thus, hepatoblasts possess bi-lineage differentiation potential into hepatocytes and cholangiocytes, a phenotypic feature that can best distinguish and define liver stem cells. Although the liver is considered not to rely on any resident stem cell population for their homeostatic maintenance, facultative stem/progenitor cells with the bi-lineage differentiation potential, referred to as oval cells in rodents, do emerge under severe damage conditions and contribute to the regenerative processes. Identification of specific markers has enabled researchers to isolate and characterize these fetal and adult stem/progenitor cell populations. *In vitro* culture systems as well as *in vivo* studies using animal models have been elucidating detailed molecular mechanisms, including intercellular signaling webs and intracellular transcriptional regulatory networks, that coordinately regulate development, differentiation and behavior of these cells. Understanding the cellular and molecular basis of liver development and regeneration from the perspective of the embryonic and adult stem/progenitor cells should make invaluable contributions to future development of technologies to produce fully functional hepatocytes *in vitro* that are applicable for cell therapy and pharmaceutical screening.

T. Itoh (✉), M. Tanaka, and A. Miyajima
Institute of Molecular and Cellular Biosciences, University of Tokyo,
1-1-1Yayoi, Bunkyo-ku, Tokyo, Japan
e-mail: itohru@iam.u-tokyo.ac.jp

G. Steinhoff (ed.), *Regenerative Medicine: From Protocol to Patient,* 327
DOI 10.1007/978-90-481-9075-1_14,

14.1 Introduction

In the adult human, the liver is the largest organ in the body, accounting for around one fiftieth of the body weight. The mammalian liver plays multiple critical roles in maintaining vital activity of the organisms, including metabolism of amino acids, lipids, and carbohydrates, serum protein synthesis, detoxification of xenobiotic compounds, production and secretion of bile, immune regulation, and so forth. To achieve these complex biological functions, the liver possesses a characteristic and sophisticated structure composed of several different types of cells. Hepatocytes, also known as the liver parenchymal cells, account for 80% of the total volume of the organ and serve as the principal cell type to execute the majority of the organ's functions. The other cell types, collectively termed as non-parenchymal cells (NPCs), include cholangiocytes (bile duct epithelial cells), Kupffer cells, hepatic stellate cells, endothelial cells, coelomic epithelial cells (mesothelial cells), and several kinds of immune cells. While each of these cell types has its own embryonic origin, hepatocytes and cholangiocytes, the two epithelial lineages in the organ, derive from a common precursor cell population, so-called hepatoblasts, in the developing liver (Lemaigre 2009; Tanimizu and Miyajima 2007; Zhao and Duncan 2005) (Fig. 14.1a). Thus, the term "liver stem cell" (or "hepatic stem cell") is most generally applied to represent this type of bi-potential progenitor cells that can differentiate to both hepatocytes and cholangiocytes. Hepatoblasts, however, are usually considered a cell population found only during the fetal period, and it is not clear whether and how these cells are related to the putative stem/progenitor cell populations in the adult liver. In other words, the self-renewal capacity of hepatoblasts *in vivo* remains undetermined. Thus, it would be safer to denote these cells as the fetal liver "stem/progenitor" cells, and we would like to adopt this description in this chapter.

In contrast to the situation in the developing liver, where hepatoblasts are fairly well established as the bipotential liver stem/progenitor cell, the one regarding the adult liver still has considerable controversy. In many other organs, such as the hematopoietic and epidermal systems and the small intestine, the tissue stem cells can be defined, and have indeed been isolated and/or anatomically located, as the cells that are responsible for normal tissue turnover. Thus, those stem cells, under the physiological condition, continue self-renewal while producing the progenies that give rise to the mature cell types and eventually replace the expired cells in the organ to maintain homeostasis. In the liver, hepatocyte turnover occurs very slowly, and it is still unclear and under debate whether such a kind of "stem cell" also exists and is actively involved in homeostatic maintenance of the organ.

Nevertheless, apart from this relatively complicated situation regarding the *bona fide* stem cell in the adult liver, researchers in this field have been quite successful in identifying and characterizing several different classes of putative adult liver stem cells, which should be of significant importance particularly in view of therapeutic applications. Classification of these different adult liver stem cells will be briefly described in Sect. 14.2.2.

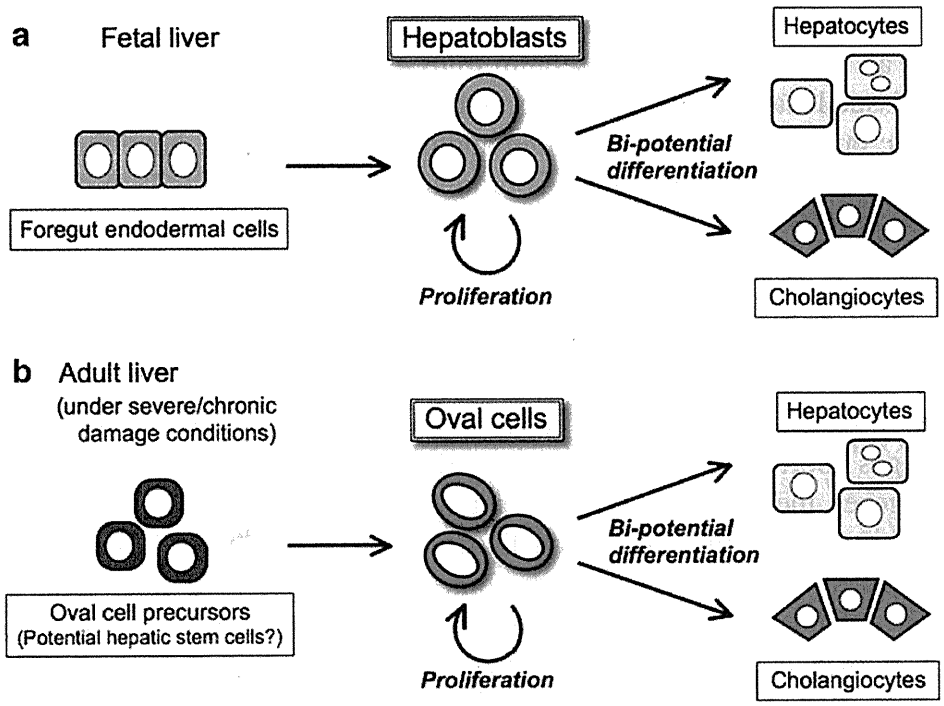

Fig. 14.1 Stem/progenitor cell populations in the fetal and adult livers. In the course of fetal liver development (**a**), hepatoblasts are derived from foregut endodermal cells, proliferate, and then undergo differentiation into two epithelial cell lineages, hepatocytes and cholangiocytes (bile duct epithelial cells). This bi-lineage differentiation potential is regarded as the hallmark of liver stem/progenitor cells. In the adult liver, regeneration can usually be achieved by replication of differentiated, mature cell populations (not shown here). Under severe/chronic liver damage conditions (**b**), however, facultative stem/progenitor cells, called oval cells in rodents, emerge from hitherto unidentified precursor cells and expand. These cells also possess bi-lineage differentiation potential and are considered to contribute to the regeneration process

14.2 Derivation/Classification

14.2.1 Hepatoblasts

Among the three germ layers generated during gastrulation (i.e., ectoderm, mesoderm, and endoderm), the liver derives principally from the endoderm. The endoderm differentiates into the primitive gut, which in turn gives rise to the gastrointestinal tract as well as various associated organs, including the thyroid, lung, pancreas, as well as liver. During the early stage of the liver development, interaction between the endoderm and the adjacent mesoderm plays a key role in induction of the organ.

The processes and mechanisms of mammalian liver development have been most extensively studied in the mouse embryos. Liver organogenesis starts at embryonic day (E) 8.0–8.5 in mice, which corresponds to about 3 weeks of gestation in humans. At this stage, the ventral foregut endoderm, a part of the endoderm from which the liver forms, faces the developing heart and receives inductive signals for the hepatic fate from the cardiac mesoderm. The fibroblast growth factor

(FGF) family of secreted proteins has been shown to mediate the inductive signals (Jung et al. 1999). In addition, septum transversum mesenchyme (STM), a mesodermal tissue locating adjacently to both of these organ primordia, also contributes to hepatic fate induction by providing another soluble factor, bone morphogenetic protein (BMP) (Rossi et al. 2001). Coordinated action of both FGF and BMP drives the liver developmental program with concomitant induction of several hepatic lineage-specific genes, such as *Albumin* and *Transthyretin*. This process is called "hepatic specification" and leads to generation of hepatoblasts, the fetal liver stem cells, initially lining up to form the hepatic endoderm.

Soon after the hepatic endoderm formation, hepatoblasts undergo dynamic changes in their morphology and localization, the process that can be divided into three stages (Bort et al. 2006). In stage I (E8.5), along with the expression of liver-specific genes, the initially cuboidal hepatoblasts become columnar in shape, leading to the formation of a thickened epithelium. In stage II (E9.0–E9.5), the hepatoblasts further change their morphology to become a pseudo-stratified epithelium. A study using mice deficient of the hematopoietically expressed homeobox (Hhex, or Hex) gene has shown that this transcription factor is critical for this stage (Bort et al. 2006). In stage III (E9.5+), the basal lamina that has covered the epithelium breaks down and the hepatoblasts start to delaminate and then migrate into the surrounding stroma, the STM, to form the liver bud. This step is also controlled by the functions of homeobox transcription factors, prospero-related homeobox 1 (Prox1), hepatocyte nuclear factor (HNF) 6 (HNF6; also known as Onecut-1 or Oc1) and Onecut2 (Oc2) (Margagliotti et al. 2007; Sosa-Pineda et al. 2000).

After the liver bud formation, hepatoblasts continuously proliferate throughout the embryonic days. Proliferation and survival of hepatoblasts is known to be regulated by various extracellular signals, such as hepatocyte growth factor (HGF), transforming growth factor beta (TGFbeta), Wnt/beta-catenin, and Sonic hedgehog (Hirose et al. 2009; Micsenyi et al. 2004; Schmidt et al. 1995; Tanimizu and Miyajima 2007; Weinstein et al. 2001). These signals may act on hepatoblasts either in an autocrine fashion, or by being supplied from the surrounding mesenchymal cells. Endothelial cells also play a critical role in hepatoblast regulation, as mice lacking endothelial cells show a defect in liver bud outgrowth (Matsumoto et al. 2001). The molecular nature of the signal provided by endothelial cells still remains elusive.

14.2.2 Adult Liver Stem/Progenitor Cells

In the field of liver biology, the term "liver stem cells" (or "hepatic stem cells") have been defined and used by researchers in several different ways. As exemplified by Grompe (Grompe 2003), the definitions can include, but may not be limited to, the following:

(a) Cells responsible for normal tissue turnover
(b) Cells which give rise to regeneration after partial hepatectomy (PH)