(c) Cells responsible for progenitor-dependent regeneration
(d) Transplantable liver repopulating cells
(e) Cells which result in hepatocyte and bile duct phenotype *in vitro*

Notably, these definitions are not mutually exclusive, and a given "liver stem cell" population may fulfill some of them simultaneously. For each definition, stem cells are accredited according to different and specific types of assays, either *in vitro* or *in vivo*.

Hepatocyte turnover under the physiological condition is relatively slow, which makes it quite difficult to investigate the cellular behavior in the course of homeostatic maintenance of the liver. Nevertheless, hepatocyte replacement does occur, and there must be some mechanism that ensures this tissue turnover. One of the long-standing model is the so-called "streaming liver hypothesis", where a "flow" of hepatocytes is assumed, just analogous to the well appreciated crypt-to-villi movement of the intestinal epithelial cells. In this model, hepatocytes are newly formed in the periportal region and then gradually move, while undergoing lineage maturation, toward the central vein (Zajicek et al. 1985). Although appealing, much of the evidence accumulated so far argues against this hypothesis, and it is more favorably considered that the liver maintenance is rather achieved by simple division of the preexisting hepatocytes. Very recently, however, a study in the human liver using mitochondrial mutations as a genetic marker identified that clonal patches of hepatocytes did emerge from the periportal regions and extended toward the central veins, supporting the presence of the hepatocyte flow as assumed by the streaming liver hypothesis (Fellous et al. 2009). Studies using mouse models that are suitably engineered to track the behavior of a particular hepatocyte would be helpful to solve this controversy.

The characteristic feature of the liver is its unique and remarkably high capacity to regenerate upon various injuries, such as those caused by partial hepatectomy or toxic insults. In rodent models, for example, after 70% partial hepatectomy, the liver can completely recover its initial volume and function within a week or so. During this recovery process, hepatocytes, as well as cholangiocytes, in the remaining liver undergo a few cycles of cell division to sufficiently restore the lost tissue (Michalopoulos and DeFrances 1997). Thus, the liver regeneration can usually be achieved by proliferation of the differentiated, postmitotic hepatocytes that remain intact, without necessitating an involvement of stem/progenitor cell populations. When the liver suffers from severe and/or chronic damages, however, hepatocyte proliferation is suppressed. It is under this condition when the facultative stem/progenitor cells are known to emerge and contribute to the liver regeneration process. Those stem/progenitor cells, referred to as oval cells in rodent models, are characterized by their potentials to proliferate as well as to differentiate into both hepatocytes and cholangiocytes, the two epithelial lineages in the liver (Grompe 2003; Matthews and Yeoh 2005; Newsome et al. 2004; Tanimizu and Miyajima 2007) (Fig. 14.1b). The most popular model to induce oval cells is the 2-acetylaminofluorene (2-AAF)/PH system in rats, where hepatocyte proliferation is blocked by 2-AAF prior to PH. This model, however, is not applicable to induce oval cells in mice. Other procedures,

such as the administration of a 3,5-diethoxycarbonyl-1,4-dihidro-collidine (DDC)-containing diet (Preisegger et al. 1999) and a choline-deficient ethionine-supplemented diet (CDE) (Akhurst et al. 2001) have been established and used in mice, as well as in rats (Fig. 14.2). Notably, most of the experimental procedures used to induce oval cell emergence and proliferation in the rodent liver eventually lead to tumorigenesis.

Although the term "oval cells" are used specifically in rodents, cells with similar characteristics have also been reported in various human liver diseases, such as chronic viral hepatitis, alcoholic liver disease (ALD), and nonalcoholic fatty liver disease (NAFLD), and are also implicated in tumorigenesis (Fausto 2004; Lee et al. 2006; Roskams et al. 2003). In humans, these cells are usually referred to as "hepatic progenitor cells" or "Intermediate hepatobiliary cells".

While oval cells are well known to emerge always from the periportal area, the cellular origin of oval cells is still not clarified (Fig. 14.3). Ever since their initial characterization, phenotypic resemblance between oval cells and bile duct epithelial cells has suggested that they presumably originate from the biliary tree. The fact that most of the molecular markers for oval cells are also expressed in cholangiocytes supports this notion. It is not clear, however, whether most if not all cholangiocytes can equally or similarly behave as progenitors for oval cells, or there is a certain type of specialized "origin-of-oval cells" located somewhere among cholangiocytes. Potentially lying on an extension of the latter possibility is the model that the canal of Hering, a structure where interlobular bile ducts and hepatocytes are connected, is the origin of oval cells (Paku et al. 2001). Given its anatomical location in between cholangiocytes and hepatocytes, it appears reasonable to assume that this structure may serve as a niche for putative stem cells for these two cell lineages. Unfortunately, a direct proof for this model is hampered by lack of any specific marker for cells constituting the canal of Hering. Identification of such a

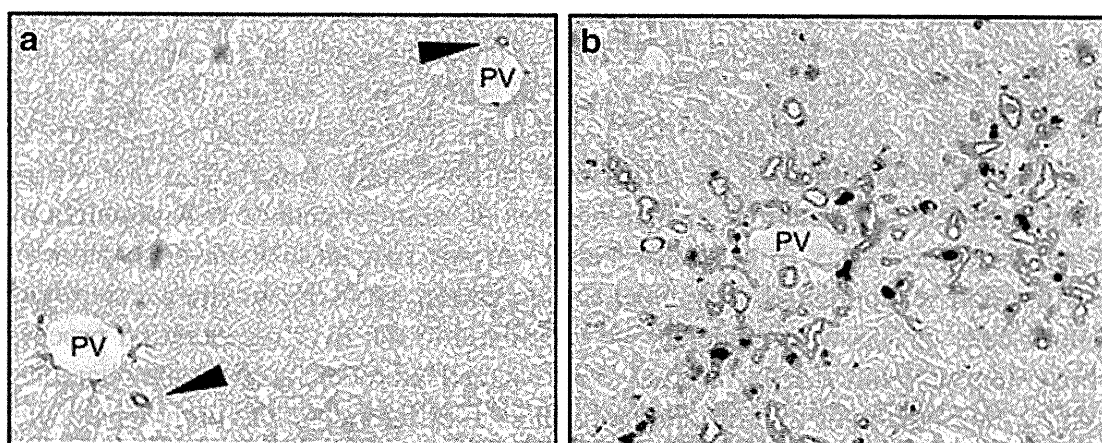

Fig. 14.2 Emergence of oval cells in a mouse model of chronic liver injury. Sections of the liver prepared from a normal mouse (**a**) and a mouse fed DDC diet for 8 weeks (**b**) were immunostained with anti-CK19 antibody. In the liver of the mouse fed DDC diet, CK19+ oval cells emerge from the periportal area, forming duct-llike structures (**b**; *brown signals*). Note that CK19 marks cholangiocytes comprising bile ducts in the normal liver (**a**; *arrowheads*). *PV* portal vein

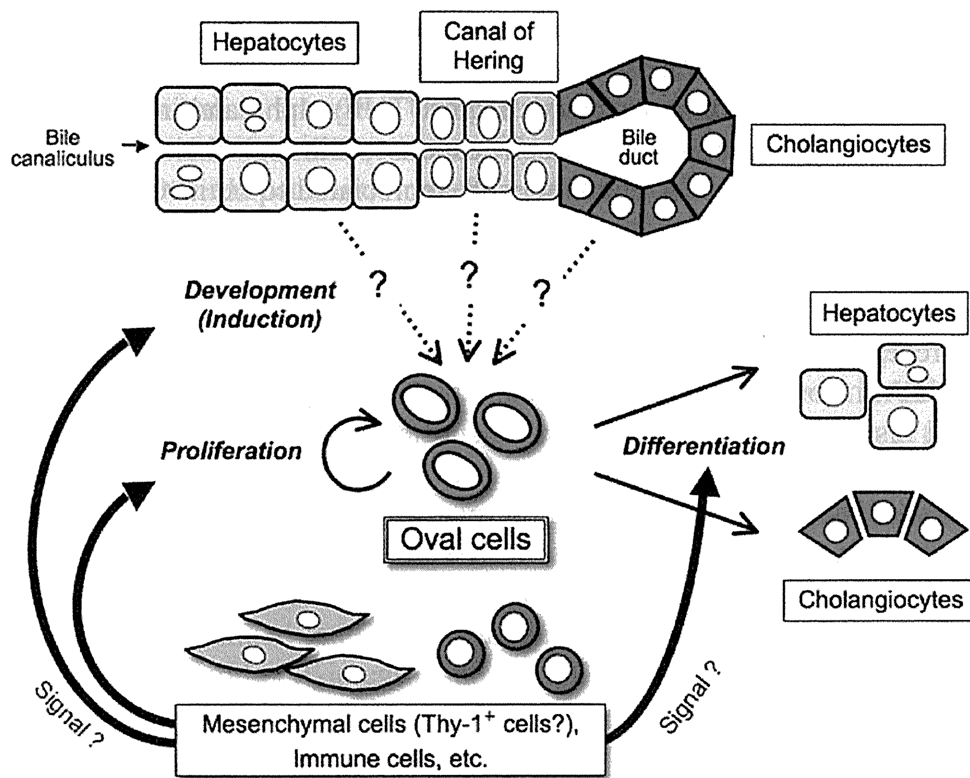

Fig. 14.3 Relationship among cells involved in oval cell response. Upon severe/chronic liver damages, oval cells emerge from the periportal region. The exact origin of oval cells has not yet been identified, but is supposed to be the canals of Hering, cholangiocytes, or heptocytes. Together with oval cells, several types of mesenchymal cells as well as immune cells accumulate in the injured liver and are often observed surrounding oval cells. These cells are likely to modulate induction, proliferation, migration, and/or differentiation of oval cells by means of various signaling mechanisms including direct cell-cell interaction, secretion of soluble factors (cytokines), and deposition of extracellular matrices, thereby playing key roles in regulation of oval cell response

molecule and a subsequent genetic lineage tracing study should help clarifying this issue. Although a possible contribution of hepatocytes as an origin of oval cells can also be considered, a study using mice with chimeric livers have suggested that this is not likely the case (Wang et al. 2003).

As a matter of course, emergence and expansion of oval cells upon liver injury is not an autonomous process within these cells but involves various other types of cells, which interact either directly or indirectly with oval cells, and also possibly with their putative precursor cells, and together shape the entire phenomenon often termed as "oval cell response" (Fig. 14.3). Mesenchymal cells such as stellate cells have long been suggested to physically interact with oval cells and exert some signals on them. A recent study has highlighted that a population of mesenchymal cells expressing thymus cell antigen 1 (Thy-1), which is distinct from stellate cells or myofibroblasts, reside in close proximity to oval cells in rat liver (Yovchev et al. 2009). Further characterization of this unique population may provide a clue to understand the nature of signals controling oval cell behaviors. Chronic injury conditions in the liver are usually associated with induction of inflammation, and the

role of lymphocytes and inflammatory responses have been suggested (Knight et al. 2007; Strick-Marchand et al. 2008). In accord with this notion, several inflammatory cytokines, such as tumor necrosis factor (TNF)-alpha and interferon-gamma, have been shown to modulate oval cell response, although their modes of action remain not fully clarified. A cytokine well appreciated to be involved in oval cell regulation is TNF-like weak inducer of apoptosis (TWEAK); transgenic mice overexpressing TWEAK in the liver exhibit periportal oval cell hyperplasia, while DDC diet-induced oval cell expansion was significantly reduced in mice lacking the Tweak receptor Fn14 as well as in wild-type mice administrated with a blocking anti-TWEAK monoclonal antibody (Jakubowski et al. 2005). As a signal related to oval cell response, recent studies have identified activation of the canonical Wnt/beta-catenin pathway in oval cells (Apte et al. 2008; Hu et al. 2007; Itoh et al. 2009; Yang et al. 2008). The Wnt/beta-catenin pathway is well known to be involved in stem cell regulation in various organs and tissues, and further characterization of the role of this pathway in oval cells, including its relevant target genes and interaction with other signaling pathways, is awaited.

14.3 Characteristics/Properties

14.3.1 Hepatoblasts

In order to characterize a particular cell population, the cell sorting method using antibodies against specific surface markers expressed on that population is a powerful tool, as the cells can be viably isolated and thus can be subjected to *in vitro* culture and/or *in vivo* transplantation experiments. In the last decade, much effort has been made to identify such specific cell surface antigens expressed on fetal hepatoblasts, leading to successful identification of several markers as well as establishment of protocols to isolate these cells.

Delta-like 1 homolog (Dlk1; also known as Pref-1 or fetal antigen 1) was initially identified as a marker for mouse hepatoblasts, and has later been shown to be useful to enrich and purify rat fetal liver progenitor cells with the liver repopulating activity (Oertel et al. 2008; Tanimizu et al. 2004). DLK1 is also known to be expressed in human fetal liver (Floridon et al. 2000). In mouse embryos, Dlk1 expression in the liver is initially observed around E9.0 in the developing liver bud and is maintained at a high level until E16, which then declines significantly and disappears at the neonatal and adult stages. Dlk1$^+$ cells isolated from E14.5 livers expressed albumin and formed colonies composed of hetpatocyte (Albumin$^+$) and cholangiocyte (Cytokeratin [CK] 19$^+$) lineages *in vitro*. Moreover, 7% of the colony-forming Dlk1$^+$ cells formed large colonies containing more than 100 cells during 5 days of culture, thus indicating that Dlk1 serves as a useful marker to enrich highly proliferative, bipotential hepatoblasts from fetal liver.

E-cadherin is also widely used as a fetal hepatoblast marker (Nitou et al. 2002), although its expression is not necessarily limited in these cells but persists even in the differentiated epithelial lineages. Using a specific monoclonal antibody against E-cadherin, hepatoblasts could be isolated form E12.5 mouse liver with 90% purity and 40% yield. Other hepatoblast markers include Liv-2 in the mouse, whose antigen has not yet been molecularly identified (Watanabe et al. 2002).

In addition to these relatively simple isolation of hepatoblasts based on the expression of single positive selection markers, several other groups have established well elaborated protocols employing combinations of multiple markers, sometimes including those for negative selection. In the rat system, the $RT1A1^-$ $OX18^{low}$ $ICAM1^+$ fraction of E13 fetal liver has been shown to contain hepatoblasts (Kubota and Reid 2000). Suzuki et al. designated as "hepatic colony-forming unit in culture (H-CFU-C)" a putative self-renewing stem cell population in the developing liver. Thus, based on an *in vitro* single cell-based assay of sorted cells, clonogenic cells capable of both self-renewal and multilineage differentiation were sought to be identified. They separated fetal liver cells based on expression of several markers including alpha6- and beta1-integrin subunits (CD49f and CD29, respectively) and demonstrated that the $CD45^-$ $Ter119^-$ $c\text{-}Kit^-$ $CD29^+$ $CD49f^+$ and $CD45^-$ $Ter119^-$ $c\text{-}Kit^-$ $c\text{-}Met^+$ $CD49f^{+/low}$ fractions of E13.5 mouse liver contained the H-CFU-C activity (Suzuki et al. 2000, 2002).

Using a combination of surface markers, a recent study has elucidated the phenotypic transition of hepatoblasts in the course of mouse fetal liver development (Tanaka et al. 2009). Thus, upon liver bud formation at around E9, hepatoblasts expressing both Dlk1 and epithelial cell adhesion molecule (EpCAM; also known as CD326, Tacstd1, or Trop1), a known marker for cholangiocytes and oval cells (see below), emerge from $EpCAM^+Dlk1^-$ foregut endodermal cells. The $EpCAM^+Dlk1^+$ cells contain highly proliferative hepatoblasts at E11.5, and thereafter undergo dramatic reduction in expression of EpCAM concomitantly with losing proliferative potential. At around E16.5, EpCAM expression is upregulated in ductal plates around the portal vein, while absent in immature hepatocytes.

14.3.2 Adult Liver Stem/Progenitor Cells

Oval cells were initially described by Farber, using a rat model of liver carcinogenesis, as "small oval cells about the ducts and vessels in the portal areas" having "scanty, lightly basophilic cytoplasm and pale blue-staining nuclei (by hematoxylin and eosin stain)" (Farber 1956). Since then, many studies have further characterized these cells and have established them as facultative liver stem/progenitor cells that are likely to play a relevant role in liver regeneration from various types of injuries (Grompe 2003; Matthews and Yeoh 2005; Newsome et al. 2004; Tanimizu and Miyajima 2007). Thus, oval cells are considered to be capable of differentiating into two hepatic epithelial lineages, i.e., hepatocyte and cholangiocyte (Fig. 14.1b). In possible relation to this notion, oval cells express both hepatocyte (Albumin) and cholangocyte (CK19)

markers. The immature hepatocyte marker alpha-fetoprotein (Afp) is known to be expressed in oval cells in rats, but not in mice (Jelnes et al. 2007). Similarly, expression of the hepatoblast marker Dlk1 has been shown in a subpopulation of rat oval cells, but is not found in mouse oval cells (Jelnes et al. 2007; Jensen et al. 2004; Tanimizu et al. 2004). There are several monoclonal antibodies that have long been used as "golden standards" to recognize oval cell markers, such as OV-1 and OV-6 in rats (Dunsford and Sell 1989) and A6 in mice (Engelhardt et al. 1990). OV-1 antibody reacts with an unknown antigen expressed on the surface of oval cells and thus can be used to isolate these cells, while OV-6 antibody recognizes a common epitope in the cytoskeleton components CK14 and CK19 (Bisgaard et al. 1993). Unfortunately, the A6 antibody used for mouse studies recognizes some intracellular antigen and thus is not suitable to be used for sorting of viable oval cells.

Similar to the situation with fetal liver hepatoblasts, much effort has been made in recent years to explore cell surface molecules that can be used to identify and isolate oval cells. This has led to the identification of EpCAM and CD133 (also known as prominin 1) as novel oval cell markers in both mice and rats (Okabe et al. 2009; Rountree et al. 2007; Suzuki et al. 2008b; Yovchev et al. 2007). The oncofetal protein glypican-3 has also been documented as a rat oval cell marker (Grozdanov et al. 2006). Notably, however, these molecules, as well as the OC-1/OC-6 and A6 antigens, are all expressed also in cholangiocytes in the normal liver. This fact strongly implies a close relationship between cholangiocytes and oval cells as mentioned earlier, with the former possibly being an origin of the latter (Fig. 14.3).

Interestingly, Trop2 (Tacstd2), a transmembrane molecule that is structurally related to EpCAM, has been found to be expressed exclusively in oval cells in the injured liver, but not in cholangiocytes in the normal liver (Okabe et al. 2009). Thus, Trop2 may serve as a genuine "oval cell marker" and would be advantageous for further characterization of oval cells. Similarly, a recent study identified a transcription factor, Foxl1, as another oval cell-specific marker (Sackett et al. 2009). Although this molecule is not a cell-surface antigen, a transgenic (Tg) mouse line expressing the Cre recombinase under the control of the Foxl1 promoter has been made and proven to be quite useful. Thus, a lineage tracing study using this Foxl1-Cre Tg mice demonstrated that both hepatocytes and cholangiocytes were found as descendants of Foxl1^{+} oval cells. This does not necessarily indicate that single oval cells can clonally differentiate into these two lineages, but strongly supports the notion that oval cells are bipotential progenitors for hepatocytes and cholangiocytes.

A recent study has established a panel of surface reactive monoclonal antibodies that can each detect different populations of ductal and periductal cells in the mouse oval cell response (Dorrell et al. 2008). Intriguingly, some of them seem to label cell populations that are apparently enriched or reside specifically in the oval cell-induced livers, with little or no reactivity shown in the normal liver. Identification of the corresponding antigen molecules, as well as further characterization of these cell populations, should expedite our understanding of the mechanisms of the oval cell response at the cellular and molecular levels.

Using flow cytometry-based cell separation methods in combination with the aforementioned cell surface markers, oval cells can be viably isolated and subjected

to *in vitro* culture to evaluate their proliferation and differentiation potentials. In general, oval cells isolated from injured livers proliferate to form colonies *in vitro* in the presence of certain combinations of growth factors, and the clonally expanded cells are capable of differentiating into both hepatocyte and cholangiocyte lineages under appropriate culture conditions. These results strongly suggest that oval cells indeed possess clonal bi-lineage differentiation potential, at least *in vitro*, a notion which needs to be evaluated using *in vivo* experimental systems as well. As oval cell antigens like EpCAM and CD133 are also expressed in cholangiocytes under uninjured conditions, the cells positive for these markers were also isolated from normal adult livers and similarly subjected to *in vitro* culture experiments (Okabe et al. 2009; Suzuki et al. 2008b). Interestingly, both EpCAM$^+$cells and CD133$^+$ cells isolated from the normal liver also formed colonies as well as underwent differentiation into hepatocytes and cholangiocytes. Thus, the normal adult liver harbors "potential hepatic stem cells", which can be defined as those with clonogenicity and bi-lineage differentiation potential *in vitro*, similar to H-CFU-C in the embryonic liver (Fig. 14.1b). Notably, EpCAM$^+$cells isolated from human postnatal livers, as well as fetal livers, have also been found to contain closely related hepatic stem cells (hHpSCs) that can be defined *in vitro* (Schmelzer et al. 2007). The exact location and character of these potential hepatic stem cell populations *in vivo*, as well as their possible contribution to homeostasis and/or regenerative process of the liver, remain to be elucidated. In particular, it is tempting to speculate that these cells may serve as the precursors for oval cells, which needs to be addressed in future studies.

14.4 Differentiation Capacity and Their Precursors

As has been mentioned repeatedly in the preceding sections, the characteristic feature of the liver stem/progenitor cells is their potential to differentiate into two lineages, i.e., hepatocytes and cholangiocytes (Fig. 14.1). In addition to these two hepatic cell lineages, much evidence has been accumulated supporting that the liver stem/progenitor cells are also capable of differentiating into pancreatic and other cell lineages both *in vitro* and *in vivo* under appropriate experimental settings. So far, circumstantial understanding of the mechanisms of liver stem/progenitor cell differentiation have been accomplished with regard to hepatoblasts in the developing liver, and herein we will focus mostly on this issue. Although oval cells have been shown to possess differentiation capacity to hepatocytes, cholangiocytes and other cell types, the underlying mechanisms have little been addressed.

14.4.1 Differentiation into Hepatocytes

By definition, hepatoblasts undergo during their development a fate decision between the hepatocyte and cholangiocyte lineages. The molecular basis of this hepato-biliary

lineage decision still remains largely unknown. Several molecules have been implicated in differentiation of hepatoblasts into the cholangiocyte lineage, which will be discussed in the next section.

While the adult liver exerts various metabolic functions, the fetal liver lacks such functions and instead serves as a hematopoietic organ. Around E10 in the mouse embryo, hematopoietic stem cells immigrate into the fetal liver from the aorta-gonad-mesonephros region and the placenta, and expand their population tremendously in the microenvironment provided by the fetal liver till birth. During this period of time, hematopoietic cells enhance differentiation of hepatoblasts into hepatocytes by producing cytokines (Kinoshita et al. 1999). As hematopoiesis switches from the fetal liver to the bone marrow, liver organogenesis progresses to become a center for metabolism.

Several *in vitro* primary culture systems for fetal liver cells, and more specifically for sorted hepatoblasts, have been established and extensively used to characterize the cellular and molecular mechanisms of hepatocyte diffirentiation. In many cases, oncostatin M (OSM), one of the interleukin 6-famiy cytokines, shows potent activity to induce differentiation of hepatoblasts and immature hepatocytes to functional hepatocytes, as evidenced by expression of various hepatocyte-specific marker genes and acquisition of metabolic functions such as cytosolic glycogen accumulation and ammonia clearance from the culture medium (Kamiya et al. 1999). OSM transduces signals through a specific receptor complex containing the gp130 subunit, and the liver of mice lacking gp130 show defects in functional differentiation of hepatocytes (Kamiya et al. 1999). As OSM receptor-deficient mice exhibit no obvious anomaly in the liver development, other cytokines may play a similar or redundant role (Tanaka et al. 2003). In addition to the OSM signals, HGF, extracellular matrices (ECMs), and cell-to-cell contacts have also been implicated in stimulating hepatocyte differentiation (Kamiya et al. 2002; Kojima et al. 2000; Suzuki et al. 2003). On the other hand, TNF-alpha has been shown to antagonize the differentiation-promoting activities of OSM and control the timing of hepatocyte maturation (Kamiya and Gonzalez 2004). Thus, TNF-alpha expression is detected in the liver until perinatal stages, and then decreases after birth. Concomitantly with this transition, hepatocytes are relieved from inhibitory effect of TNF-alpha and then strongly promoted to acquire mature metabolic functions.

In addition to these extracellular signals, hepatocyte differentiation and maturation are regulated by cell-intrinsic machineries involving various transcription factors. A set of transcription factors, such as HNF1alpha, HNF4, and CCAAT/enhancer binding protein (C/EBP) alpha, are known to be abundantly and characteristically expressed in hepatocytes and thus are collectively termed as "liver-enriched transcription factors". While studies using gene knockout mice have elucidated that each of these molecules has its own unique functions as manifested by observed specific phenotypes (Costa et al. 2003; Schrem et al. 2002, 2004), it has become evident that they function cooperatively to form a dynamic transcriptional network of autoregulatory and cross-regulatory loops (Kyrmizi et al. 2006; Lemaigre 2009). In addition, these liver-enriched transcription factors also interact with various other transcription factors and/or regulatory molecules in a context-dependent manner to

achieve specific target gene expression. For example, C/EBPalpha is an essential factor for glucose metabolism during the perinatal stage, and mice lacking this transcription factor die soon after birth due to hypoglycemia caused by defective gluconeogenic gene expression (Wang et al. 1995). Despite of this specific functional requirement at the perinatal stage, C/EBPalpha is already expressed in E14.5 fetal liver, suggesting that an additional factor may function cooperatively to ensure its temporally-regulated activity. Indeed, the forkhead family transcription factor Foxo1 has been found to be inducibly expressed in the perinatal liver, physically interact with C/EBPalpha, and augment C/EBPalpha-dependent transcription of a gluconeogenic gene, phosphoenolpyruvate carboxykinase (PEPCK) (Sekine et al. 2007). On the other hand, C/EBPalpha is also critical for ammonia detoxification activity of hepatocytes, as the knockout mice lack expression of carbamoyl phosphate synthetase-I (CPS1), a key enzyme in the urea cycle, leading to hyperammonemia (Kimura et al. 1998). Again, CPS1 is expressed only after the neonatal stage, and hence an involvement of some regulatory factor for C/EBPalpha-dependent CPS1 expression was suspected. In this case, Y-box binding protein-1 (YB-1) has been identified to be a molecule that suppresses C/EBPalpha function and negatively regulates CPS1 expression in the fetal liver (Chen et al. 2009). YB-1 is highly expressed in E14.5 fetal liver, and the expression significantly declines before birth. This results in the release of C/EBPalpha from YB-1-mediated suppression on the CPS1 promoter, leading to expression of CPS1 and ammonia clearance activity. Taken together, transcriptional activities of C/EBPalpha are differently controlled by expression and cooperative function of specialized "gatekeeper" molecules, Foxo1 and YB-1, for gluconeogenic and urea cycle enzymes, respectively.

As hepatocytes acquire mature metabolic functions, they also undergo structural maturation and establish specialized tissue architectures that are associated with their functions (Tanimizu and Miyajima 2007). They construct so-called "hepatocyte-type" epithelial polarity, where the apical surface, termed the bile canaliculus, is formed between neighboring hepatocytes, and the polarized hepatocytes are organized to form a cord-like structure. A study using gene knockout mice has suggested that the small GTPase ARF6 plays a critical role in the latter process (Suzuki et al. 2006).

14.4.2 Differentiation into Cholangiocytes

Cholangiocytes are epithelial cells that line the biliary tract. The biliary tract can be separated ontogenetically into two parts, that is, the extrahepatic bile duct and the intrahepatic bile duct (IHBD). The extrahepatic bile duct is comprised of the hepatic ducts, the cystic duct, the common bile duct, and the gallbladder, and develops from the endoderm independently of the hepatoblast formation. On the other hand, cholangiocytes forming the intrahepatic bile ducts derive from hepatoblasts, as mentioned earlier. It is not clear how the extrahepatic and intrahepatic biliary tracts, developing separately, eventually anastomose.

The process of IHBD formation from hepatoblasts involves cholangiocyte differentiation (lineage specification) and morphogenesis of ductal structures. In mouse embryos, the initial sign of cholangiocyte specification can be recognized at E11.5, when the cholangiocyte marker SRY-box containing gene 9 (Sox9) is expressed in liver cells that are located a short distance from the branches of the portal vein (Antoniou et al. 2009). These cells align around the portal vein to form a single-layered structure, called the ductal plate. At E15.5, the ductal plate becomes focally bilayered to form the primitive ductal structures (PDS), and lumens can be detected between the two layers. A recent study has suggested that the PDS are transiently asymmetrical, in that the cells on the portal-side layer express Sox9 but not the hepatoblast marker HNF4, while that those on the parenchymal-side layer express HNF4 but not Sox9 (Antoniou et al. 2009). By E18.5, the hepatoblasts lining the parenchymal side of the PDS differentiate to cholangiocytes, leading to formation of radially symmetrical duct structures entirely delineated by cholangiocytes. During this process, the ductal plate cells that are not involved in tubulogenesis regress and eventually disappear, and the remaining ducts become surrounded by periportal mesenchymal cells.

With regard to the molecular mechanisms involved in cholangiocyte differentiation, the roles of several transcription factors have been implicated, such as T-box transcription factor 3 (Tbx3), the Onecut transcription factors HNF6 and OC2, and HNF1beta. In Tbx3 knockout mouse embryos, hepatoblast proliferation is severely impaired and biliary differentiation is promoted at the expense of hepatocyte differentiation, suggesting that Tbx3 plays a role in hepato-biliary lineage decision (Ludtke et al. 2009; Suzuki et al. 2008a). Gene expression analyses have shown that Tbx3 functions to maintain expression of hepatocyte transcription factors, HNF4alpha and C/EBPalpha, while suppressing that of cholangiocyte transcription factors, HNF6 and HNF1beta (Ludtke et al. 2009). Mice deficient of HNF6 shows bile duct malformation, and this phenotype is further enhanced by combined knockout of HNF6 and OC2 (Clotman et al. 2002, 2005). A direct and critical target of HNF6 is HNF1beta, and mice with liver-specific inactivation of HNF1beta shows defect in bile duct development (Coffinier et al. 2002). The Onecut transcription factors also regulate hepato-biliary lineage decision of hepatoblasts by modulating transforming growth factor (TGF) beta signaling. In the normal liver, TGFbeta signaling is found to be strongly activated near the portal veins but weakly in the rest of the parenchyma. In the liver of HNF6 and OC2 double knockout mice, increased TGFbeta signaling is observed in the parenchymal region, where "hybrid" hepatic cells that display characteristics of both hepatocytes and cholangiocytes are generated (Clotman et al. 2005). Thus, the Oncecut transcription factors play a role in shaping the proper gradient of TGFbeta signaling activity to ensure induction of cholangiocytes only in the periportal region.

Another molecular mechanism well known to be involved in bile duct formation is the Notch signaling pathway. In humans, mutations in JAGGED 1 (JAG1), a ligand for the Notch receptors, are associated with Alagille syndrome (ALGS or ALGS1; Online Mendelian Inheritance in Man #118450), an autosomal dominant disorder characterized by multiple developmental defects including neonatal cholestasis caused by a paucity of IHBD (Li et al. 1997; Oda et al. 1997). In addition, another form of Alagille syndrome has been found to be caused by mutations in the NOTCH2 gene (ALGS2;

Online Mendelian Inheritance in Man #610205) (McDaniell et al. 2006). In accord with these notions, mice doubly heterozygous for a Jag1 null allele and a Notch2 hypomorphic allele recapitulate most of the human syndrome phenotypes, including the bile duct paucity (McCright et al. 2002). A study using an *in vitro* culture of mouse hepatoblasts has shown that activation of the Notch signaling pathway promotes differentiation of hepatoblasts into the cholangiocyte lineage by coordinating a network of liver-enriched transcription factors including HNF1alpha and beta, HNF4, and C/EBPalpha (Tanimizu and Miyajima 2004). More recently, *in vivo* studies using the aforementioned compound (doubly heterozygous) mouse mutant for Jag1 and Notch2, or the liver-specific Notch2 knockout mice, have suggested that the Notch2 signaling is rather required for bile duct morphogenesis, but is likely dispensable for cholangiocyte cell specification (Geisler et al. 2008; Lozier et al. 2008). Similarly, in fetal livers of mice lacking hairy and enhancer of split 1 (Hes1), a target of the Notch signaling, the ductal plate formation occurs normally but the subsequent remodeling and tubular structure formation is completely blocked (Kodama et al. 2004). Together with the fact that Jag1 is expressed in portal fibroblasts in the periportal region (Suzuki et al. 2008c), it is currently considered most plausible that portal fibroblasts stimulate cholangiocytes via the Jag1/Notch2 interaction and the downstream Hes1 expression, which leads to induction of ductal morphogenesis (Lemaigre 2009).

14.4.3 Differentiation into Non-hepatic Lineages

The liver and the pancreas share a common developmental origin, and a bipotential precursor cell population for these organs has been identified within the embryonic endoderm (Deutsch et al. 2001). In addition, hepatocytes and pancreatic beta-cells are known to have similarities in gene expression profiles and possess similar inherent glucose sensing systems, thereby being capable of responding to changes in blood glucose concentrations. Consistent with these facts, many studies have demonstrated that liver stem/progenitor cells from both embryonic and adult origins as well as hepatocytes can be converted to insulin-producing cells, functional pancreatic beta-cell-like cells, and/or to islet-like cell clusters containing other pancreatic lineages under certain conditions.

Clonally expanded H-CFU-C derived from fetal mouse liver show expression of pancreatic endocrine and exocrine lineage markers in culture, and can be integrated into and formed pancreatic ducts and acinar cells when transplanted into pancreas of recipient mice (Suzuki et al. 2002). Notably, H-CFU-C is also shown to be capable of differentiating into gastric and intestinal cells *in vivo*. Purified adult rat hepatic oval cells can be differentiated into pancreatic endocrine hormone-producing cells when cultured in a high-glucose environment (Yang et al. 2002). Rat liver epithelial WB cells, which represent the cultured counterpart of stem-like cells derived from normal adult liver, can be reprogrammed into functional insulin-producing cells by stable expression of pancreatic duodenal homeobox 1 (Pdx1) or its super-active form (Pdx1-VP16) (Tang et al. 2006). Epithelial progenitor cells derived from human fetal

liver (FH cells) can also be induced to differentiate into insulin-producing cells after expression of the PDX1 gene (Zalzman et al. 2003).

In addition to these *in vitro* experiments, several studies employing *in vivo* gene delivery systems have shown that adenoviral vector-mediated transduction of pancreatic transcription factors, such as Pdx1, Neurogenin3 (Ngn3), NeuroD, and MafA, can induce formation of ectopic islet-like cells and production of insulin in the adult liver (Ferber et al. 2000; Kojima et al. 2003; Song et al. 2007; Wang et al. 2007). Although these phenomena have been considered to represent trans-differentiation of mature hepatocytes into pancreatic cells, a recent study employing Ngn3 gene transfer in combination with a genetic lineage tracing have suggested an alternative possibility. Thus, introduction of this transcription factor can sufficiently induce emergence of ectopic, periportal islet-like cell clusters in streptozotocin (STZ)-induced diabetic model mice, and these clusters do not originate from differentiated hepatocytes but are rather likely produced by "trans-determination" of oval cell-like progenitor cells, which are lineage-determined but not terminally differentiated (Yechoor et al. 2009). In view of this, it is noteworthy that DDC-induced activation of hepatic oval cells *in vivo* has been reported to ameliorate STZ-induced diabetes in mice (Kim et al. 2007).

14.5 Potential Application for Therapies

At present, orthotopic liver transplantation is the most commonly used procedure to treat various liver diseases. This, however, has always been hampered by persistent shortage of donor organs. Although isolated mature hepatocytes when transplanted have been shown to successfully repopulate the recipient liver with considerably high efficiency at least in rodent models, the rates of engraftment and survival of transplanted hepatocytes in human liver is often very limited. Furthermore, despite that mature hepatocytes show tremendous proliferative activity in response to regenerative stimuli *in vivo*, they usually lose this capacity immediately once isolated and subjected for *in vitro* cultures. Thus, the ability to obtain an unlimited supply of human hepatocytes from any expandable source should significantly improve the development and clinical application of hepatocyte transplantation. In addition, it will also facilitate the studies on the basic mechanisms of human liver diseases, as well as evaluation of drugs for their actions and toxicities due to the metabolism of xenobiotics in hepatocytes. Considering the strong proliferative potential and amenability for *in vitro* manipulation, the liver stem/progenitor cells may be attractive candidates for these applications. These cells may also be useful for cell therapy to treat diabetic patients, given their potential to be effectively reprogrammed toward pancreatic lineages. However, isolation of fetal hepatoblasts and adult hepatic stem/progenitor cells from human liver for therapeutic use should be practically quite difficult.

In recent years, much effort and concomitant progress have been made in establishing the protocols to generate various types of functionally differentiated cells, including mature hepatocytes, *in vitro* from pluripotent stem cells such as

embryonic stem (ES) cells and induced pluripotent stem (iPS) cells (Basma et al. 2009; Snykers et al. 2009). This has led to the notion that application of the precise conditions that recapitulate the normal developmental process within the embryo is generally the best way to achieve highly functional derivatives. Thus, to produce hepatocytes for example, these pluripotent stem cells can be sequentially induced to differentiate to the definitive endoderm, then the hepatic lineage cells with the character of hepatoblasts, and finally to functional hepatocytes, directed by the timed use of appropriate amounts and combinations of cytokines (Gouon-Evans et al. 2006). In view of this, studies elucidating the mechanisms of the normal liver organogenesis, and particularly of hepatoblast development and differentiation, should offer an important clue to future development of a better protocol to induce functional hepatocytes *in vitro*. Notably, use of specific surface markers for hepatoblasts or other hepatic cells to enrich particular cell lineages in the course of induced differentiation should be advantageous to obtain hepatocytes with better quality and quantity. Moreover, this will also beneficial to eliminate undifferentiated stem cells that remain contaminated, as these cells may potentially cause tumors such as teratoma upon transplantation into recipients.

14.6 Conclusion and Future Development in Research

In recent years, considerable progress has been made in our understanding of the mechanisms of liver development at the cellular and molecular levels. Establishment of methods for *in vitro* culture of fetal liver cells, in particular the isolated hepatoblasts, as well as various mouse models with genetic modifications have invaluably contributed to identify and elucidate the role of genes involved in fetal liver development. Although characterization of adult liver stem/progenitor cells represented by oval cells has been less achieved up to the present compared to that of hepatoblasts, it has become much accelerated by the finding and availability of useful marker molecules. Further analyses on the extrinsic signals and the intrinsic genetic programs regulating these cell populations should lead to clarifying the molecular basis of liver regeneration, as well as its similarities and differences with that of liver development. In-depth understanding of the mechanisms governing these complicated and elaborated processes should definitely help establish a better protocol to generate functional hepatic cells amenable to therapeutic cell transplantation and pharmaceutical drug development.

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Retention in the Golgi apparatus and expression on the cell surface of Cfr/Esl-1/Glg-1/MG-160 are regulated by two distinct mechanisms

Yuichiro MIYAOKA*[1], Hidenori KATO*[1], Kazuki EBATO*[1], Shigeru SAITO*, Naoko MIYATA*, Toru IMAMURA† and Atsushi MIYAJIMA*[2]

*Laboratory of Cell Growth and Differentiation, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan, and †Signaling Molecules Research Group, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan

Cfr (cysteine-rich fibroblast growth factor receptor) is an Fgf (fibroblast growth factor)-binding protein without a tyrosine kinase. We have shown previously that Cfr is involved in Fgf18 signalling via Fgf receptor 3c. However, as Cfr is also known as Glg (Golgi apparatus protein)-1 or MG-160 and occurs in the Golgi apparatus, it remains unknown how the distribution of Cfr is regulated. In the present study, we performed a mutagenic analysis of Cfr to show that two distinct regions contribute to its distribution and stability. First, the C-terminal region retains Cfr in the Golgi apparatus. Secondly, the Cfr repeats in the extracellular juxtamembrane region destabilizes Cfr passed through the Golgi apparatus. This destabilization does not depend on the cleavage and secretion of the extracellular domain of Cfr. Furthermore, we found that Cfr with a GPI (glycosylphosphatidylinositol) anchor was predominantly expressed on the cell surface in Ba/F3 cells and affected Fgf18 signalling in a similar manner to the full-length Cfr, indicating that the interaction of Cfr with Fgfs on the cell surface is important for its function in Fgf signalling. These results suggest that the expression of Cfr in the Golgi apparatus and on the plasma membrane is finely tuned through two distinct mechanisms for exhibiting different functions.

Key words: cysteine-rich fibroblast growth factor receptor (Cfr), E-selectin ligand (Esl), fibroblast growth factor (Fgf), Golgi apparatus, mutagenesis.

INTRODUCTION

Cfr (cysteine-rich fibroblast growth factor receptor) was identified originally as a transmembrane protein with affinity for Fgf (fibroblast growth factor) 1 and Fgf2 by biochemical screening [1]. It binds to Fgfs via a large extracellular domain comprising 16 repeats of a unique motif called the Cfr repeat, but has a short intracellular domain consisting of only 13 amino acid residues [2]. Cfr has no homology with known Fgf-binding molecules including Fgfrs (Fgf receptors)with a tyrosine kinase, and its physiological function had remained unknown. Recently, we generated *Cfr*-deficient mice, and found that they died in the perinatal period and show growth retardation and skeletal phenotypes. Because these phenotypes were similar to those of *Fgf18*-deficient mice, we examined the interaction between Cfr and Fgf18 biochemically and genetically, and demonstrated that Cfr binds to Fgf18 to positively regulate Fgf18 signalling through Fgfr3c [3].

However, Cfr is also known as Glg (Golgi apparatus protein)-1 or MG-160, and accumulating evidence indicates that it occurs predominantly in the Golgi apparatus in various cells [4–6]. Furthermore, Cfr has been shown to bind at least two other molecules, E-selectin and TGFβ (transforming growth factor β), and is also known as Esl (E-selectin ligand)-1 and Ltbp (latent TGFβ-binding protein)-1 [7,8]. It was reported recently that Cfr binds to the TGFβ precursor in the Golgi apparatus to modify its maturation and secretion, regulating TGFβ function [9]. Because Cfr acts as an Fgf-binding protein, a ligand of E-selectin and a TGFβ-binding protein, its cellular position is critical for its function. To interact with extracellular cytokines Cfr must be on the cell surface, but it can also interact with cytokines produced in the cell in the Golgi apparatus as reported for TGFβ. To bind E-selectin, Cfr must be on the cell surface. Thus Cfr is a unique multifunctional molecule and its intracellular distribution must be regulated.

Previous studies have suggested that the C-terminal cytoplasmic tail is important for the intracellular distribution of Cfr. Truncation of the cytoplasmic tail caused partial translocation of rat Cfr on to the cell surface in CHO (Chinese hamster ovary) cells [10]. Moreover, there exists an alternatively spliced variant of Cfr with an extension of 24 amino acid residues to the cytoplasmic tail in primates and humans. Human Cfr with or without the extension is located in the Golgi apparatus and on the cell surface respectively [11]. However, the involvement of other regions of Cfr in the regulation of its intracellular localization has not been examined.

To address this issue, we first confirmed that the cytoplasmic tail is important for the expression of Cfr in the Golgi apparatus, but noticed that without the tail the protein was extremely unstable. A GPI (glycosylphosphatidylinositol)-anchored Cfr mutant was directed to the cell surface and enhanced Fgf18 signalling via Fgfr3c, suggesting that Cfr positively regulates Fgf signalling on the cell surface rather than in the Golgi apparatus. However, it was also unstable and deletion mutants revealed that the Cfr repeats in the juxtamembrane domain contribute to the instability. Although it is known that Cfr is proteolytically cleaved at the juxtamembrane region and its extracellular domain is secreted [8,12], this cleavage is not involved in the instability. Furthermore, we demonstrated that the insertion of the Cfr repeats into the juxtamembrane region of EpCAM (epithelial cell-adhesion

Abbreviations used: Cfr, cysteine-rich fibroblast growth factor receptor; EGFP, enhanced green fluorescent protein; EpCAM, epithelial cell-adhesion molecule; Esl, E-selectin ligand; Fgf, fibroblast growth factor; Fgfr, Fgf receptor; Glg, Golgi apparatus protein; GPI, glycosylphosphatidylinositol; GST, glutathione transferase; IRES, internal ribosomal entry site; LFA, leucocyte function-associated antigen; TGFβ, transforming growth factor β; Ltbp, latent TGFβ-binding protein; TGOLN2, *trans*-Golgi network protein 2; WST-1, water-soluble tetrazolium salt 1.

[1] These authors equally contributed to this work.

[2] To whom correspondence should be addressed (email miyajima@iam.u-tokyo.ac.jp).

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