eradication. Several recent studies have provided evidence of the utility and limitation of the cancer stem cell differentiation strategy by modulating the signaling pathways responsible for the differentiation of normal stem/progenitor cells.

2.2.1 Bone morphogenic protein signaling

Bone morphogenic protein (BMP) signaling is known to be activated during embryogenesis and to play a pivotal role in the differentiation of neural and intestinal stem cells (Varga and Wrana, 2005). BMPs belong to a subgroup of the transforming growth factor-β superfamily and activate signaling through the BMP-receptor (BMPR)-mediated phosphorylation of Smad proteins. Interestingly, recent studies have suggested the utility of BMPs to induce the differentiation of brain cancer stem cells and facilitate brain tumor eradication (Lee et al., 2008; Piccirillo et al., 2006). More recently, colorectal cancer stem cells have been shown to lack the expression of BMP4, and the administration of BMP4 enhanced the terminal differentiation, apoptosis, and chemosensitization of colorectal cancer stem cells (Lombardo et al., 2011). Interestingly, the effects of BMP4 on the differentiation of colorectal cancer stem cells appeared to be independent of the phosphorylation status of Smad, suggesting the importance of non-canonical signaling pathways activated by BMP4 for the differentiation of these cells.

2.2.2 Oncostatin M signaling

Oncostatin M (OSM) is a pleiotropic cytokine that belongs to the IL-6 family, which includes IL-6, IL-11, and leukemia inhibitory factor (LIF). These cytokines share the gp130 receptor subunit as a common signal transducer, and activate Janus tyrosine kinases and the signal transducer and activator of transcription 3 (STAT3) pathways. However, gp130 forms a heterodimer with a unique partner, for example, the IL6 receptor, LIF receptor, or OSM receptor (OSMR); thus, each cytokine uniquely induces a certain signaling pathway (Heinrich et al., 2003), and OSM is known to exploit distinct signaling in an OSMR-specific manner (Kinoshita and Miyajima, 2002). Of note, OSM is known to activate the hepatocytic differentiation program in hepatoblasts in an OSMR-specific manner (Kamiya et al., 1999; Kinoshita and Miyajima, 2002).

We recently identified that OSMR is expressed in a subset of liver cancer stem cells (Yamashita et al., 2010). Interestingly, OSMR-positive hepatocellular carcinoma (HCC) was characterized by the abundant expression of stem cell markers and poorly differentiated morphology, suggesting that OSMR is more likely to be expressed in HCC with stem/progenitor cell features (Yamashita et al., 2008a). Of note, the OSM-OSMR signaling pathway was maintained in these HCCs, and OSM induced hepatocytic differentiation in liver cancer stem cells (Fig. 5).

Unexpectedly, we identified that the hepatocytic differentiation of liver cancer stem cells by OSM resulted in enhanced cell proliferation *in vitro* and modest anti-tumor activity *in vivo* when administered alone. However, we have further demonstrated that OSM-mediated hepatocytic differentiation of liver cancer stem cells effectively suppresses HCC growth when combined with conventional chemotherapy. It is possible that OSM may boost the anti-tumor activity of 5-FU by "exhausting dormant cancer stem cells" through hepatocytic differentiation and active cell division (Fig. 6). A similar chemosensitization effect was observed in colorectal cancer stem cells differentiated by BMP4 administration (Lombardo et al., 2011).

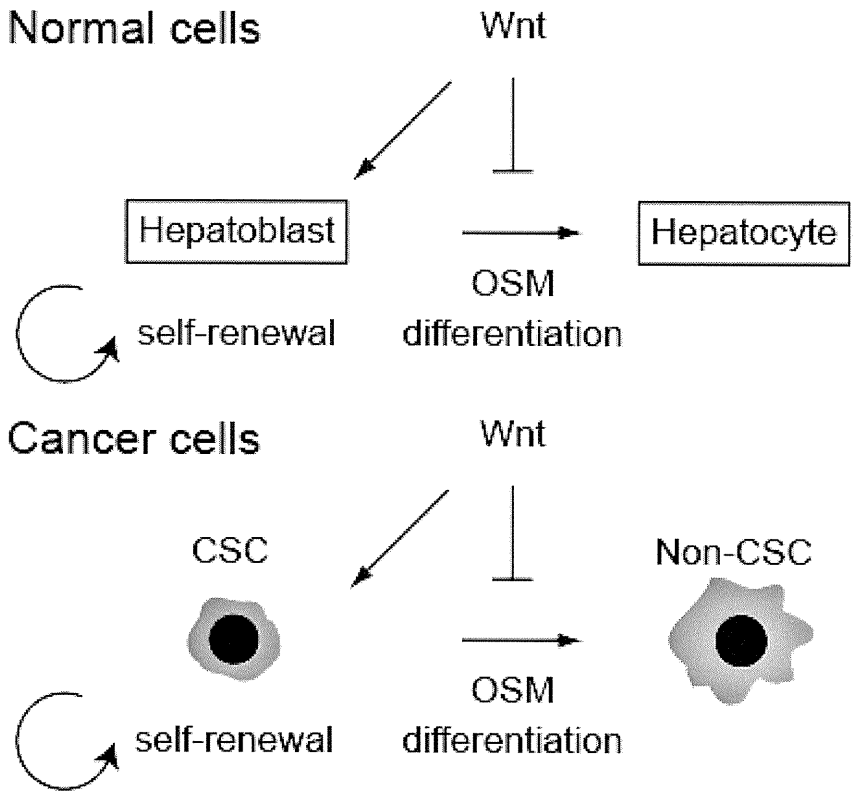

Fig. 5. Signaling pathways responsible for the self-renewal and differentiation of liver cancer stem cells. CSC, cancer stem cell; OSM, oncostatin M

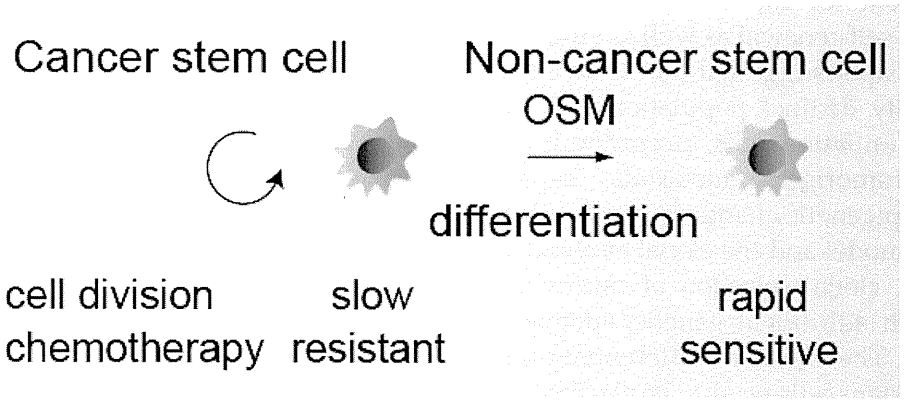

Fig. 6. Effect of oncostatin M (OSM) on exhausting dormant liver cancer stem cells

3. Limitation of cancer stem cell differentiation

As described above, some of the signaling pathways for the differentiation of normal stem cells may be maintained in cancer stem cells. To induce the differentiation of cancer stem cells by specific ligands, the expression of the corresponding receptors bound to ligands is clearly required, suggesting the importance of clarifying the mechanisms for receptor expression regulation. Interestingly, BMPRs and OSMR were detected in colorectal and liver

cancer stem cells, respectively, suggesting the possibility of ligand-induced differentiation therapy in the clinic. However, the expression of these receptors might be transcriptionally suppressed in a subset of cancers through methylation of their promoter regions (Deng et al., 2009; Kim et al., 2009; Lee et al., 2008). Indeed, a recent study suggested that BMP-mediated brain cancer stem cell differentiation failed in a subset of brain tumors in which BMP receptor promoters were methylated and silenced (Lee et al., 2008). Therefore, cancer stem cells may acquire resistance against differentiation therapy by additional epigenetic changes during the differentiation treatment.

It has been postulated that both normal stem cells and cancer stem cells are dormant and show slow cell cycles. Consistently, cancer stem cells are considered to be more resistant to conventional cytotoxic chemotherapeutic agents than non-cancer stem cells, possibly due to slow cell cycles as well as the increased expression of ATP-binding cassette (ABC) transporters, robust DNA damage responses, and activated anti-apoptotic signaling (Bao et al., 2006; Dean et al., 2005; Viale et al., 2009). Therefore, the induction of differentiation programs in cancer stem cells may result in cell proliferation of the tumor. Indeed, we recently demonstrated that differentiation of liver cancer stem cells by OSM increased cell proliferation, at least *in vitro* (Yamashita et al., 2010). Our data clearly suggested the necessity of conventional chemotherapy in addition to differentiation therapy to eradicate non-cancer stem cells originating from cancer stem cells. Furthermore, although the combination of OSM and conventional chemotherapy effectively inhibited tumor growth in our model, we did not observe tumor shrinkage (Yamashita et al., 2010). If both progenitors derived from a cancer stem cell lose their self-renewal capacity by the induction of differentiation, the tumor should subsequently shrink following the depletion of cancer stem cells. However, it is possible that ligand-based differentiation programs cannot completely inhibit the self-renewal programs of target cancer stem cells. Thus, the induction of differentiation in cancer stem cells with the eradication of non-cancer stem cells might not be sufficient for the eradication of the tumor, which may suggest the importance of inhibiting self-renewal as well as stimulating the differentiation of cancer stem cells.

A recent paper suggested that leukemia-initiating cells are composed of genetically diverse, functionally distinct populations (Notta et al., 2011), suggesting the clonal evolution of leukemia-initiating cells. Accordingly, cancer stem cells in solid tumors may also have a distinct tumorigenic/metastatic capacity as well as chemoresistance with certain genetic/epigenetic changes in each subclone as a result of clonal evolution. Thus, the cancer stem cell model and the clonal evolution model are not considered to be mutually exclusive. Therefore, clonal selection of cancer stem cells resistant to differentiation therapy might occur with additional genetic/epigenetic changes during treatment as a result of clonal evolution. The effects of differentiation therapy on the clonal evolution or genetic diversity of cancer stem cells need to be clarified in the future.

4. Conclusion

The recent re-emergence of the cancer stem cell hypothesis has provided novel insights on the effect of differentiation programs on cancer stem cells for the potential eradication of tumors. Although the activation of several signaling pathways by certain cytokines may be effective for the differentiation of cancer stem cells, their utility and limitation for tumor eradication should be clarified in future to provide novel therapeutic opportunities for cancer patients.

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Chapter 16
Heterogeneity of Liver Cancer Stem Cells

Taro Yamashita, Masao Honda, and Shuichi Kaneko

Abstract Hepatocellular carcinoma (HCC) is an aggressive disease with a dismal outcome. Although considered to be monoclonal in origin, HCC has heterogeneous pathologies and genetic/genomic profiles, suggesting that HCC may initiate in different cell lineages. Recent advances in cancer and stem-cell biology have revealed similarities between organogenesis and tumorigenesis, including hierarchical organization dictated by a subset of cells with stem-like features termed cancer stem cells (CSCs). Several hepatic stem/progenitor markers have been shown to be useful for the isolation of putative CSCs from HCC, although the expression patterns and phenotypic diversity of CSCs purified by these markers are still elusive. Here, we summarize the current knowledge of liver CSCs and discuss their heterogeneity and commonality.

Keywords Cancer Stem Cell · Liver Development · Hepatocellular Carcinoma · Epithelial-Mesenchymal Transition

1 Introduction

Embryogenesis and tumorigenesis share similar features including autonomous cell proliferation, motility, homing, dynamic morphologic changes, cellular heterogeneity, and interactions with the microenvironment. Indeed, carcinogenesis could be described as deregulated malignant organogenesis mediated by abnormally proliferating and/or metastatic cancer cells and activated stromal cells that trigger angiogenesis, fibrosis, and inflammation at the site. Cancer cells and stem cells have similar capabilities with respect to self-renewal, limitless division, and generation of heterogeneous cell populations. These observations have resulted in the hypothesis that cancers are transformed stem cells with arrest of maturation (Potter 1978; Sell 1993). Although the origin of cancer is still a controversial issue, the

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cancer stem-cell (CSC) concept, i.e., that a subset of cells bearing stem-cell like features are indispensable for tumor development and perpetuation, has recently been revived and supported by accumulating evidence (Clarke et al. 2006). Because both embryogenesis and tumorigenesis are a continuous process of self-renewal, asymmetric division, and differentiation, various molecules are thought to be concomitantly and gradually regulated, which results in the generation of heterogeneous populations expressing various stem/maturation markers. In this chapter, we would like to discuss the heterogeneity and phenotypic diversity of liver CSCs as well as hepatic stem/progenitor cells.

2 Liver Development and Stem-Cell Marker Expression

2.1 Early Stages of Embryogenesis

Embryogenesis is characterized by the ordered emergence of an organism made up of a multitude of stem and differentiated cells, and various signaling pathways play crucial roles in organogenesis where dynamic cell proliferation and motility arises (Slack 2008). The first differentiation event during mammalian development is the formation of the inner cell mass at the blastula stage (Fig. 16.1). Embryonic stem cells (ES cells) are located and can be successfully isolated from the inner cell mass of the blastula (Murry and Keller 2008). Before the blastula stage, early embryonic cells appear to have no capability for self-renewal; therefore, the most primitive markers of fetal tissue stem cells should be expressed first in this inner

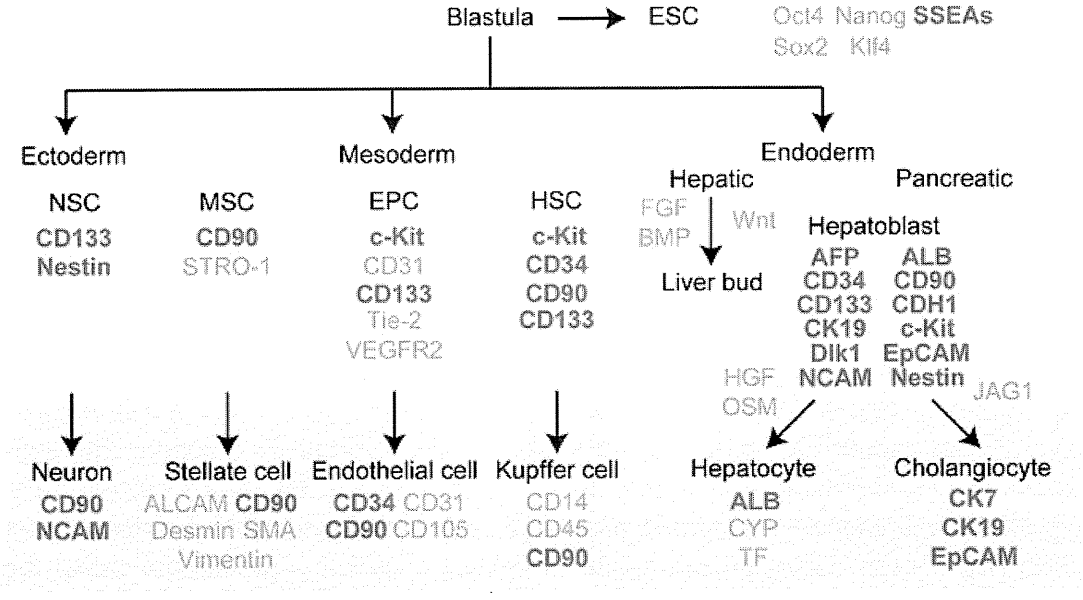

Fig. 16.1 Liver development, stem/progenitor cell markers, and activated signaling. Stem/progenitor cell markers expressed in various cells constituting the liver (neurons, stellate cells, endothelial cells, Kupffer cells, hepatocytes, and cholangiocytes) are shown. Reported hepatic stem/progenitor cell markers are indicated as bold. ESC: embryonic stem cell; NSC: neural stem cell; MSC: mesenchymal stem cell; EPC: endothelial progenitor cell; HSC: hematopoietic stem cell

cell mass at the blastula stage (Slack 2008). Several markers are reported to be expressed at this stage by ES cells, including OCT4, Klf4, Sox2, SSEAs, and Nanog (Graf and Stadtfeld 2008). The importance of these genetic regulators was dramatically demonstrated by their induction of pluripotency in fibroblasts (Takahashi et al. 2007a, 2007b).

2.2 Hepatic Specification

The formation of tissue-specific stem cells is believed to occur at the stage when the three germ layers (endoderm, mesoderm, and ectoderm) are developed from the blastula (Fig. 16.1). The primitive endodermal cells are thought to generate both hepatic and pancreatic stem cells (Murry and Keller 2008). Liver specification signaling is activated at the ventral endoderm (hepatic endoderm) by paracrine secretion of fibroblast growth factor (FGF) and bone morphogenic protein (BMP) from the cardiac mesoderm and septum transversum, respectively (Calmont et al. 2006; Rossi et al. 2001; Zaret and Grompe 2008). Recent findings suggest that Wnt/β-catenin signaling also induces hepatic specification (Ober et al. 2006). Activation of these signaling pathways results in the formation of the liver bud from the hepatic endoderm. The liver bud is considered to be the earliest developmental stage of liver organogenesis, and albumin and alpha-fetoprotein (AFP) are known to be expressed at this stage (Dabeva and Shafritz 2003). Once the hepatic endoderm is specified and the liver bud begins to grow, the cells are called hepatoblasts and have the ability to differentiate into hepatic and biliary lineages. Thus, hepatoblasts are at least bipotent progenitors developed from hepatic endoderm (Fausto 2004).

2.3 Hepatocytic Differentiation

Several cytokines/growth factors are known to be involved in the differentiation of hepatoblasts into hepatocytes (Kinoshita and Miyajima 2002). Oncostatin M (OSM), an interleukin 6-related cytokine produced by CD45+ hematopoietic cells, enhances glucocorticoid mediated hepatocytic differentiation through the activation of the signal transducer and activator of transcription 3 (STAT3) pathway (Kamiya et al. 1999). Hepatocyte growth factor (HGF) is also known to be activated during the process of liver regeneration; and treatment with HGF induces hepatocytic differentiation in hepatoblasts, although the detailed mechanism is still unclear (Kinoshita and Miyajima 2002).

Hepatocytes adjacent to the periportal area are more likely to be responsible for the regeneration and spread of the liver into the pericentral area, and hepatocytes adjacent to the centrilobular region are considered to have a more mature hepatocyte-like phenotype that includes production of serum proteins, e.g., albumin (ALB), alpha-1 antitrypsin, and transferrin (TF), and activation of enzymes involved in xenobiotic metabolism, e.g., cytochrome P450 (CYP) (Fig. 16.1). Thus, markers associated with hepatic liver function, such as production of serum proteins and metabolism of various substrates, are generally used to evaluate liver maturation.

2.4 Hepatocytes as Stem Cells

Once fully developed, hepatocytes in the adult liver have a life expectancy of over a year; and most of hepatocytes remain quiescent and stay in the G0 phase. However, when parenchymal cells are lost, hepatocytes exit the G0 phase and start to proliferate. Hepatocytes are known to have the ability to proliferate almost indefinitely in rodents (Oertel and Shafritz 2008; Overturf et al. 1997). Furthermore, hepatocytes have the potential to differentiate into biliary lineages under special conditions with activation of hepatocyte growth factor/epidermal growth factor (HGF/EGF) signaling (Limaye et al. 2008; Michalopoulos et al. 2005). Thus, in light of the definition of stem cells, hepatocytes have similar features in terms of the potential for self-renewal, differentiation, and unlimited cell proliferation. However, transplanted hepatocytes cannot repopulate the liver without injury and do not behave as stem cells do under normal conditions (Oertel and Shafritz 2008).

2.5 Biliary Differentiation

Cholangiocytes are bile duct epithelial cells developed from hepatoblasts, and defects in bile duct formation result in the impairment of bile flow, or cholestasis. Previous studies demonstrated that mutations in the Jagged1 (JAG1) gene cause Alagille syndrome, which is characterized by cholestasis and jaundice due to intrahepatic bile duct abnormalities (Li et al. 1997; Oda et al. 1997). Consistently, several studies have demonstrated that the JAG1–Notch signaling pathway plays a crucial role in the differentiation of hepatoblasts into cholangiocytes (Lozier et al. 2008; Tanimizu and Miyajima 2004). Once developed, cholangiocytes are considered to be mitotically dormant and express specific cytokeratins such as CK7 and CK19. However, in the course of chronic liver diseases, cholangiocytes as well as hepatic progenitor cells may start to proliferate in response to stimuli to form ductular reactions in the periportal area (Roskams et al. 2004b), although phenotypes as well as markers expressed on cells of the various cholangiocyte lineages are largely unknown.

3 Heterogeneity of Stem-Cell Marker Expression in Hepatic Progenitor Cells

3.1 Putative Hepatic Stem/Progenitor Cell Markers

Hepatoblasts are considered to have the features of stem cells with respect to self-renewal and asymmetric division, and can repopulate normal and injured liver. Similar small primitive epithelial cells are known to emerge in the periportal area of the injured adult liver when hepatocyte replication is blocked, and these cells are called oval cells (in rodents) or hepatic progenitor cells (Roskams et al. 2003).

Hepatoblasts and hepatic progenitor cells express the biliary markers cytokeratin 19 (CK19) and epithelial cell adhesion molecule (EpCAM) as well as the hepatocyte markers albumin and AFP (Oertel and Shafritz 2008; Schmelzer et al. 2006, 2007; Sell 2003). In addition, numerous studies have demonstrated that hepatic progenitor cells express a variety of markers putatively detected in various ectodermal or mesodermal lineages, including nestin [neural/mesenchymal] (Koenig et al. 2006; Niki et al. 1999; Roskams et al. 2004a), NCAM [neural/mesenchymal] (Roskams et al. 2004a; Schmelzer et al. 2006), CD34, and c-Kit [hematopoietic] (Crosby et al. 2001), CD133 [neural/hematopoietic/mesenchymal] (Kordes et al. 2007; Suzuki et al. 2008), CD90 (Thy-1) [hematopoietic/mesenchymal] (Masson et al. 2006; Weiss et al. 2008), E-cadherin [epithelial] (Nitou et al. 2002), and Dlk1 [epithelial/hematopoietic] (Jensen et al. 2004; Khurana and Mukhopadhyay 2008; Oertel et al. 2008; Tanimizu et al. 2003) (Fig. 16.1). Indeed, these markers are expressed in neural, hematopoietic, mesenchymal, and epithelial stem/progenitor cells that can give rise to neurons, stellate cells, Kupffer cells, endothelial cells, hepatocytes, and cholangiocytes (Dudas et al. 2009; Escribano et al. 1998; Gangenahalli et al. 2006; Gilyarov 2008; Wauthier et al. 2008). Thus far, it is unclear how these markers are expressed in hepatic stem/progenitor cells at a particular developmental stage or whether the expression status of these markers is associated with their functional phenotypes. It is also unclear which would be the most primitive marker detected in hepatic stem cells that can generate relatively differentiated hepatic progenitor cells.

3.2 Heterogeneity of Hepatic Progenitor Cells

Hepatic stem/progenitor cells are considered a heterogeneous population (Jelnes et al. 2007) that is potentially organized in a hierarchical manner with various degrees of differentiation that may be related to their expression of stem-cell markers. Furthermore, the expression status of the stem/maturation markers would be altered to form a gradient, which potentially makes any isolated cell population arbitrary and heterogeneous. Therefore, identification of hepatic progenitor cells using a robust marker is crucial to understanding the heterogeneity of liver lineages during the development/regeneration processes. However, there is a great deal of controversy about the status of marker expression and the cellular phenotypes of stem/progenitor cells.

For example, AFP is one of the earliest markers detected in the liver bud, suggesting that AFP is detected in the most primitive hepatic stem cells. However, recent publications indicate that AFP might not be expressed in EpCAM-positive putative hepatic stem cells from adult and fetal liver that can give rise to AFP-positive hepatoblasts (Schmelzer et al. 2006, 2007). CD90 is a marker detected in both hepatic and hematopoietic stem/progenitor cells, and a recent report suggested that CD90+ cells from adult human livers showed the immunophenotype of CD34+ c-Kit+ CK14+ CK19+ HepPar1+ hepatic progenitors with the capability to engraft in the liver of immunodeficient mice (Weiss et al. 2008). However, another recent study investigating the characteristics of oval cells using EpCAM and CD90

reported that CD90+ cells are more likely to have the features of hepatic stellate cells or activated myofibroblasts in a 2-acetylaminofluorene/partial hepatectomy rat model (Yovchev et al. 2008). Another group also raised a question about the capability of CD90-positive embryonic day (ED) 14 fetal liver cells to repopulate the liver (Oertel et al. 2007). Intriguingly, a recent paper identified a human fetal liver cell population of CD90+ EpCAM+ cells that can give rise to both mesenchymal and hepatic lineages (human fetal liver multipotent progenitor cells (hFLMPCs)) with the immunophenotype of CD34+, CD90+, c-kit+, EpCAM+, c-met+, SSEA-4+, CK18+, CK19+, albumin−, AFP−, CD44h+, and vimentin+ (Dan et al. 2006). Thus, even with the robust stem-cell markers that are widely used for the isolation of hepatic progenitor cells, there are many controversial issues including the differentiation status and the repopulation capability of hepatic progenitors expressing a certain stem-cell marker.

3.3 Factors Affecting the Heterogeneity of Putative Hepatic Stem/Progenitor Cells

Several factors may contribute to the controversy over the phenotypes of the hepatic stem/progenitor cells described above. First, the expression status of stem-cell markers is thought to be gradually altered in hepatic progenitor cells of various developmental stages in the fetal liver. Given this situation, purification of stem-cell marker-positive cells in the fetal liver could be arbitrary and may produce mixtures of hepatic stem/progenitor cells if different thresholds are used. Therefore, the cellular phenotypes of isolated cells could be stem- or progenitor-like depending on the abundance of each cell type, which may be related to the developmental stage of the fetal liver used for isolation.

Second, hematopoiesis takes place in the fetal liver, and various stem/progenitor cells including hematopoietic and mesenchymal cells have emerged and could be co-isolated to various degrees according to the developmental stage of the fetal liver. Indeed, all markers currently used for the isolation of hepatic stem/progenitor cells are not specific to a certain lineage (e.g., c-Kit and CD34 serve as hematopoietic markers; CD90, as a hematopoietic and mesenchymal marker; nestin, as a neural marker; AFP, as a hepatocytic marker; and EpCAM, as a biliary marker) (Fig. 16.1). Therefore, purified putative hepatic stem/progenitor cells may include cells of various lineages, making analysis of the phenotypes of the isolated cells difficult and controversial.

Third, hepatic stem/progenitor cells as well as hematopoietic/mesenchymal stem cells may exhibit cellular plasticity. If hepatic/mesenchymal/hematopoietic stem cells can trans-differentiate into one another, a hepatic stem-cell population isolated using a certain marker could easily be a mixture of hepatic, mesenchymal, and hematopoietic lineages depending on their culture conditions. Consistently, recent papers have suggested the phenotypic reversion of fetal human liver epithelial cells and hematopoietic cells into mesenchymal and epithelial lineages, respectively (Inada et al. 2008; Khurana and Mukhopadhyay 2008).

Fourth, although stem cells' self-renewal and differentiation are tightly regulated by the microenvironment (called the stem-cell niche), the phenotypes as well as potential markers of niche cells (presumed to be located in the "canals of Hering," bile canaliculi lined partially by hepatocytes and partly by cholangiocytes) are still under debate (Kuwahara et al. 2008). The phenotypes of isolated hepatic stem/progenitor cells may be different if these cells are isolated from different niches.

Taken together, the expression patterns of stem-cell markers currently used for the isolation of hepatic stem/progenitor cells are very heterogeneous and may be distinct and time-dependent at the various developmental stages of the liver lineages. Cellular plasticity of isolated stem/progenitor cell populations as well as co-purification of mesenchymal/hematopoietic stem cells may further complicate the results of liver transplantation experiments even if rigorously purified cells are used. Accurate phenotyping of fetal liver cells using robust markers and reliable isolation/culture systems may provide more detailed information about the process of human liver lineages differentiation.

4 Liver Cancer as a Disease of Deregulated Stem Cells

4.1 The CSC Concept

Although considered monoclonal in origin, tumor cells are heterogeneous in terms of morphology, clinical behavior, and molecular profiles (Fialkow 1976; Vogelstein and Kinzler 2004). This heterogeneity has been explained by the clonal evolution of tumor cells resulting from the progressive accumulation of multiple genetic changes (Hanahan and Weinberg 2000). However, recent data have suggested that heterogeneity may also be due to derivation of the tumor cells from stem/progenitor cells residing in the organ (Jordan et al. 2006). The concept of cancer as an abnormal stem-cell disease was proposed many years ago on the basis of the similar capabilities of cancer cells and normal stem cells to self-renew, produce heterogeneous progeny, and divide in an unlimited fashion (Wicha et al. 2006).

The CSC concept, that a subset of cells bearing stem-cell like features are indispensable for tumor development, has recently been revived by the advancement of stem-cell biology (Clarke et al. 2006). Accumulating evidence suggests the involvement of CSCs in the perpetuation of various cancers including leukemia, breast cancer, brain cancer, and colon cancer (Al-Hajj et al. 2003; Bonnet and Dick 1997; Lessard and Sauvageau 2003; O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Singh et al. 2004). Experimentally, putative CSCs have been purified using cell-surface markers specific for normal stem cells, and stem-cell like features have been confirmed by in vitro clonogenicity and in vivo tumorigenicity assays. CSCs are considered more metastatic and resistant to drugs and radiation than non-CSCs within the tumor, and these findings warrant the development of treatment strategies that can specifically eradicate CSCs (Dean et al. 2005; Rich, 2007).

4.2 Hepatocellular Carcinoma as a Disease of Stem Cells

Recent results of cancer genome sequencing revealed that about ten or more genes are mutated and may be responsible for the development of colorectal cancers (Sjoblom et al. 2006; Wood et al. 2007), which could hardly happen in differentiated colonic epithelial cells because the majority of these cells are exfoliated in a short period of time (Boman and Huang 2008). Thus, it is now widely believed that gastrointestinal cancer is a disease of stem cells with aberrant genetic/epigenetic changes (Takaishi et al. 2008; Zou 2008). However, the cellular origin of HCC is still a controversial issue because both hepatocytes and stem/progenitor cells may reside in the injured liver for a long period to acquire genetic/epigenetic changes.

In rodents, accumulating evidence suggests that HCC may originate from oval cells as well as hepatocytes. For example, HCC developed in the "Solt-Farber" model is thought to originate from oval cells, whereas HCC arising from diethylnitrosamine (DEN) treatment appears to originate from hepatocytes (Sell 2002). Transgenic models that used the albumin promoter to express c-Myc, E2F1, TGF-α, or c-Myc/TGF-α resulted in the formation of HCC in mice, suggesting a role for these genes in the development of HCC that mainly originates from hepatocytes (Calvisi and Thorgeirsson 2005). On the other hand, when fetal hepatoblasts were isolated from TP53-/- mice by cell sorting using E-cadherin antibodies and transformed by c-Myc, Akt, or Ras, they developed HCCs with a mixture of HCC and CC cellular types (Zender et al. 2006). Furthermore, single hepatoblasts transformed by β-catenin or Bmi1 also developed HCC with mixed cellular types in immunodeficient mice (Chiba et al. 2007).

Altogether, the above observations strongly suggest that HCC may originate from stem/progenitor cells as well as hepatocytes. HCC developed from progenitor cells appears to be a mixed population of HCC and CC with various degrees of expression of hepatic and biliary lineage markers, suggesting that these HCCs continue to maintain the ability to differentiate into both hepatic and biliary lineages.

4.3 Putative Liver Cancer Stem-Cell Markers

The generally acknowledged definition of a CSC is as a cell within a tumor that possesses the capability to self-renew and to give rise to the heterogeneous lineages of cancer cells that comprise tumors in immunodeficient mice (Clarke et al. 2006). Thus far, the expression of six markers, i.e., side population (SP), ATP-binding cassette protein (ABC) G2, CD133, CD90, OV6, and EpCAM, has been experimentally proven in the population of CSCs in human HCCs; and these markers have been used for the isolation of putative liver CSCs.

4.3.1 SP Fraction

The ability to effectively carry out efflux of dyes was first demonstrated in bone marrow cells, and these cells were termed SP cells because they were found to the side

of the peak containing the bulk of dye-positive cells in fluorescent activated cell sorting (FACS) analysis plots (Goodell et al. 1996). These bone marrow cells are highly enriched for long-term repopulating hematopoietic stem cells, and since then, SP cells have been identified in a variety of normal and tumor tissues (Challen and Little 2006). The ability to regulate the efflux of Hoechst dyes appears to be conferred in part through the expression of ATP-binding cassette protein (ABC) transporters because treatment with the ABC transporter inhibitor verapamil reduces the number of cells in the SP fraction (Wu and Alman 2008). The degree of efflux activity appears to correlate inversely with the maturation state, and the cells exhibiting the highest efflux activity appear to be the most primitive. Chiba et al. investigated the existence of the SP fraction in four HCC cell lines using Hoechst 33342 dye and identified the SP fraction in HuH7 and PLC/PRL5 cells (Chiba et al. 2006). They demonstrated that AFP+ CK19+ cells are enriched in the SP fraction and form tumors more efficiently compared with non-SP cells in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. SP cells isolated from HCC cell lines may be related to the metastatic and chemoresistant capability of these tumors (Shi et al. 2008), and may have activation of anti-apoptotic signaling through Bcl-2 and Bax regulation (Fan et al. 2007).

4.3.2 ABCG2

Resistance to chemotherapeutic agents is one of the hallmarks of CSCs (Dean et al. 2005), and ABC transporters are believed to play a central role in the efflux of chemical reagents. Especially, the ABC transporter ABCG2 is believed to be essential to pump out Hoechst dyes and maintain the SP fraction (Zhou et al. 2001). Zen et al. (2007) investigated the expression of ABCG2 in two human HCC cell lines and showed a hierarchy of cancer cells with respect to ABCG2 expression. Expression of AFP and CK19 is mainly detected in ABCG2+ HCC cells, and ABCG2+ cancer cells are detected in clinical HCC specimens. ABCG2 expression may be related to doxorubicin resistance, and Akt signaling may affect chemoresistance through alteration of the subcellular localization of ABCG2 (Hu et al. 2008).

4.3.3 CD133 (Prominin 1)

CD133 was the first identified member of the prominin family of pentaspan membrane proteins recognized by an AC133 monoclonal antibody and was originally classified as a marker of primitive hematopoietic stem cells (Yin et al. 1997). In addition, CD133 is known to be expressed in neural, hepatic, colonic, and endothelial stem/progenitor cells (Mizrak et al. 2008). Discrepancies exist concerning the gene expression status (AC133 mRNA) and the protein levels recognized by AC133 antibody, possibly because of the glycosylation status of CD133 and the existence of a splice variant (termed as AC133-2).

Expanding evidence highlights the utility of CD133 as a marker for CSCs in various human tumors including brain, colon, pancreas, and prostate cancers (Visvader and Lindeman 2008). Ma et al. (2007) investigated the processes of liver regeneration using a partial hepatectomy model to identify the emergence of CD133+ cells

in the resected liver. They further discovered that CD133+ cells act as CSCs that are chemoresistant in HCC (Ma et al. 2007, 2008b). Similar findings were reported by several groups (Suetsugu et al. 2006; Yin et al. 2007), and a recent paper further suggested that an aldehyde dehydrogenase (ALDH) + cell population among the CD133+ cells may be more tumorigenic CSCs (Ma et al. 2008a).

4.3.4 CD90 (Thy-1)

CD90 is a glycosylphosphatidylinositol (GPI)-anchored protein that is particularly abundant on the surface of thymocytes and T cells; but CD90 is also expressed in various cell types including fibroblasts, endothelial cells, neurons, and hematopoietic cells (Rege and Hagood 2006). Yang et al. (2008b, 2008c) recently investigated the expression of CD90 and CD44 in CD45-depleted primary HCC cells and peripheral blood mononuclear cells and identified that CD45– CD90+ tumor cells were more tumorigenic if they expressed CD44, Oct4, Bmi1, Albumin, AFP, Wnt3a, stat3, and HIF-1α . They further demonstrated that CD90+ CD44+ cells were more aggressive than CD90+ CD44– cells, and CD44 blockage prevented tumor formation by the CD90+ cells. On the basis of these data, the authors suggested that the presence of CD45– CD90+ cells in a population could be used as a marker for human liver cancer and as a target for the diagnosis and therapy of HCC.

4.3.5 OV6

Anti-OV6 is one of the monoclonal antibodies previously developed against cells isolated from carcinogen-treated rat liver (Dunsford and Sell 1989) and is known to react with oval cells and normal bile duct epithelial cells (Van Den Heuvel et al. 2001). The antigen recognized by anti-OV6 monoclonal antibodies in human liver has not yet been determined (Strain et al. 2003). Yang et al. (2008a) recently isolated OV6+ cells from human HCC to demonstrate that OV6+ cells have CSC-like features such as high tumorigenic capability and chemoresistance (Yang et al. 2008a). The authors further showed that OV6+ cells were characterized by the activation of Wnt/β-catenin signaling and inactivation of this signaling pathway resulted in a decrease in the OV6+ cell population. These results suggest that Wnt/β-catenin signaling may be a good target for eradication of OV6+ liver CSCs.

4.3.6 EpCAM (Epithelial Cell Adhesion/Activating Molecule, CD326)

EpCAM is one of the first tumor-associated antigens identified (Herlyn et al. 1979) and has numerous synonyms including 17-1A, HEA125, MK-1, GA733-2, EGP-2, EGP34, KSA, TROP-1, ESA, and KS1/4. EpCAM is expressed in a large variety of human adenocarcinomas and squamous cell carcinomas (Went et al. 2006), but the function as well as the regulatory mechanisms of EpCAM expression remained largely unknown to date (Balzar et al. 1999). We recently showed that the expression of the EpCAM gene (TACSTD1) is activated by Wnt/β-catenin signaling in a cis-regulatory mechanism (Yamashita et al. 2007). Furthermore, a very recent paper

suggested that the EpCAM intracellular domain is cleaved at the cell membrane and associates with β-catenin and Lef-1 in the nucleus to activate Wnt/β-catenin signaling (Maetzel et al. 2009). These data suggest that EpCAM is not just a cell-surface molecule, but a signal transducer regulated by Wnt/β-catenin signaling in a positive-feedback manner.

EpCAM is used for the isolation of CSCs from various tumors including colonic and pancreatic cancers (Visvader and Lindeman 2008). We recently used EpCAM and AFP to identify the novel prognostic HCC subtypes related to a certain developmental stage of human liver lineages (Yamashita et al. 2008). Furthermore, we isolated EpCAM+ HCC cells from primary HCC samples and cell lines to show that EpCAM+ cells have the features of CSCs (Yamashita et al. 2009). Activation of Wnt/β-catenin signaling enriched the population of EpCAM+ CSCs, and blockage of EpCAM expression resulted in the inhibition of tumor formation by EpCAM+ cells in NOD/SCID mice. Thus, EpCAM seems a potentially useful marker and a good target for isolation and elimination of liver CSCs.

4.4 Heterogeneity of Liver Cancer Stem Cells

Although the markers listed above have been shown to be useful for the isolation of putative CSCs, it is unclear how these markers are expressed in primary HCC tissues or in HCC cell lines. It is also unclear whether the CSCs expressing these markers exist in all or are restricted to a certain subtype of HCCs. Furthermore, primary HCC tissues are composed of mixtures of mesenchymal/endothelial/inflammatory cells as well as tumor epithelial cells, and isolation of CSCs using such markers may result in the isolation of mixtures of tumor epithelial and stromal cells, a problem similar to that observed in the isolation of normal hepatic stem/progenitor cells (Fig. 16.2). Because recent findings have suggested the significance of stromal cells in tumorigenesis and metastasis of cancer (Dome et al. 2009; Karnoub et al. 2007; Mishra et al. 2008), it is possible that co-isolation of stromal cells may result in an enhanced tumorigenicity in immunodeficient mice that may not be related to the stem-like traits of tumor epithelial cells.

We recently investigated the expression of EpCAM (epithelial), CD133 (epithelial/hematopoietic/neural/endothelial), and CD90 (hematopoietic/mesenchymal/endothelial) in six HCC cell lines (Yamashita et al. 2009). Interestingly, AFP+ HCC cell lines (Hep3B, HuH1, and HuH7) have a subpopulation of EpCAM+ CD133+ cells but no CD90+ cells. In contrast, AFP– HCC cell lines (SK-Hep-1, HLE, and HLF) have a subpopulation of CD90+ cells but no EpCAM+ or CD133+ cells. Thus, AFP+ HCC cells may be more likely to have a subpopulation of epithelial CSC-markers+ cells (epithelial CSCs), whereas AFP– HCC cells may have a subpopulation of mesenchymal-markers+ cells (mesenchymal CSCs) (Fig. 16.2). These data suggest that CSC markers may not be equally expressed in all HCCs. Instead, the expression patterns of CSC markers in liver CSCs may be different in each HCC subtype, possibly due to the heterogeneity of activated signaling