別紙５

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Yamashita T, Honda M, Kaneko S.（金子）	Heterogeneity of Liver Cancer Stem Cells.	Xin W. Wang Joe W. Grisham Snorri S. Thorgeirsson	Molecular Genetics of Liver Neoplasia	Springer	New York	2010	301-318

雑誌

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V. 研究成果の刊行物・別刷

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

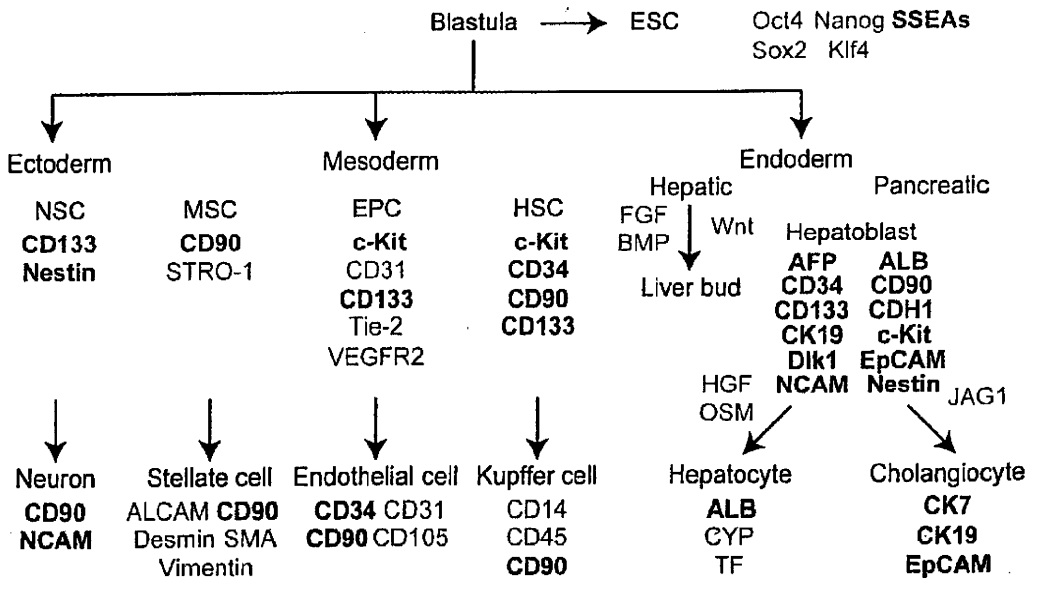

Fig. 16.1 Liver development, stem/progenitor cell markers, and activated signaling. Stem/progenitor cell markers expressed in various cells constituting the liver (neurons, stellate cells, endothelial cells, Kupffer cells, hepatocytes, and cholangiocytes) are shown. Reported hepatic stem/progenitor cell 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 (Deán 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