Engels, B., Engelhard, V.H., Sidney, J., Sette, A., Binder, D.C., Liu, R.B., Kranz, D.M., Meredith, S.C., Rowley, D.A., and Schreiber, H. (2013). Cancer Cell *23*, this issue, 516–526.

Johnson, L.A., Morgan, R.A., Dudley, M.E., Cassard, L., Yang, J.C., Hughes, M.S., Kammula, U.S., Royal, R.E., Sherry, R.M., Wunderlich, J.R., et al. (2009). Blood *114*, 535–546.

Schreiber, H. (2013). Cancer Immunology. In Fundamental Immunology, Seventh Edition, W.E. Paul, ed. (Philadelphia, PA, USA: Lippincott Williams and Wilkins, a Wolters Kluwer business), pp. 1200–1234.

Schreiber, H., and Rowley, D.A. (2008). Science *319*, 164–165.

Schumacher, T.N. (2002). Nat. Rev. Immunol. *2*, 512–519.

Stone, J.D., Chervin, A.S., and Kranz, D.M. (2009). Immunology *126*, 165–176.

Standing Out from the Crowd: Cancer Stem Cells in Hepatocellular Carcinoma

Bruno Sainz, Jr.[1] **and Christopher Heeschen**[1,*]
[1]Stem Cells and Cancer Group, Molecular Pathology Programme, Spanish National Cancer Research Centre (CNIO), Madrid 28029, Spain
*Correspondence: cheeschen@cnio.es
http://dx.doi.org/10.1016/j.ccr.2013.03.023

Cancer stem cells (CSCs) drive solid tumor formation. In this issue of *Cancer Cell*, Zhao and colleagues identify the calcium channel α2δ1 subunit as a new functional hepatocellular carcinoma (HCC) CSC biomarker, which is vital for CSC biology as blocking α2δ1 in combination with doxorubicin treatment hinders HCC tumor formation.

Hepatocellular carcinoma (HCC) accounts for 90% of primary liver cancers and is the third most common cause of cancer-related deaths worldwide (Edwards et al., 2010). Unlike most other carcinomas, where mutations in specific oncogenes or tumor suppressors drive tumor initiation and progression, the majority of HCCs are multifactorial and primarily due to infections with hepatitis B virus (HBV) or hepatitis C virus (HCV). However, worldwide cases of nonviral HCC are on the rise due to growing numbers of patients with metabolic liver diseases (Alberti et al., 2005; Van Thiel and Ramadori, 2011). This multi-causality makes identification and subsequent targeting of a common HCC-specific alteration or even a cell-of-origin virtually impossible. Fortunately, where consensus does exist is in the concept that the majority of HCC arise from a subpopulation of cancer cells referred to as tumor-initiating cells (TICs) or cancer stem cells (CSCs) (Majumdar et al., 2012). Thus, identifying and therapeutically targeting these cells represents a more feasible approach for treating HCC regardless of the underlying cause.

CSCs are believed to possess stem cell-like properties such as unlimited self-renewal, exclusive in vivo tumorigenicity, and subsequent generation of differentiated progeny recapitulating the parental tumor phenotype (Figure 1). Evidence for their existence in several solid tumors has been experimentally demonstrated (reviewed in Hermann et al., 2010). For HCC, cells expressing diverse markers such as CD133, CD13, CD24, CD90, and EpCAM as well as cells defined as the side population have all been demonstrated to bear CSC characteristics. Apparently, the utility of these different markers across established cell lines and primary tumors varies significantly, and their suitability for therapeutic targeting has not been extensively evaluated. Therefore, the identification of markers, preferably a single marker, for efficient isolation of CSCs from the complex tumor cellular environment across different HCC tissues is still critically needed.

In this issue of *Cancer Cell*, Zhao et al. (2013) report that HCC CSCs can be specifically isolated with a new antibody (1B50-1) identified using a whole-cell subtractive immunization approach that recognizes the isoform 5 of the cell surface calcium channel α2δ1 subunit. 1B50-1 binds a subpopulation of HCC cells, hereafter termed $\alpha2\delta1^+$ cells, exhibiting stem cell-like properties, such as increased invasiveness, expression of stem cell-associated genes (*OCT4*, *SOX2*, *NANOG*, and *BMI1*), increased self-renewal, and the ability to give rise to both $\alpha2\delta1^+$ and $\alpha2\delta1^-$ cells. More importantly, the authors showed that subcutaneously injected $\alpha2\delta1^+$ cells from cell lines and primary HCC tumors were more tumorigenic in NOD/SCID mice compared to their $\alpha2\delta1^-$ counterparts. Although the increased tumorigenic potential of $\alpha2\delta1^+$ cells was evident with as little as 10^3 cells, limiting dilution assays (injection with less than 100 cells were not performed) revealed that not all $\alpha2\delta1^+$ cells were tumorigenic (TIC frequency in primary cases: 1 in 458 [748-281]), and higher numbers of $\alpha2\delta1^-$ cells were also capable of forming tumors (TIC frequency: 1 in 1,957 [3,785-1,012]) (calculated from Table 1 in Zhao et al., 2013). Therefore, $\alpha2\delta1^+$ cells from primary tumors were enriched for CSCs 4-fold.

Unlike many normal tissues where a stringent unidirectional hierarchy and strict balanced asymmetric division preserve tissue integrity (Jan and Jan, 1998), data in solid tumors are generally not as clear cut. On the one hand, this might be related to our still limited ability

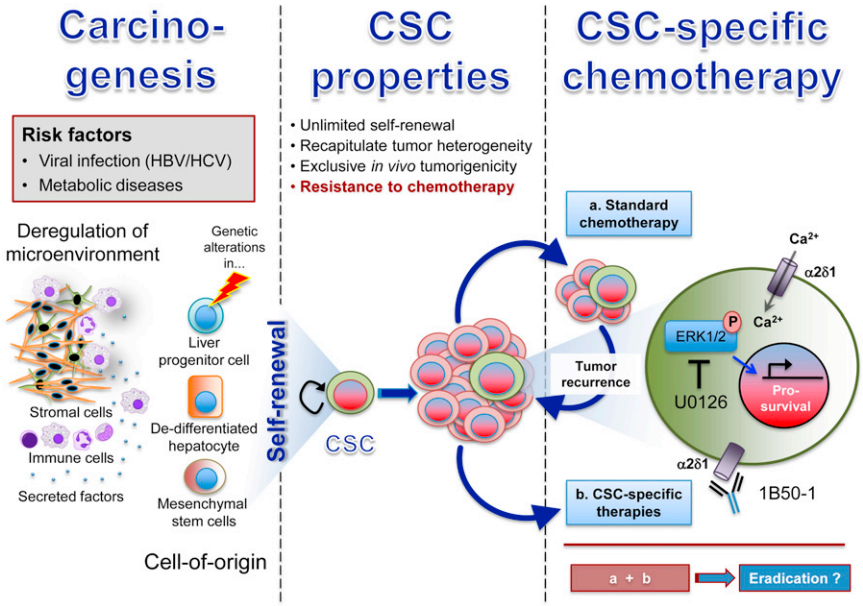

Figure 1. The CSC Concept

HCC, like many other solid tumors, is composed of a heterogeneous population of cells that contain CSCs. Although the process of hepatocarcinogenesis is multi-causal and multi-factorial, HBV and HCV infection, as well as metabolic liver diseases are the primary mediators of HCC. During tumor initiation, liver progenitor cells, hepatocytes, or circulating bone marrow-derived cells are believed to suffer genetic and epigenetic changes, which, together with a deregulation of the microenvironment (e.g., chronic inflammation and cirrhosis), eventually give rise to a distinct subpopulation of CSCs that have stem-like properties. CSCs can survive current standard therapies, resulting in tumor recurrence and disease relapse. The work by Zhao et al. (2013) suggests that HCC CSCs overexpress the calcium channel α2δ1 subunit, and thus targeting α2δ1 (e.g., using 1B50-1), or its downstream mediators (e.g., inhibition of ERK1/2), and in combination with standard chemotherapy may eradicate HCC.

for thoroughly dissecting the hierarchical organization of solid tumors. Indeed, Zhao et al. (2013) also showed a clear hierarchical organization within the $\alpha2\delta1^+$ sub-population for HCC, demonstrating that $\alpha2\delta1^+$ cells additionally expressing CD133, CD13, or EpCAM were even more tumorigenic in vivo. However, these marker combinations remain challenging, because $\alpha2\delta1^+$ cells that are negative for the second marker and vice versa also result in a moderate enrichment for CSC, and double negative populations are still not void of CSC activity (Figure 1H in Zhao et al., 2013). On the other hand, the border between tumorigenic CSCs and their nontumorigenic progenies might be more dynamic than in normal tissue, and intermediate progenitors in cancer tissue might be capable of replenishing tumorigenic CSCs. The latter should be more stringently tested in vitro and in vivo at the single cell level in order to identify the specific subset of $\alpha2\delta1^+$ cells with exclusive tumorigenic potential as well as to thoroughly evaluate the possibility that a subset of $\alpha2\delta1^-$ cells is capable of replenishing $\alpha2\delta1^+$ CSCs. Furthermore, we also should not underestimate the important role of the tumor microenvironment that could dramatically alter a cell's in vivo tumorigenic potential (Lonardo et al., 2012; Quintana et al., 2008).

While these studies remain pending, Zhao et al. (2013) further showed that in 86 paired HCC and paracancerous tissue samples, $\alpha2\delta1^+$ cells could be found not only in the majority of primary HCC samples but also in many of the corresponding paracancerous tissues. Moreover, when sorted, only $\alpha2\delta1^+$ cells from both the primary tumor and paracancerous tissues were tumorigenic in vivo (even though the number of tested samples was very small) and could recapitulate the parental tumor phenotype. Interestingly, although $\alpha2\delta1^+$ staining in the primary tumor did not correlate with any clinicopathological factor, $\alpha2\delta1^+$ staining in the paracancerous tissue strongly correlated with cirrhosis, quicker recurrence, and shorter survival, supporting the potential use of $\alpha2\delta1^+$ staining in paracancerous tissue as a prognostic HCC biomarker. Although the present study was not really powered to model the multifactorial panorama of HCC and therefore more extensive studies are still warranted, this study clearly supports the hypothesis that $\alpha2\delta1^+$ cells in paracancerous tissue represent a putative cell-of-origin for HCC recurrence.

Next, the authors determined the function of α2δ1 in HCC CSC biology. α2δ1 is a protein component of the voltage-dependent calcium channel complex, of which there exist several types. Calcium influx is an essential cellular process mediating a plethora of intracellular signaling cascades such as MAPK signaling. Not only was α2δ1 over-expressed in $\alpha2\delta1^+$ cells, but also intracellular calcium concentrations and calcium oscillations were higher in $\alpha2\delta1^+$ cells and could be modulated by 1B50-1 treatment or α2δ1 silencing. In addition, ERK1/2 phosphorylation was suppressed by α2δ1 inhibition, suggesting that α2δ1 may potentiate HCC CSC by activating pro-survival pathways mediated by MAPKs via a calcium-dependent mechanism. Indeed, the authors show that $\alpha2\delta1^+$ cells were also susceptible to the ERK1/2 inhibitor U0126. Therefore, these findings highlight not only α2δ1 as a marker amenable for HCC CSC isolation but also a previously unappreciated role for Ca^{2+} influx in CSC biology.

Finally, one of the most interesting aspects of the present study centered on the therapeutic potential of targeting α2δ1. Zhao et al. (2013) convincingly demonstrate that treatment of HCC cells with 1B50-1 or silencing of α2δ1 decreases CSC activity via induction of cellular apoptosis by downregulation of BCL2 and upregulation of BAX and BAD. Importantly, 1B50-1 not only reduced tumor size and induced cell apoptosis but also affected the CSC content, as determined by loss of $\alpha2\delta1^+$ cells and loss of serial transplantation capacity in NOD/SCID mice. Importantly, the effects could be further augmented by the addition of doxorubicin, supporting a bimodal treatment approach in which CSCs and their progenies are simultaneously targeted. This kind of combinatorial approach has also been proposed for other solid tumors, such as pancreatic ductal adenocarcinoma (Lonardo et al., 2011), and may also reflect the need for blocking replenishment of CSCs from their more

differentiated $\alpha2\delta1^-$ progenies. Based on these promising results, studies focusing on the use of 1B50-1 or other agents that target $\alpha2\delta1$ in HCC and potentially in other solid tumors should be extensively pursued.

REFERENCES

Alberti, K.G., Zimmet, P., and Shaw, J. (2005). Lancet *366*, 1059–1062.

Edwards, B.K., Ward, E., Kohler, B.A., Eheman, C., Zauber, A.G., Anderson, R.N., Jemal, A., Schymura, M.J., Lansdorp-Vogelaar, I., Seeff, L.C., et al. (2010). Cancer *116*, 544–573.

Hermann, P.C., Bhaskar, S., Cioffi, M., and Heeschen, C. (2010). Semin. Cancer Biol. *20*, 77–84.

Jan, Y.N., and Jan, L.Y. (1998). Nature *392*, 775–778.

Lonardo, E., Hermann, P.C., Mueller, M.T., Huber, S., Balic, A., Miranda-Lorenzo, I., Zagorac, S., Alcala, S., Rodriguez-Arabaolaza, I., Ramirez, J.C., et al. (2011). Cell Stem Cell *9*, 433–446.

Lonardo, E., Frias-Aldeguer, J., Hermann, P.C., and Heeschen, C. (2012). Cell Cycle *11*, 1282–1290.

Majumdar, A., Curley, S.A., Wu, X., Brown, P., Hwang, J.P., Shetty, K., Yao, Z.X., He, A.R., Li, S., Katz, L., et al. (2012). Nat Rev Gastroenterol Hepatol *9*, 530–538.

Quintana, E., Shackleton, M., Sabel, M.S., Fullen, D.R., Johnson, T.M., and Morrison, S.J. (2008). Nature *456*, 593–598.

Van Thiel, D.H., and Ramadori, G. (2011). J Gastrointest Cancer *42*, 191–194.

Zhao, W., Wang, L., Han, H., Jin, K., Lin, N., Guo, T., Chen, Y., Cheng, H., Lu, F., Fang, W., et al. (2013). Cancer Cell *23*, this issue, 541–556.