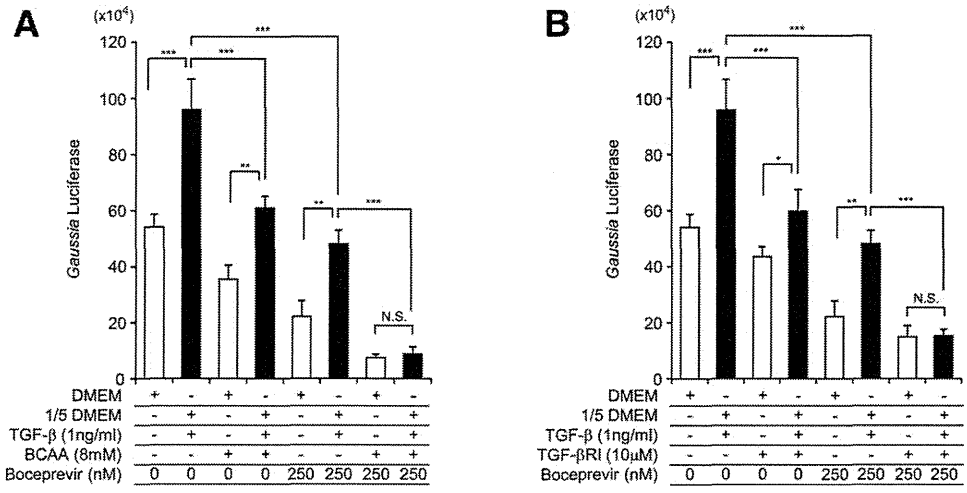

Fig. 8. Anti-HCV activity of boceprevir in combination with BCAAs (A) and TGF-β1 RI (B). HCV replication in Huh-7.5 cells was deduced by *Gaussia* luciferase activity. Boceprevir in combination with BCAAs (A) and TGF-β1 RI (B) efficiently repressed HCV replication in Huh-7.5 cells treated with amino acid depletion (1/5 DMEM) and TGF-β1. The experiments were performed in triplicate and repeated 3 times ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

therapy comprising PEG-IFN, RBV, and DAA (e.g., telaprevir or boceprevir) has significantly increased SVR rates; however, its efficacy is poor in difficult-to-cure patients such as those with cirrhosis and the IL28B treatment-resistant genotype.[2,4] An IFN-free regimen using a combination of DAAs would be effective to treat these difficult-to-cure patients; however, the emergence of multiple drug resistant viruses and the high cost of these therapies should be considered carefully in the future. Therefore, standard PEG-IFN plus RBV combination therapy is still useful as an alternative therapy for CH-C.

Previously, we reported that malnutrition in patients with the advanced fibrosis stage of CH-C is associated with IFN resistance and impaired IFN signaling by inhibiting mTORC1 and activating Socs3-mediated IFN inhibitory signaling through the nutrition-sensing transcriptional factor Foxo3a.[6] However, the effect of profibrosis signaling on IFN signaling was not addressed in our previous study. In the present study, using clinical samples and cell lines, we clearly showed that TGF-β signaling inhibits IFN signaling by activating Foxo3a-Socs3-mediated IFN inhibitory signaling (Figs. (1 and 4)) and inhibiting mTORC1 signaling (Fig. 5).

Using Foxo3a promoter-luciferase reporter constructs, we showed that TGF-β1 activated Foxo3a promoter activity through an AP1 transcription factor binding site. Among the components of AP1, c-Jun and probably ATF2, but not c-Fos, were involved in this induction. Previous reports showed that c-Jun and ATF2 were induced by amino acid depletion[13,14] and TGF-β1 treatment,[15,16] although the induction of c-Jun by amino acid depletion was not obvious in PHH in this study. It could be considered that malnutrition and profibrotic signaling cooperatively activated the Foxo3a promoter through the AP1 site and that c-Jun induction was more specifically regulated by TGF-β1 in normal hepatocytes. Mutation of the AP1 binding site (pGL4-FOXO3a [-1340-MT]) abolished the response to amino acid depletion (1/5 DMEM) and TGF-β1 treatment (Fig. 3E; Supporting Fig. 2). Conversely, c-Jun overexpression combined with amino acid depletion (1/5 DMEM) and TGF-β1 treatment activated the Foxo3a promoter by 32-fold (Fig. 3F). In addition, we showed that TGF-β1 inhibited mTORC1 signaling, as demonstrated by the decreased expression of RHEB, p-mTOR, and p-p70S6K (Fig. 5A).

These results were in concordance with gene expression in the liver of CH-C patients. The expression of c-Jun and ATF2 was significantly correlated with Smad2 and Foxo3a expression, respectively (Fig. 4), while the expression of RHEB was significantly negatively correlated with Smad2 expression in the liver of CH-C patients (Fig. 5C). In this study, TGF-β1 and TGF-β2 expression was up-regulated in advanced liver fibrosis, and the expression of TGF-β2 was well correlated with the downstream signaling molecule Smad2 (Fig. 1B-D). Although we could not address the biological differences in TGF-β isoforms in this study, TGF-β1 and TGF-β2 reportedly mediate a similar signaling pathway to induce profibrotic responses.[17] Collectively, TGF-β signaling inhibited IFN signaling by activating Foxo3a-Socs3 IFN inhibitory signaling and

inhibiting mTORC1-IFN stimulating signaling *in vitro* and *in vivo*. Recently, Lee et al. showed that Foxo3a regulates the TGF-β1 promoter directly.[18] Combining their data and ours, there must be positive feedback regulation between TGF-β1 and Foxo3a. Moreover, they identified a polymorphism in Foxo3a (rs12212067: T > G) in which the minor (G) allele was involved in the increased production of TGF-β1 and associated with the inflammatory response.[18] We genotyped the Foxo3a rs12212067 polymorphism in three cell lines and observed TT in Huh-7 and Huh-7.5 and GG in TTNT (Supporting Table 3). Although we could not find a significant difference in Foxo3a promoter activity in response to TGF-β1 among these cell lines (Supporting Fig. 2), further studies should be performed to compare Foxo3a-Socs3 IFN inhibitory signaling among them. Furthermore, it is worthwhile to examine the relationship between the genotype at rs12212067 and treatment response and severity of liver disease in CH-C patients in the future.

Another interesting finding in this study was that TGF-β signaling was related to the IL28B genotype (Fig. 6). The expression of c-Jun was significantly higher in IL28B treatment-resistant minor genotype (TG/GG at rs8099917) patients than in IL28B treatment-sensitive major genotype (TT) patients. Moreover, the expression of c-Jun, Smad2, ATF2, and Socs3 was up-regulated more in IL28B minor genotype patients than in IL28B major genotype patients, especially in those with early stage liver fibrosis (F1-2). The underlying mechanisms of these findings are not known so far; however, we recently reported that the noncanonical WNT signaling ligand WNT5A is up-regulated in the liver of IL28B minor genotype patients and plays a role in treatment resistance.[19] WNT5A reportedly mediates downstream signaling through c-Jun and ATF2 in *Xenopus* cells and human osteosarcoma cells.[20,21] It could be speculated that WNT5A potentiates TGF-β signaling through these transcription factors, although this hypothesis should be tested in the future.

We examined whether BCAAs and TGF-β RI improve the IFN inhibitory signaling induced by malnutrition and TGF-β signaling (Fig. 7). Previously, we demonstrated that BCAAs improved the IFN signaling that was inhibited by malnutrition.[6] In the present study, we found that BCAAs blocked TGF-β signaling by decreasing the levels of p-Smad3L, p-JNK, and c-Jun (Fig. 7A). Consequently, BCAAs decreased the expression of Foxo3a, Socs3, and HCV core protein (Fig. 7). In addition, we found that the combination of BCAAs or TGF-β RI and the NS3 protease inhibitor boceprevir efficiently inhibited HCV replication and canceled the positive effects of malnutrition and TGF-β1 on HCV replication (Fig. 8). A recent report showed that the NS3 protease of HCV mimics TGF-β2 and activates the TGF-β type I receptor.[22] Therefore, the anti-HCV effect of boceprevir could be potentiated in combination with BCAAs or TGF-β RI, which blocked TGF-β signaling and increased IFN signaling. Therefore, the combination of BCAAs or TGF-β RI with DAAs could be useful for the treatment of difficult-to-cure CH-C patients with advanced liver fibrosis and the IL28B treatment-resistant genotype.

In conclusion, we clarified that TGF-β signaling inhibits IFN signaling and is related to the treatment-resistant phenotype of CH-C patients with advanced liver fibrosis and the IL28B treatment-resistant genotype. Furthermore, blocking TGF-β signaling by BCAAs or TGF-β RI could potentiate the anti-HCV effect of DAAs. An oral TGF-β RI small compound, LY2157299, is now being assessed in a phase II trial for the treatment of advanced-stage HCC. Further studies should be performed to address the significance of these compounds for the eradication of HCV in patients with advanced liver fibrosis for preventing HCC.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website.

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REVIEW

Orchestration of hepatocellular carcinoma development by diverse liver cancer stem cells

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Abstract Hepatocellular carcinoma (HCC) is one of the world's most aggressive diseases and carries a poor prognosis for patients. Recent evidence suggests that HCC is organized by cancer stem cells (CSCs), which are a subset of cells with stem cell-like features. CSCs are considered a pivotal target for the eradication of cancer, and liver CSCs have been investigated using various stem cell markers. Several hepatic stem/progenitor markers have been shown to be useful for isolating putative CSCs from HCC, although the expression patterns and phenotypic diversity of CSCs purified by these markers remain obscure. Recently, we found that liver CSCs defined by different markers show unique features of tumorigenicity and metastasis, with phenotypes closely associated with committed liver lineages. Furthermore, our data suggest that these distinct CSCs collaborate to orchestrate the tumorigenicity and metastasis of HCC. In this review article, we summarize the recent advances in understanding the pathogenesis and heterogeneity of liver CSCs.

Keywords Hepatocellular carcinoma · Cancer stem cell · Tumorigenicity · Metastasis

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death from cancer worldwide [1]. Its prevalence is mostly attributed to hepatitis B virus or hepatitis C virus infection, and high incidence is observed in Asia and Africa [2]. Increasing occurrences and mortality from HCC have also been observed in most industrialized countries [3]. Therefore, there is an urgent need to develop effective diagnostic and treatment strategies against this disease.

HCC is a heterogeneous disease in terms of morphology, biological behavior, response to treatment, and molecular profile [4]. This heterogeneity has traditionally been explained by the clonal evolution of tumor cells resulting from the progressive accumulation of multiple genetic and epigenetic changes [5, 6]. However, recent studies suggest that its heterogeneity may result from the hierarchical organization of tumor cells by a subset of cells with stem and progenitor cell features known as cancer stem cells (CSCs) [7]. CSCs are highly tumorigenic, metastatic, chemo- and radiotherapy resistant, responsible for tumor relapse after therapy, and able to divide symmetrically or asymmetrically to orchestrate the tumor mass [8]. Therefore, they are considered to be a pivotal target for eradicating HCC [9]. In this review, we summarize recent findings on liver CSCs in terms of heterogeneity and discuss an HCC treatment strategy that targets them.

CSC hypothesis

Cancer cells and stem cells have similar capabilities with respect to self-renewal, limitless division, and the generation of heterogeneous cell populations. The observation of these similarities many years ago led to the proposal that

cancer might be a type of abnormal stem cell disease [10], a concept which has recently been revisited [11]. The generally acknowledged definition of a CSC is a cell within a tumor that possesses the ability to self-renew and to give rise to heterogeneous lineages of cancer cells that comprise tumors in immunodeficient mice [11]. Experimentally, putative CSCs have been isolated using cell surface markers specific for normal stem cells. Stem cell-like features of CSCs have been confirmed by functional in vitro clonogenicity and in vivo tumorigenicity assays. Moreover, accumulating evidence suggests that CSCs play a role in perpetuating various cancers including leukemia and solid tumors [12–18].

In HCC, several markers are reported to enrich the CSC population, including the epithelial cell adhesion molecule (EpCAM), CD133, CD90, CD44, CD24, CD13, and oval cell marker OV6, as well as Hoechst dye efflux or aldehyde dehydrogenase activities [19–25]. Most of these markers are expressed in normal hepatic progenitors known as oncofetal markers [20–22, 26–35]. These marker-positive cells were experimentally confirmed to be more tumorigenic than marker-negative cells in immunodeficient mice using cell lines [9]. Among them, calcium channel α2δ1 isoform5, EpCAM, CD90, and CD133 are the markers confirmed thus far to enrich CSCs from primary HCCs [36, 37]. Recent studies have shown that some of these liver CSC markers are also functionally involved in the maintenance of CSC features (Table 1). EpCAM enhances Wnt signaling in ES cells and cancer [38, 39], and CD133 expression may maintain $CD133^+$ liver CSCs through the activation of neurotensin/IL-8/CXCL1 signaling [40]. CD44 regulates the redox status [41], while CD13 decreases cell damage induced by oxidative stress after exposure to genotoxic reagents [19]. Furthermore, a recent study demonstrated that the calcium channel α2δ1 isoform5, recognized by a monoclonal antibody 1B50-1, is expressed in liver CSCs and regulates calcium influx and ERK signaling [37]. Thus, the functional involvement of most liver CSC markers potentially makes them a good target for the eradication of liver CSCs. In particular, cell surface markers detected in liver CSCs may be good targets for immunotherapy.

Table 1 Cell surface markers in liver CSCs

Cell surface markers	Function in CSCs
Calcium channel α2δ1 isoform5	Calcium influx and activation of ERK signaling
CD13	ROS-induced DNA damage reduction
CD133	Neurotensin-interleukin-8-CXCL1 signaling
CD24	STAT3 mediated NANOG regulation
CD44	Regulation of redox status through xCT
CD90	Unknown
DLK1	Unknown
EpCAM	Activation of Wnt signaling
OV6	Unknown

Heterogeneity of liver CSCs

As described above, various hepatic progenitor markers have been detected in the population of liver CSCs. Purified cell populations using certain stem cell markers show CSC features such as high tumorigenicity, an invasive nature, and chemo- and radiotherapy resistance. However, it is unclear how these markers are expressed in primary HCC tissues or HCC cell lines. It is also unclear whether the CSCs expressing these markers exist in all HCCs or are restricted to a certain subtype. This is an especially important issue when treating HCC patients using molecularly targeted therapy against certain marker-positive CSCs.

In normal fetal livers, hepatoblasts express the biliary markers CK19 and EpCAM, as well as the hepatocyte markers albumin and alpha fetoprotein (AFP) [26, 27, 42, 43]. 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, NCAM, CD34 and c-Kit, CD133, CD90, E-cadherin, and Dlk1 [44]. Hepatoblasts are also considered a heterogeneous population potentially organized in a hierarchical manner with various degrees of differentiation that may be related to their expression of stem cell markers [45]. Indeed, recent studies demonstrated that the characteristics of hepatic progenitors expressing different markers show distinct natures [32, 46]. Normal $EpCAM^+$ and $CD90^+$ oval cells represent two distinct populations: the former expresses classical oval cell markers such as AFP, OV-1, and CK19, and the latter expresses desmin and a-SMA but not AFP, OV-1, or CK19, which indicates that $CD90^+$ populations are more likely to be mesenchymal cells.

We explored the expression patterns of the representative liver CSC markers CD133, CD90, and EpCAM in primary HCC, and found that $EpCAM^+$ and $CD90^+$ CSCs show different gene expression patterns and cell morphology [36]. We further explored the tumorigenic capacity of sorted cells isolated from 15 primary HCCs and 7 liver cancer cell lines [36]. Although the number of samples analyzed was small, tumorigenic $EpCAM^+$, $CD133^+$, or $CD90^+$ CSCs were obtained in 26.6 % ($n = 4$), 20 % ($n = 3$), and 13.3 % ($n = 2$) of 15 HCCs, respectively, when xenotransplanted into NOD/SCID mice.

Interestingly, no EpCAM/CD90 double positive cells were detected in primary HCC, and $EpCAM^+$ and $CD90^+$ cells were distinctive with different tumorigenic/metastatic

capacities; that is, $EpCAM^+$ cells were associated with a high tumorigenic capacity and hepatic epithelial stem cell features, while $CD90^+$ cells had a metastatic propensity with mesenchymal vascular endothelial cell features. Importantly, the existence of $EpCAM^+$ cells correlated with high serum AFP values with a tendency for portal vein invasion, whereas the existence of $CD90^+$ cells was associated with a high incidence of distant organ metastasis. Furthermore, $CD90^+$ CSCs abundantly expressed c-Kit and showed chemosensitivity against the c-Kit inhibitor imatinib mesylate, whereas $EpCAM^+$ CSCs showed no such chemosensitivity. These data demonstrate that liver CSCs are not a single entity but exist heterogeneously with distinct CSC marker expression, suggesting that no common liver CSCs expressing particular stem cell markers exist in all HCCs. Our data also indicate that the presence of distinct CSCs is a key determinant of cancer phenotypes in terms of tumorigenicity and metastatic propensity, which may influence the clinical outcome of HCC.

The distinct nature of $EpCAM^+$ and $CD90^+$ liver CSCs raises the question whether these different types of CSCs originate from the same or different type of cells. This question remains elusive, but a recent study investigating three independent cell clones established from the same HCC specimen revealed that these clones maintain common karyotype abnormality but express EpCAM, CD90, and CD133 distinctively with different chemosensitivities against sunitinib [47], suggesting that distinct liver CSCs expressing different markers may originate from the same type of cells. In terms of liver CSC origin, a recent study demonstrated that acquisition of liver CSC properties is independent of the cell of origin, and liver CSCs can originate from hepatic progenitor cells, hepatoblasts, or adult hepatocytes in mice by forced H-Ras/SV40LT induction and subsequent oncogenic reprogramming [48]. In addition, another study has demonstrated the unexpected plasticity of normal mature hepatocytes to dedifferentiate into progenitor cells in rats [49], and this type of plasticity has also been reported in breast non-CSCs [50, 51]. Given the cellular plasticity reported in normal and cancer cells described above, it is reasonable to speculate that a similar plasticity may exist in $EpCAM^+$ and $CD90^+$ CSCs that can convert their tumorigenic/metastatic phenotypes and marker expression status. Further studies are required to clarify the role of cell plasticity on heterogeneity of HCC [36].

Interaction of distinct cell lineages in liver organogenesis and hepatocarcinogenesis

Embryogenesis is characterized by the ordered emergence of an organism made up of a multitude of stem and differentiated cells. Various signaling pathways play crucial roles in the dynamic cell proliferation and motility of organogenesis [52]. For example, in liver organogenesis, liver specification signaling is activated at the ventral endoderm (hepatic endoderm) by the paracrine secretion of fibroblast growth factor (FGF) and bone morphogenic protein (BMP) from the cardiac mesoderm and septum transversum, respectively [53–55]. Wnt/beta-catenin signaling may also induce hepatic specification [56]. 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, which coincides with the expression of albumin and AFP [57].

Once the hepatic endoderm is specified and the liver bud begins to grow, the cells become hepatoblasts and have the ability to differentiate into hepatic and biliary lineages as bipotent progenitors. Epithelial and mesenchymal cells located in the endoderm and/or mesoderm collaborate to orchestrate liver organogenesis [58] (Fig 1a). The importance of this was elegantly demonstrated in a recent in vitro study generating liver buds using induced pluripotent stem cells, human umbilical vascular endothelial cells, and mesenchymal stem cells [59].

Embryogenesis and tumorigenesis share similar features including autonomous cell proliferation, motility, homing, dynamic morphologic changes, cellular heterogeneity, and interactions with the microenvironment. Liver cancer development may partially recapitulate fetal liver development in terms of the emergence of cells expressing certain stem cell markers and the activation of signaling pathways during liver development (Fig 1b). Indeed, signaling pathways activated in normal liver development are known to be activated and may be involved in the development and maintenance of liver CSCs. FGF and Wnt signaling has also been implicated in the development of HCC [60–63], with the latter shown to regulate the self-renewal of hepatoblasts and liver CSCs [20, 31, 64–68].

Moreover, as observed in the process of normal liver development, the collaboration of CSCs with epithelial or mesenchymal cell features may play an important role in the tumorigenicity and metastasis of HCC (Fig 1b). Our data indicate that $EpCAM^+$ CSCs have no metastatic capacity for distant sites when subcutaneously injected into NOD/SCID mice. However, when $CD90^+$ CSCs were co-injected with $EpCAM^+$ CSCs, $EpCAM^+$ cells could metastasize to the lung, whereas subcutaneous primary tumors showed no difference in size [36]. Furthermore, although imatinib mesylate treatment had little effect on the size of primary subcutaneous tumors, it significantly suppressed lung metastasis potentially through the suppression of $CD90^+$ CSCs.

We found that the effect of $CD90^+$ CSCs on the enhanced cell motility of $EpCAM^+$ cells was mediated, at least in part,

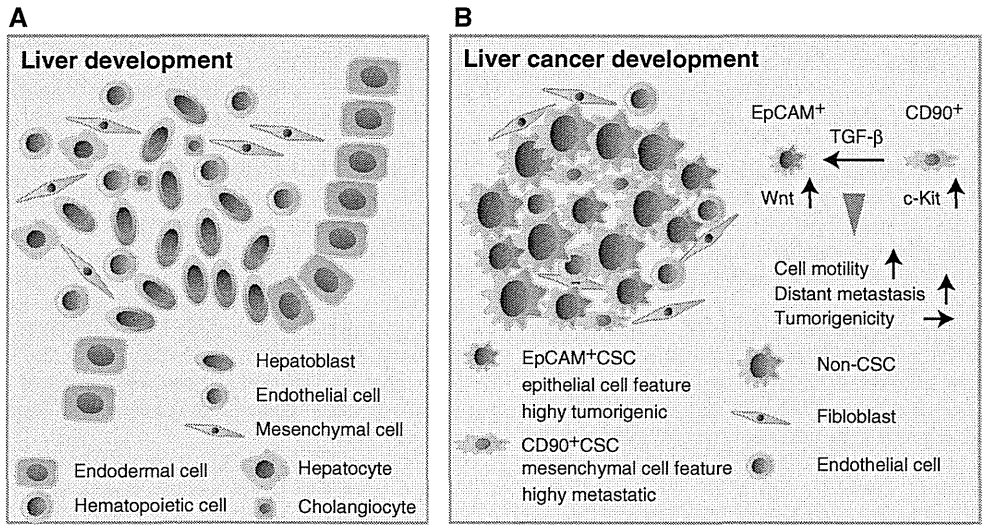

Fig. 1 Interaction of epithelial and mesenchymal cells in liver development and liver cancer development. **a** Liver bud formation is regulated by the activation of FGF, BMP, and Wnt signaling through the interaction of endodermal cells, endothelial cells, and mesenchymal cells. **b** Liver cancer development is regulated by the interaction of $EpCAM^+$ and $CD90^+$ CSCs. In primary HCC, $EpCAM^+$ and $CD90^+$ CSCs distinctively exist. $EpCAM^+$ CSCs show epithelial cell features with a high tumorigenic capacity and activated Wnt signaling, whereas $CD90^+$ CSCs show mesenchymal cell features with a highly metastatic capacity and activation of c-Kit signaling. In primary HCC where $EpCAM^+$ and $CD90^+$ CSCs co-exist, $CD90^+$ CSCs regulate distant organ metastasis through the activation of TGF-β signaling, but have no effect on tumorigenicity at primary sites which is mediated by $EpCAM^+$ CSCs

through the activation of TGF-β signaling by $CD90^+$ CSCs (Fig 1b) [36]. This suggests that $CD90^+$ cells are not only metastatic to the distant organ but also help the metastasis of $CD90^-$ cells, including $EpCAM^+$ cells, which have no distant metastatic capacity of their own. Our data further suggest that imatinib mesylate inhibits distant organ metastasis by suppressing $CD90^+$ metastatic CSCs, albeit with little effect on $EpCAM^+$ tumorigenic epithelial stem-like CSCs, which indicates the importance of $EpCAM^+$ and $CD90^+$ CSC interaction in the process of HCC development, especially in distant organ metastasis. These data suggest the limitations of a treatment strategy targeting only certain CSC marker-positive cells to eradicate HCC, as it is highly possible that marker-positive CSCs exist in each HCC patient with different chemosensitivities against molecularly targeted therapy. Interestingly, we have recently identified that EpCAM+ HCC cell lines show abundant expression of the transcription factor SALL4 and high histone deacetylase activity, and the histone deacetylase inhibitor successfully suppressed proliferation of EpCAM+ HCC cell lines but showed little effect on CD90+ HCC cell lines [69]. Further studies of liver CSC heterogeneity are required to provide better treatment strategies for HCC patients.

Conclusions

There is accumulating evidence that liver CSCs play a key role in the development and perpetuation of HCC, and the importance of targeting CSCs has become clearer. Understanding the diversity of liver CSCs will further the development of personalized medicine targeting patient-specific liver CSCs.

Conflict of interest The authors declare that they have no conflict of interest.

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Efficient generation of highly immunocompetent dendritic cells from peripheral blood of patients with hepatitis C virus-related hepatocellular carcinoma☆,☆☆

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ABSTRACT

Background & aims: Immunotherapy using dendritic cells (DCs) is a promising cancer therapy. The success of this therapy depends on the function of induced DCs. However, there has been no consensus on optimal conditions for DC preparation in vitro for immunotherapy of hepatocellular carcinoma (HCC) patients. To address relevant issues, we evaluated the procedures to induce DCs that efficiently function in hepatitis C virus (HCV)-related HCC.
Methods: We studied immunological data from 14 HCC patients. The DC preparation and the surface markers were assessed by flow cytometric analysis. Four different additional activation stimuli (Method I, medium alone; Method II, with OK-432; Method III, with IL-1β + IL-6 + TNF-α; Method IV, with IL-1β + IL-6 + TNF-α + PGE2) were tested and the functions of DCs were confirmed by examination of the ability of phagocytosis, cytokine production and allogeneic mixed lymphocyte reaction (MLR).
Results: The numbers of DCs induced and their cytokine production ability were not different between healthy controls and HCC patients. T-cell stimulatory activity of DCs in MLR was significantly lower in HCC patients than in healthy controls. The maturation of DCs with OK-432 boosted production of cytokines and chemokines, such as IL-2, IL-12p70, IFN-γ, TNF-α, IL-13 and MIP1α, and restored T-cell stimulatory activity of DCs in MLR.
Conclusions: The clinically approved compound OK-432 is a candidate for highly immunocompetent DC preparation and may be considered as a key drug for immunotherapy of HCV-related HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer worldwide and the fourth leading cause of cancer-related deaths in Japan [1]. Hepatitis C virus (HCV) infection is a common cause of chronic liver disease and leads to liver cirrhosis, contributing to the incidence of HCC [2]. Although some therapies for HCC exist, tumor recurrence rates are extremely high in these patients after curative treatments, including hepatic resection and radiofrequency ablation (RFA) [3]. In terms of the reason of this, HCV-related chronic hepatitis and cirrhosis are known to have carcinogenic potential for the development of HCC [4]. For the secondary chemoprevention of HCC patients with HCV-related cirrhosis, recent studies showed the efficacy of long-term and low-dose interferon-α therapy [5]. Among many strategies for HCC recurrence, immunotherapy is considered to be an attractive strategy to eradicate tumor cells completely [6].

Until now, different immunotherapeutic approaches have been tested for patients with HCC [7]. However, tumors have evolved numerous immune escape mechanisms, including the generation of cells with immune suppressor functions, such as Tregs and myeloid-derived suppressor cells [8]. Dendritic cells (DCs) are the most potent professional antigen-presenting cells with the unique ability to initiate and maintain adaptive immunity, and are considered

Abbreviations: DC, dendritic cell; HCV, hepatitis C virus; IFN, interferon; PBMC, peripheral blood mononuclear cell.

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to overcome the immune-suppressive environment produced by tumors [9,10]. DCs are also known to enhance antitumor immunity by activating the functions of endocytosis, trafficking, maturation and cytokine production. Numerous studies have shown that DCs from peripheral blood of HCC patients are reduced in number, have an immature phenotype and an impaired function [11]. Moreover, to date, there is no consensus on optimal conditions for DC preparation in vitro for immunotherapy of HCC patients.

Recently, we have developed the combined immunotherapy of transcatheter hepatic arterial embolization (TAE) with infusion of immature and mature monocyte-derived DCs (MoDCs) for HCC [12, 13]. In this study, we evaluated the procedures to induce MoDCs that efficiently function in the immune-mediated treatments for HCC.

2. Materials and methods

2.1. Patients and healthy controls

Fourteen patients (four women and ten men) attending Kanazawa University Hospital (Ishikawa, Japan) between September 2007 and December 2008 were enrolled in this study. All patients were serologically positive for HCV. HCC was radiologically diagnosed by computed tomography (CT), magnetic resonance imaging (MRI) and CT angiography. Blood samples were taken from 14 patients with HCC and 14 healthy controls having no hepatitis history and serologically negative for both hepatitis B and C. The clinical profiles of the patients and controls analyzed in the present study are shown in Table 1. All patients gave written informed consent to participate in the study in accordance with the Helsinki Declaration and this study was approved by the regional ethics committee (Medical Ethics Committee of Kanazawa University, No. 829).

2.2. Preparation of immature DCs

Immature DCs were separated from peripheral blood mononuclear cells (PBMCs) of patients and healthy controls. PBMCs were isolated by centrifugation using Lymphoprep™ Tubes (Nycomed, Roskilde, Denmark). The cells were resuspended in serum-free medium (GMP CellGro DC Medium; CellGro, Manassas, VA) and allowed to adhere to 6-well tissue culture dishes (Costar, Cambridge, MA) at 1.4×10^7 cells in 2 mL per well. After 2 h at 37 °C, non-adherent cells were removed and adherent cells were cultured in the medium with 50 ng/mL recombinant human IL-4 (GMP grade; CellGro) and 100 ng/mL recombinant human GM-CSF (GMP grade; CellGro) for 5 days to generate immature DCs.

Table 1
Characteristics of healthy controls and patients.

	Controls	HCC patients	P
No. of patients	14	14	
Age (years)	42.6 ± 14.9	68.8 ± 7.6	<.05
Gender (M/F)	7/7	10/4	n.s.
WBC ($X10^2/\mu L$)	ND	43.5 ± 15.4	n.s.
PLT ($X10^4/\mu L$)	ND	13.1 ± 6.0	n.s.
PT (%)	ND	85.2 ± 13.3	n.s.
ALT (IU/L)	ND	59.7 ± 46.8	n.s.
Alb (g/dL)	ND	3.3 ± 0.6	n.s.
T-Bil (mg/dL)	ND	0.8 ± 0.4	n.s.
Histology of non-tumor liver			
Chronic hepatitis	ND	8	n.s.
Cirrhosis (Child-Pugh A/B/C)	ND	6 (5/1/0)	n.s.
TNM stage (I/II/IIIA/IIIB/IIIC/IV)	ND	0/11/1/2/0/0	

Data are expressed as the mean ± SD.
ND: not determined, n.s.: not significant.

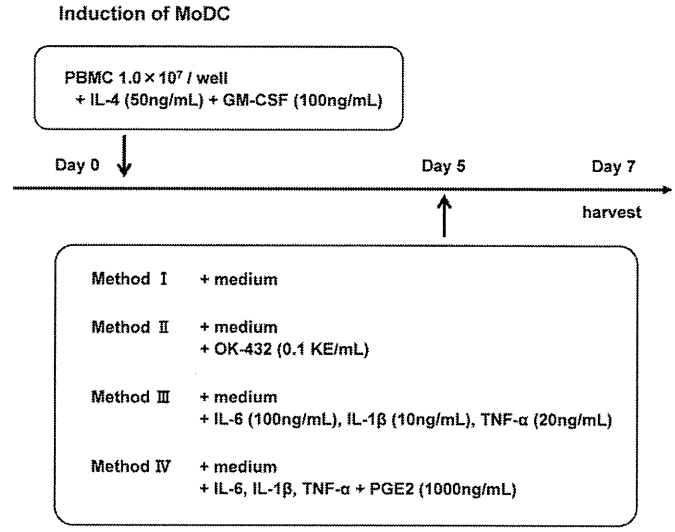

Fig. 1. Protocols for preparation of DCs. DCs, which were derived from PBMCs in the presence of IL-4 and GM-CSF for 5 days, were cultured for 2 additional days with the serum-free medium only (Method I), OK-432 (Method II), and cytokine cocktails consisting of IL-6, IL-1β and TNF-α without (Method III) or with (Method IV) PGE2. On day 7, these cells were harvested and evaluated.

2.3. Activation of immature DCs

Several activation stimuli were tested (Fig. 1). After 5 days of culture, the immature DCs induced by the above method were cultured for 2 additional days in the serum-free medium (Method I) or stimulated with 0.1 KE/mL OK-432 (Chugai Pharmaceuticals, Tokyo, Japan) (Method II), 10 ng/mL IL-1β (GMP grade; CellGro), 100 ng/mL IL-6 (GMP grade; CellGro) and 20 ng/mL TNF-α (GMP grade; CellGro) (Method III), and IL-1β, IL-6, TNF-α and PGE2 (Kaken Pharmaceuticals, Tokyo, Japan) (Method IV). On day 7, the cells were harvested.

2.4. Antibodies

The following anti-human monoclonal antibodies (mAbs) were used for flow cytometry: anti-lin1 (lineage cocktail 1; CD3, CD14, CD16, CD19, CD20 and CD56)-FITC, anti-HLA-DR-PerCP and -FITC (L243), anti-CD11c-APC (S-HCL-3), anti-CD123-PE (9F5), anti-CCR7-PE (3D12), anti-CD14-APC (MφP9) (BD Biosciences Pharmingen, San Jose, CA), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5.2B7) (Beckman Coulter, Fullerton, CA).

2.5. Flow cytometric analysis

Surface markers on DCs were evaluated using flow cytometric analysis. Cells were analyzed on a FACSCalibur™ for four-color flow cytometry. Data analysis was performed using CELLQuest™ software (Becton Dickinson, San Jose, CA).

2.6. Endocytosis assay

The endocytic capacity of DCs was assessed by measurement of FITC dextran (Sigma-Aldrich, St. Louis, MO) uptake. DCs were incubated for 30 min at 37 °C in the presence of 1 mg/mL FITC dextran, washed three times and analyzed using a FACSCalibur™ cytometer.

2.7. Cytokine production assay

The concentrations of cytokines and chemokines in the supernatants of culture medium were measured using enzyme-linked immunosorbent assay (ELISA) kit for IL-12p40 and IFNγ (Biosource International,

Camarillo, CA) and the Bioplex assay (Bio-Rad, Hercules, CA) according to the manufacturers' instructions.

2.8. Allogeneic mixed lymphocyte reaction (allo-MLR)

To evaluate the immune-stimulatory function of DCs after different stimulations, allogeneic mixed lymphocyte reaction (allo-MLR) was performed. 1×10^4 irradiated DCs (25 Gy) and 1×10^5 allogeneic PBMCs from a healthy donor were suspended in 200 μL of serum-free medium (GMP CellGro DC Medium) and co-cultured in 96-well round-bottomed microplates. After 2 days at 37 °C, 1 μCi of [^{3}H]-thymidine (Amersham Pharmacia Biotech, Piscataway, New Jersey) was added to each well. The amount of incorporated [^{3}H]-thymidine was counted using a liquid scintillation counter (Beckman Coulter, Palo Alto, California). Results are expressed as the stimulation index (counts per minute in the presence of DCs divided by counts per minute in the absence of DCs).

2.9. Statistical analysis

Results are indicated as means ± SD. The statistical significance of differences between groups was determined by applying the Mann–Whitney *U* test and unpaired *t* test. Any *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Yield and phenotype of DCs in patients and healthy controls

Adherent cells separated from PBMCs were cultured in the presence of IL-4 and GM-CSF, and harvested on day 7 (Method I). These harvested cells, which showed high levels of MHC class II (HLA-DR) and the absence of marker for mature monocytes (CD14), were consistent with the cell surface markers of DCs. The yield of DCs was variable, ranging from 23% to 28% of the initial PBMC population, and indicated no

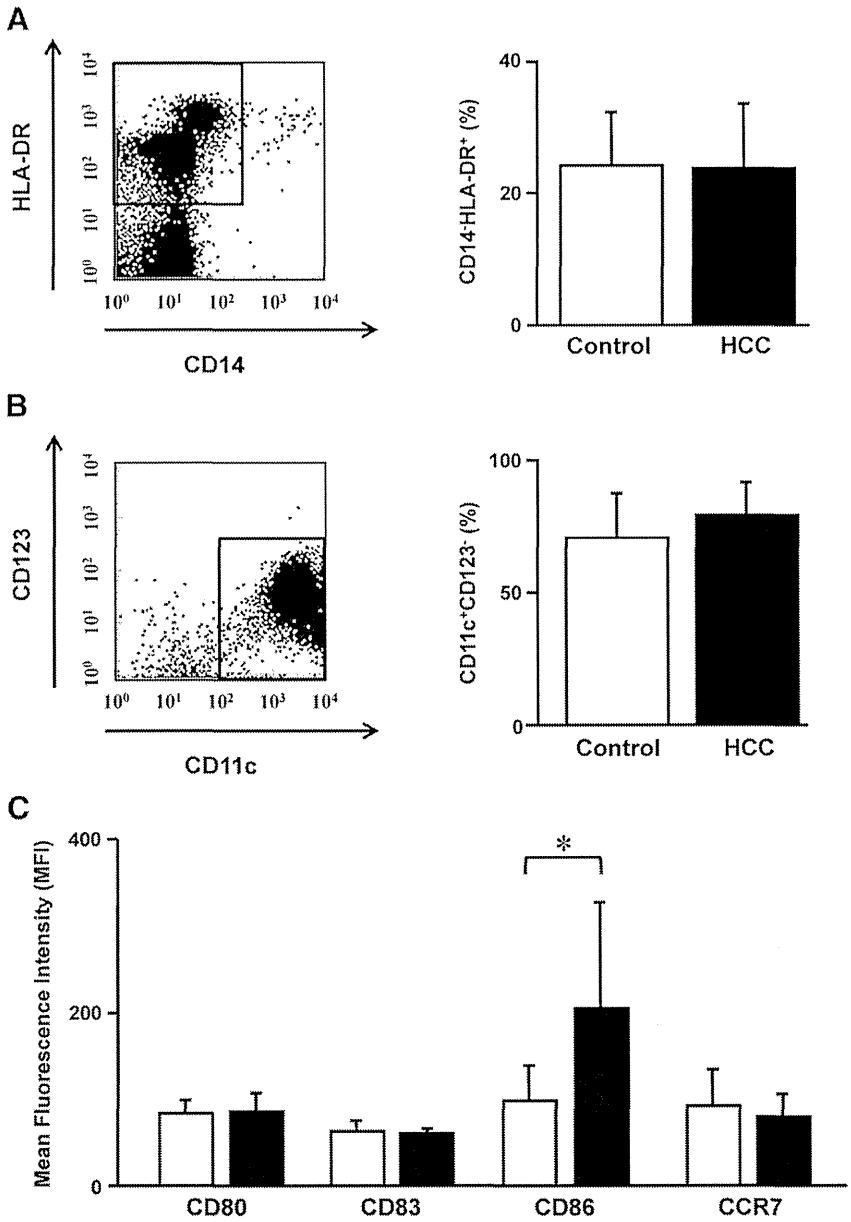

Fig. 2. The yield and characteristics of DCs derived from PBMCs in the presence of IL-4 and GM-CSF (Method I). (A) The yield of CD14$^-$HLA-DR$^+$ DCs in healthy controls and HCC patients, which was calculated as a percentage of the cells gated on side and forward scatter. (B) The yield of CD11c$^+$CD123$^-$ myeloid DCs in healthy controls and HCC patients, which was calculated as a percentage in lin1$^-$HLA-DR$^+$ cells. (C) Analysis of cell surface markers of lin1$^-$HLA-DR$^+$ DCs in healthy controls and HCC patients. Data are expressed as mean fluorescence intensity (MFI) ± SD. White and black bars indicate healthy controls and HCC patients, respectively. *$P < .05$.

significant difference between healthy controls and HCC patients (Fig. 2A). We next analyzed the surface markers, which classified DCs as myeloid or plasmacytoid DCs, on these lineage marker (lin1)-negative and HLA-DR-positive ($lin1^{-}HLA\text{-}DR^{+}$) DCs. In both patients and controls, the majority of DCs expressed CD11c and the percentages of the DCs classified under the myeloid subset ($CD11c^{+}CD123^{-}$) were not different among the two groups (Fig. 2B).

Subsequently, we analyzed the expression of co-stimulatory molecules, including B7-1 (CD80), B7-2 (CD86), an activation marker (CD83) and a chemokine receptor (CCR7) by recording geometric mean fluorescence intensities (MFIs) (Fig. 2C). Notably, the expression level of CD86 was significantly increased in HCC patients (204.5 ± 120.5, $P < .05$) compared with that of healthy controls (97.7 ± 42.3). The expression levels of CD80, CD83 and CCR7 were similar in the two groups.

3.2. Functions of DCs in patients and healthy controls

The endocytic and phagocytic capacities of DCs were assessed by measurement of FITC dextran uptake using flow cytometry. A representative result is shown in Fig. 3A. MFIs of DCs induced from PBMCs of HCC patients indicate more FITC dextran uptake than those from healthy controls (223.3 ± 82.9 vs. 113.1 ± 35.4, $P < .05$).

Next, we analyzed their ability to produce inflammatory cytokines that influence T-cell function. The spontaneous IL-12p40 production of DCs was measured using an enzyme-linked immunosorbent assay (ELISA) (Fig. 3B). The concentrations of IL-12p40 in the supernatant did not differ between HCC patients and healthy controls (11.4 ± 3.2 vs. 14.2 ± 7.5, $P = .29$).

In contrast, regarding T-cell stimulatory activity of DCs in allogeneic MLR, which was expressed as stimulation index value of [^{3}H]-thymidine incorporation at the T-cell/DC ratio of 10/1, the index values were significantly lower in HCC patients than in healthy controls (1.9 ± 1.1 vs. 3.5 ± 0.5, $P < .05$) (Fig. 3C).

3.3. Yield and phenotype of DCs with different stimulations

Considering effective antitumor immunity of DC-based immunotherapy, maturational status and T-cell stimulatory potential of DCs are important. Therefore, we next examined the effects of different maturation stimuli on the phenotypes and the functions of DCs induced from PBMCs in HCC patients. As shown in Fig. 1, PBMCs of HCC patients were differentiated into immature DCs in the presence of IL-4 and GM-CSF on day 5 and then harvested on day 7 after culturing for two additional days in several activation stimuli.

First, the expressions of HLA-DR, various costimulatory molecules and chemokine receptor, which were the indicators of DC maturation, were assessed (Fig. 4). By culturing with the additional stimuli (Methods II, III and IV), the percentage of $CD14^{-}HLA\text{-}DR^{+}$ DCs was significantly increased compared with that in the medium alone (Method I) (Fig. 4A). However, the percentage of $CD11c^{+}CD123^{-}$ DCs was not different in all groups.

In the next step, we assessed the geometric mean fluorescence intensities of CD80, CD83, CD86 and CCR7 (Fig. 4B). The expressions of CD80 and CD86 of DCs with the stimulation (Methods II, III and IV) increased significantly compared with those with medium alone (Method I). Furthermore, the DCs stimulated with OK-432 (Method II) or cytokine cocktail with PGE2 (Method IV) showed a significant increase of CD80 and CD86 in comparison with those stimulated with cytokine cocktail without PGE2 (Method III). The expression level of CD83 was increased by OK-432 (Method II) and cytokine cocktail with PGE2

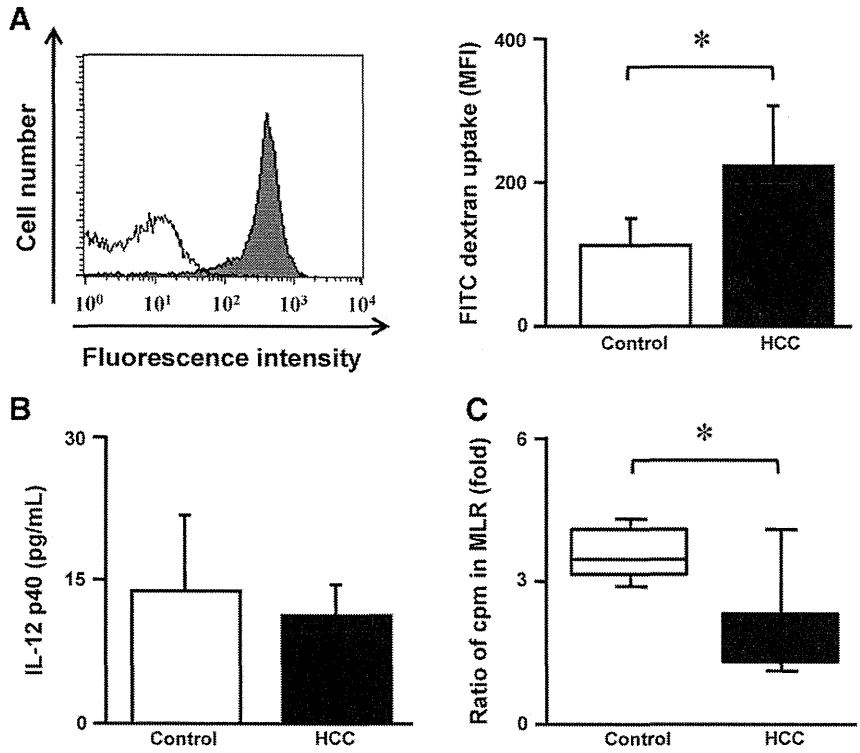

Fig. 3. Analysis of functions of DCs. (A) The phagocytic capacity of DCs was assessed by measurement of FITC dextran uptake by flow cytometry. A representative analysis is shown in the left panel. The shaded curves indicate MFIs of DCs incubated with FITC dextran for 30 min at 37 °C, and the unshaded curves are those of control DCs incubated without FITC dextran at the same time. MFIs of positive cells in healthy controls (white bar) and HCC patients (black bar) are indicated in the right panel. Data are expressed as MFI ± SD. (B) The capacity of cytokine production of DCs in healthy controls and HCC patients. (C) T-cell stimulatory capacity of DCs was evaluated by allogeneic MLR. White and black bars indicate healthy controls and HCC patients, respectively. *$P < .05$.

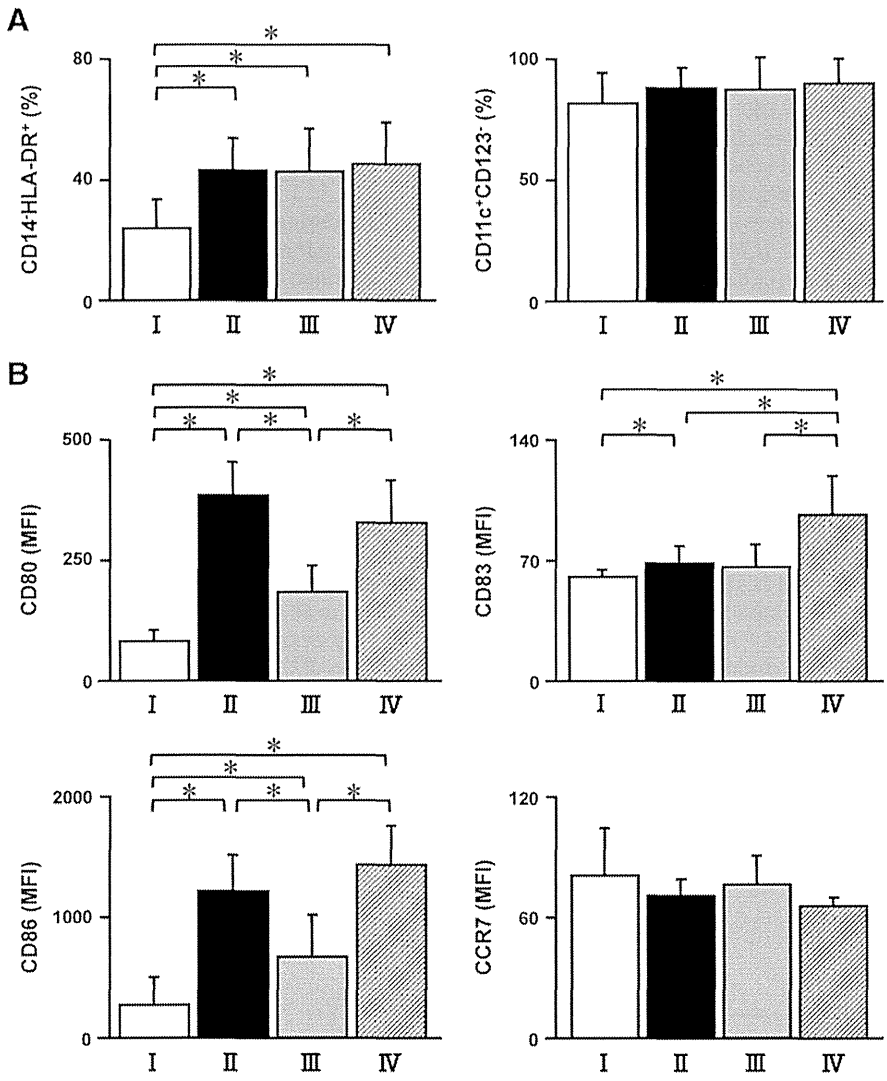

Fig. 4. Comparison of the yield and phenotype of DCs induced from PBMCs of HCC patients using 4 kinds of methods. (A) The yields of $CD14^{-}HLA\text{-}DR^{+}$ and $CD11c^{+}CD123^{-}$ DCs are shown in left and right panels, respectively. (B) The surface markers consisting of CD80, CD83, CD86 and CCR7 on DCs were evaluated by flow cytometry. White, black, gray and hatched bars indicate the methods of DC maturation with methods I, II, III and IV, respectively. The methods of DC maturation are shown in Fig. 1. *$P < .05$.

(Method IV) stimulation. The expression of CCR7 was not different among the groups.

3.4. Function of DCs with different stimulations

Initially, the change of phagocytic capacity of DCs induced from PBMCs in HCC patients was assessed by the same protocol (shown above) (Fig. 5A). The uptake of FITC dextran of DCs stimulated with OK-432, cytokine cocktail with and without PGE2 (Methods II, III and IV) was decreased significantly compared with that of DCs cultured in medium alone (Method I) ($P < .05$).

Next, we also examined cytokine production, such as IL-12p40 and IFN-γ, of DCs by ELISA (Fig. 5B). The DCs stimulated with OK-432 (Method II) produced much more IL-12p40 and IFN-γ than the DCs stimulated with cytokine cocktail with and without PGE2 (Methods IV and III, respectively) or medium alone (Method I) ($P < .05$). In the analysis of IFN-γ production, the DCs stimulated with OK-432 (Method II) produced the largest amount of IFN-γ among the groups ($P < .05$).

3.5. Allo-stimulatory capacity of DCs with different stimulations

To assess whether the enhanced expression of costimulatory molecules reflects the antigen presentation capacity, we studied the function using an allo-MLR (Fig. 5C). Stimulation with OK-432 (Method II) or cytokine cocktail with PGE2 (Method IV) was more efficient in inducing T-cell proliferation than that with cytokine cocktail without PGE2 (Method III) or medium alone (Method I). Moreover, the index value of DCs stimulated with OK-432 was significantly higher than that of DCs stimulated with cytokine cocktail with PGE2 (9.9 ± 3.9 vs. 4.7 ± 1.7, $P < .05$).

To evaluate the mechanism of strong allo-stimulatory capacity of DCs induced by OK-432 in HCC patients, the cytokine levels in allogeneic MLR supernatant were examined using Bioplex assay (Fig. 6). The levels of cytokines in the medium containing DCs with OK-432 stimulation (Method II), such as IL-2, IL-12p70, IFN-γ, TNF-α, IL-13 and MIP1α, were significantly higher than those in the medium containing DCs with medium alone (Method I) ($P < .05$) (Fig. 6A). In addition to an increase of these cytokines and chemokines, other cytokines including IL-4, IL-10 and IL-17 were also significantly increased in the medium in which DCs with OK-432 stimulation and PBMCs were co-cultured (Fig. 6B).

4. Discussion

Immunotherapy is a promising therapy for HCC patients and a number of the therapies have been evaluated [14]. Among the numerous

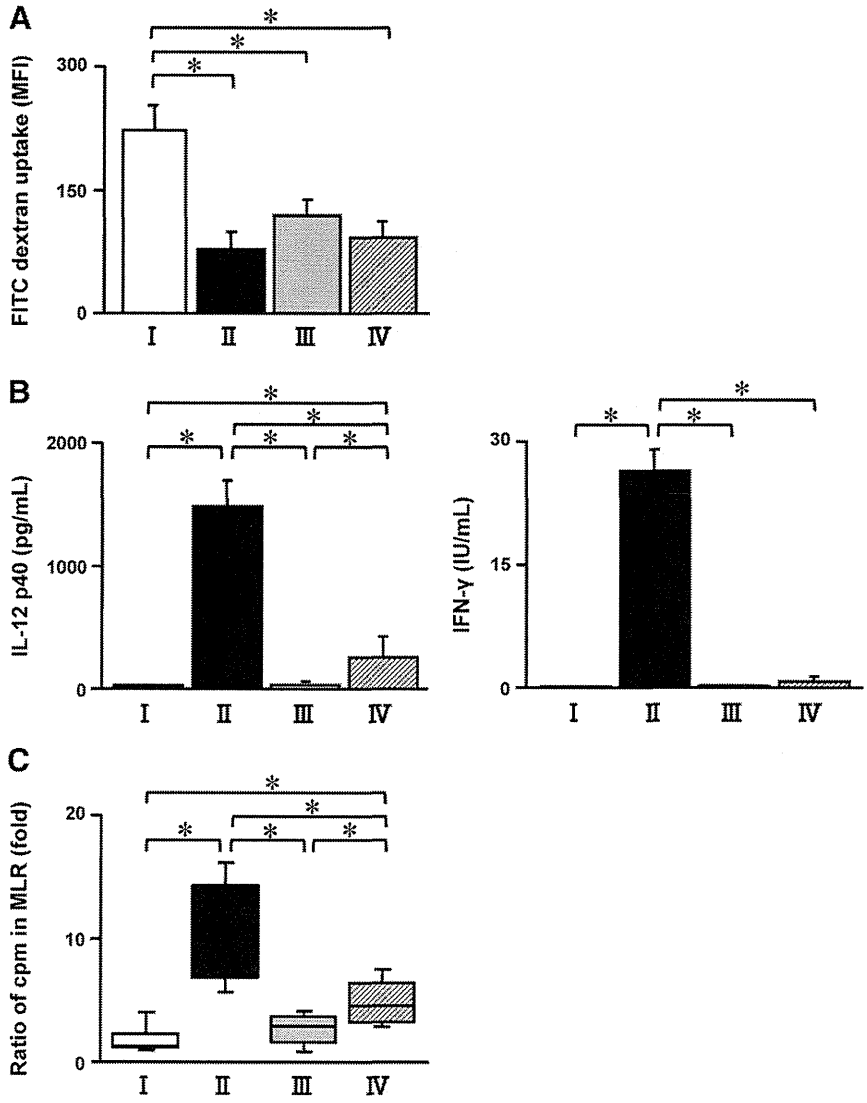

Fig. 5. Comparison of the functions of DCs induced from PBMCs of HCC patients using 4 kinds of methods. (A) The phagocytic capacity of DCs was evaluated by the uptake of FITC dextran using flow cytometry. (B) The capacity of cytokine production of DCs was measured by ELISA. (C) T-cell stimulatory capacity of DCs was evaluated by allogeneic MLR. White, black, gray and hatched bars indicate the methods of DC maturation with methods I, II, III and IV, respectively. The methods of DC maturation are shown in Fig. 1. *$P < .05$.

immune cells, DCs are the most potent type of antigen-presenting cells in the human body. However, impaired function of DCs has been implicated in the escape of the tumor from immune control in cancer patients [15]. In chronic HCV-infected patients, recent studies have shown that the function of MoDCs is not necessarily impaired [16], while several groups have reported a maturation defect and impaired function in HCC patients [17].

In the present study, the number and cell surface maturation markers of harvested DCs derived from PBMCs of HCC patients are not different from those of healthy controls. In particular, the results that the percentage of $CD11c^{+}CD123^{-}$ myeloid DCs was not different suggested that the culture method using IL-4 and GM-CSF is suitable for the induction of effective DCs in HCC immunotherapy. Because myeloid DC is a main player that produces cytokines such as IL-12, induces T-helper type 1 (Th1) response and antigen-specific cytotoxic T-cell immunity [18].

In the analysis of maturation markers, the DCs in HCC patients showed similar expression levels of CD80, CD83, CCR7 and even a high expression level of CD86 compared with the DCs in healthy donors. However, the result of FITC dextran uptake indicated that the DCs in HCC patients showed high ability, suggesting that their functional phenotype is still immature. Consistent with these results, stimulatory capacity of these DCs in MLR was lower than that of healthy controls, suggesting that additional treatment is required for optimal DC preparation.

Unfortunately, there is still no consensus on the optimal procedure for preparation of DCs using the drugs with good manufacturing practice (GMP) grade. For the clinical application of DC-based immunotherapy, it is desirable to use maturation agents with GMP grade for the safety of patients. Therefore, in this study, we evaluated the procedures using GMP-grade compounds. OK-432, a penicillin-inactivated and lyophilized preparation of *Streptococcus pyrogenes*, was reported to be immunomodulatory and have potential therapeutic properties for cancer immunotherapy [19,20]. DCs stimulated with OK-432 have been suggested to acquire a mature phenotype, produce a significant amount of T-helper type 1 (Th1) cytokines such as IL-12 and IFNγ and enhance cytotoxic T-lymphocyte activity [21]. Otherwise, many cytokines, often containing pro-inflammatory mediators, or their combinations have been tested for DC maturation. Since 1997, cytokine cocktails containing TNF-α, IL-1β, IL-6 and PGE2 have been shown to induce DC maturation

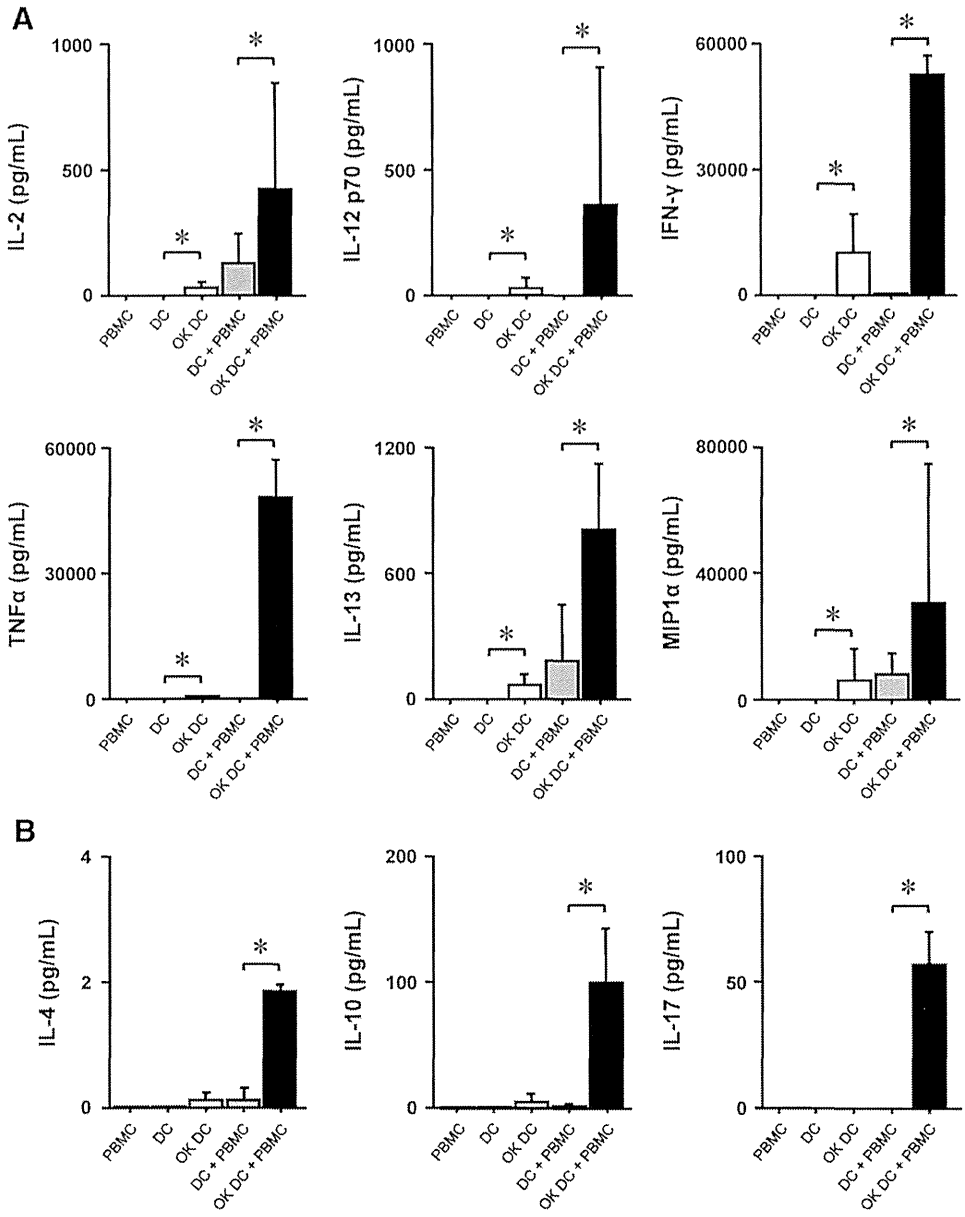

Fig. 6. Cytokine production of DCs induced from PBMCs of HCC patients with and without OK-432 stimulation in MLR. The cytokine levels of culture supernatants in allogeneic MLR were assessed by Bioplex assay. As controls, supernatants of medium cultured with PBMCs alone and DCs alone with and without OK-432 stimulation were used. (A) The concentrations of cytokines and chemokines such as IL-2, IL-12p70, IFN-γ, TNF-α, IL-13 and MIP1α in the medium cultured with 5 different conditions as indicated below. (B) The concentrations of cytokines such as IL-4, IL-10 and IL-17 were significantly higher in the medium cultured with OK-432-stimulated DCs and PBMCs than those with OK-432-unstimulated DCs and PBMCs. Dotted, hatched, white, gray and black bars indicate the cytokine levels in the medium cultured with PBMCs alone, OK-432-unstimulated DCs alone, OK-432-stimulated DCs alone, OK-432-unstimulated DCs with PBMCs and OK-432-stimulated DCs with PBMCs, respectively. *$P < .05$.

[22]. In addition, adding PGE2 to the maturation cocktail was reported to enhance further CCR7 expression, migration capacity and T-cell stimulatory activity of DCs even in patients with advanced cancer [23]. In this study, on the basis of these results, we analyzed DCs derived from PBMCs of HCV-related HCC patients by OK-432 or cytokine cocktails.

Both methods, using OK-432 or cytokine cocktails, had success in enhancing the expression levels of CD80, CD83 and CD86. The phagocytic capacity of the DCs induced by both methods was lower than that of the DCs induced by a standard method. On the other hand, the production of cytokines such as IL-12p40 and IFN-γ and allostimulatory capacity were excellent in DCs with OK-432 stimulation. These results suggest that both methods are useful for maturation, but OK-432 stimulation is the best method of DC preparation for immunotherapy in HCV-related HCC patients. Moreover, the types of cytokines and chemokines detected in allogeneic MLR were very similar to those that we previously reported in serum of patients who received immunotherapy with OK-432-stimulated DCs [13]. Taken together with these results, the DCs stimulated with OK-432 may have immunological potential in not only local but also systemic responses through cytokine production.

In conclusion, the results of the present study suggest that the clinically approved compound OK-432 is a candidate for highly immunocompetent DC preparation in HCV-related HCC patients and should provide us with a novel insight for immunotherapy of HCC.

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Review

Molecular Biology of Liver Cancer Stem Cells

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Key Words
EpCAM signaling · Hepatocellular carcinoma · Liver cancer stem cell · miR-181 · Wnt signaling

Abstract
Hepatocellular carcinoma (HCC) is one of the most common and lethal cancers worldwide. The concept of cancer stem cells (CSCs) is based primarily on the clinical and experimental observations that indicate the existence of a subpopulation of cells with the capacity to self-renew and differentiate as well as show increased resistance to radiation and chemotherapy. They are considered as the factors responsible for the cases of tumor relapse. Hepatic progenitor cells (HPCs) could form the basis of some hepatocellular carcinomas (HCC) and cholangiocarcinomas. Liver CSCs have been reported in multiple subtypes of HCC and are considered as the master regulators of HCC initiation, tumor metastasis, and progression. HPCs activators such as epithelial cell adhesion molecule (EpCAM), Wnt/β-catenin, transforming growth factor-beta (TGF-β), Notch and Hedgehog signaling systems expedite tumorigenesis or conversely, serve as a powerful cancer-prevention tool. Recent work has also identified Sal-like protein 4 (SALL4) and some epigenetic regulations as important molecules, while several therapeutic drugs that directly control HPCs have been tested both *in vivo* and *in vitro*. However, liver CSCs clearly have a complex pathogenesis, with the potential for considerable crosstalk and redundancy in signaling pathways. Hence, the targeting of single molecules or pathways may have limited benefit for treatment. In addition to the direct control of liver CSCs, many other factors are needed for CSC maintenance including angiogenesis, vasculogenesis, invasion and migration, hypoxia, immune evasion, multiple drug resistance, and radioresistance. Here, we provide a brief review of molecular signaling in liver CSCs and present insights into new therapeutic strategies for their targeting.

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Introduction

HCC affects more than half a million individuals annually and is the fifth leading cause of cancer, and ranks third in cancer mortality worldwide [1]. Despite some progress in cancer treatment, existing therapies are limited in their ability to cure malignancies and to prevent metastases and relapses. Surgery, radiofrequency ablation therapy, and chemotherapy are all directed at reducing the bulk of the tumor mass. However, in the majority of cases, tumor regrowth and relapse of disease occurs on completion of therapy. Although the concept of tumor stem cells has been proposed for a number of decades, the demonstration of their existence has only occurred within the last decade. Recently, progression of HCC is thought to be driven by cancer stem cells (CSC) through their capacity for self-renewal, the production of heterogeneous progeny, and their ability to limitlessly divide. CSCs with such characteristics have been reported for many haematological and solid human tumors. Furthermore, many potentially biologically significant surface markers and pathways that modulate these stem/progenitor cells in cancer tissue have been identified since they have dual roles in embryogenic stem cell development and tumor activation or suppression. In this review, we demonstrate a brief and uptodate review of molecular signaling in liver CSCs and present insights into new therapeutic strategies.

Liver Stem Cells in Human Liver Regeneration and Cancer Stem Cells

The liver is both an exocrine and an endocrine gland that performs complex functions and has the capacity to regenerate. This process enables the recovery of lost mass without endangering the viability of the entire organism and many studies suggest the existence of two basic types of liver regeneration. Acute liver injury is often observed in patients without liver disease, although sustaining such an injury may result in rapid liver dysfunction. Several different factors appear to be primarily responsible for injury, including drugs, toxins, chemicals, ischemia/reperfusion, and viral hepatitis. During extensive acute liver injury, there is wide-spread necrosis and apoptosis with release of cytokines, which far exceeds the capacity of the remaining healthy hepatocytes to replicate and restore the liver function. As a result, resident liver progenitor cells within the canals of Hering are activated to support or take over the role of regeneration [2].

By contrast, liver regeneration after the loss of hepatic tissue does not depend on these cell types, but rather on the proliferation of existing mature hepatocytes, the parenchymal cells of the organ. Liver regeneration in this non-toxic model of injury is a multi-step process with at least two important phases: 1) transition of quiescent hepatocytes into the cell cycle and, 2) their progression beyond the restriction point in the G1 phase of the cell cycle. Control of this process depends on a complex interaction of cytokine and growth factors released in response to liver injury. Three main growth factors: hepatocyte growth factor (HGF), epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) underpin normal hepatic regeneration through their potent mitogenic action on hepatocytes via stimulation of DNA synthesis. Termination of hepatocyte proliferation at the end of regeneration is an important part of this process which is regulated by TGF-β and activin, which serve as negative feedback mechanisms. Termination of hepatocyte proliferation is regulated by the ratio of liver to body mass rather than liver mass *per se*, thus providing a remarkable check on the extent of liver regeneration [3].

Hepatocytes are capable of large-scale clonal expansion within a diseased liver. Following very extensive liver damage or in situations in which hepatocyte regeneration after damage is compromised, a potential stem cell component located within the smallest branches of

the intrahepatic biliary tree is activated. HPCs amplify a biliary population of transit amplifying cells that are at least bipotential, namely, capable of differentiating into either hepatocytes or cholangiocytes. HPCs are induced during chronic liver inflammation, replacing damaged hepatocytes and cholangiocytes in various liver diseases including alcoholic and non-alcoholic fatty liver disease, and HBV- and HCV-induced hepatitis. HPCs are almost always accompanied by an inflammatory reaction, which is located directly adjacent to the inflammatory cells. HPCs are strongly associated with liver regeneration following acute and chronic damage through cellular interactions with liver immune cells involving paracrine signals, especially from growth factors that are released as part of the regeneration process. However, during regeneration, HPCs are considered a dangerous target in hepatocarcinogenesis by the interaction or modulating inflammation niche involved in tissue repair. HPCs have also been reported to initiate HCC and intrahepatic cholangiocarcinoma (ICC), and their function in carcinogenesis is supported by a histological investigation of liver cancer that exhibits features of both HCC and ICC accompanied by the presence of numerous HPCs. Detailed immunophenotyping of HCCs has revealed that 28-50% express markers of progenitor cells such as cytokeratin 7 and 19, respectively. These tumors also consist of cells that have an intermediate phenotype between progenitors and mature hepatocytes. In fact, patients with HCCs that express hepatocyte and biliary cell markers have a significantly poorer prognosis and a higher recurrence rate after surgical resection and liver transplantation. A small subset of cancer cells with CSC properties has been identified and characterized from HCC cell lines, animal models, and human primary HCCs, which can be identified by several cell surface antigens including CD133, CD90, CD44, EpCAM, and CD13 [4, 5], respectively.

Stem cells in the liver are proposed to be from two origins, either endogenous or intrahepatic, and exogenous or extrahepatic. Included in the intrahepatic stem cell category are the numerous HPCs with short-term proliferative capacity that localize within the canals of Hering and interlobular bile ducts.

Extrahepatic stem cells include cells derived from bone marrow and peripheral blood cells; these cells are limited in number but have a long-term proliferation capacity [6].

Molecular Signaling of Liver Cancer Stem Cells

Liver CSCs are likely to require a multitude of signals to maintain a phenotype characterized by self-renewal and pluripotency. These signals include the EpCAM, Wnt/β-catenin pathway, the Sonic Hedgehog pathway, and the Notch pathway, which play a decisive role in the regulation and maintenance of stemness and in tumor formation. The uncontrolled activation of these and other pathways is thought to lead to the initial formation of liver CSCs, and therefore tumorigenesis in general. As these pathways are frequently involved in the regulation of various stem cell phenotypes, it is tempting to speculate that gain of function mutations of members of these pathways are instrumental in the formation of liver CSCs. Wnt pathway proteins are a group of evolutionarily conserved intracellular signaling molecules that regulate the cellular fate and are implicated in the self-renewal of stem cells. The evolutionarily conserved Notch pathway is involved in many developmental processes such as differentiation, fate decision, proliferation, apoptosis, and cell adhesion. In the liver, Notch signaling contributes to the formation of a network of transcription factors involved in cholangiocyte differentiation [7, 47].