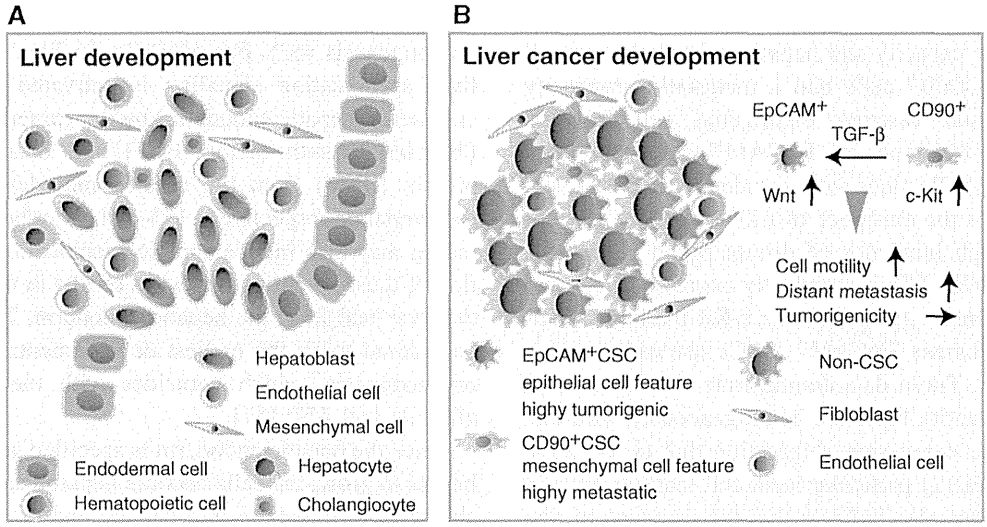

Fig. 1 Interaction of epithelial and mesenchymal cells in liver development and liver cancer development. **a** Liver bud formation is regulated by the activation of FGF, BMP, and Wnt signaling through the interaction of endodermal cells, endothelial cells, and mesenchymal cells. **b** Liver cancer development 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 ($X10^2$/μL)	ND	43.5 ± 15.4	n.s.
PLT ($X10^4$/μL)	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.

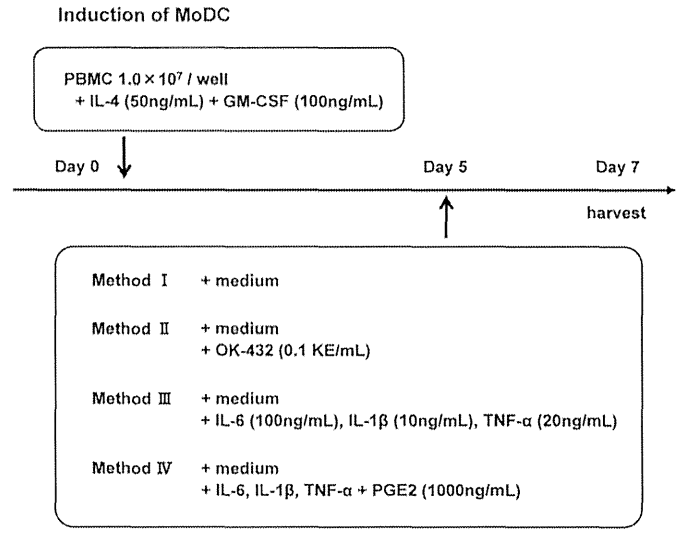

Fig. 1. Protocols for preparation of DCs. DCs, which were derived from PBMCs in the presence of IL-4 and GM-CSF for 5 days, were cultured for 2 additional days with the serum-free medium only (Method I), OK-432 (Method II), and cytokine cocktails consisting of IL-6, IL-1β and TNF-α without (Method III)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

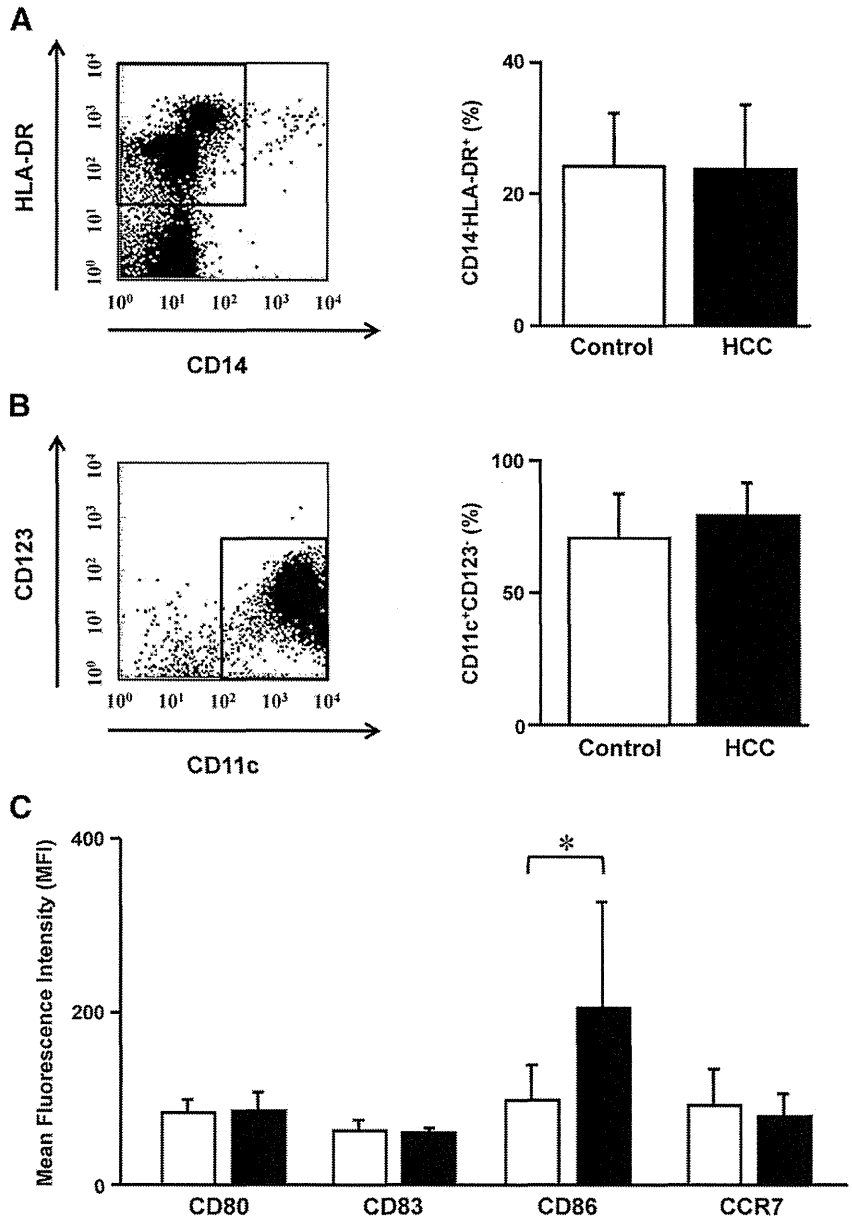

Fig. 2. The yield and characteristics of DCs derived from PBMCs in the presence of IL-4 and GM-CSF (Method I). (A) The yield of CD14$^-$HLA-DR$^+$ DCs in healthy controls and HCC patients, which was calculated as a percentage of the cells gated on side and forward scatter. (B) The yield of CD11c$^+$CD123$^-$ 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

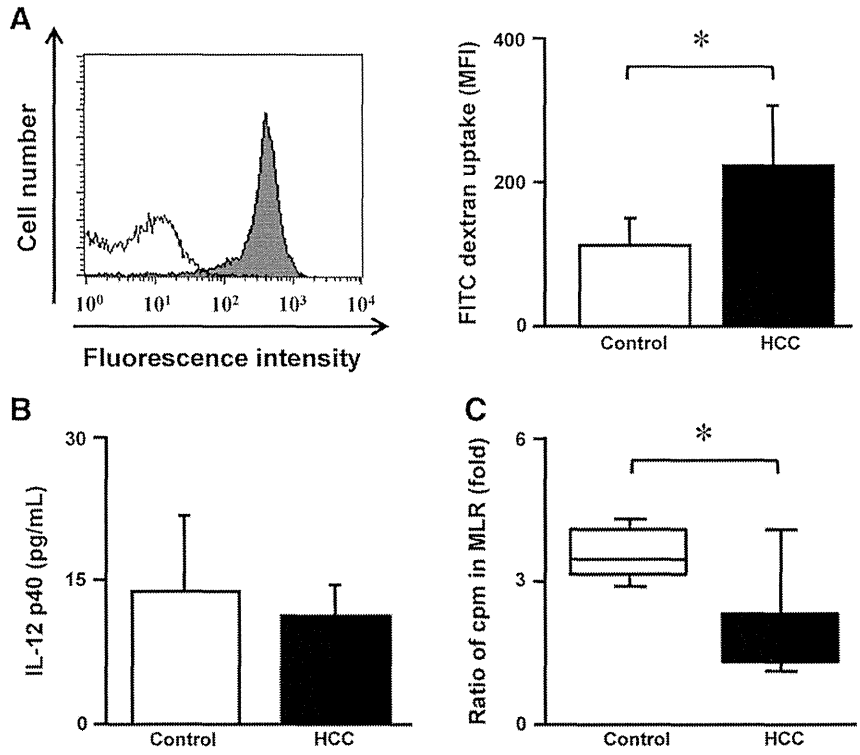

Fig. 3. Analysis of functions of DCs. (A) The phagocytic capacity of DCs was assessed by measurement of FITC dextran uptake by flow cytometry. A representative analysis is shown in the left panel. The shaded curves indicate MFIs of DCs incubated with FITC dextran for 30 min at 37 °C, and the unshaded 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. *$P < .05$.

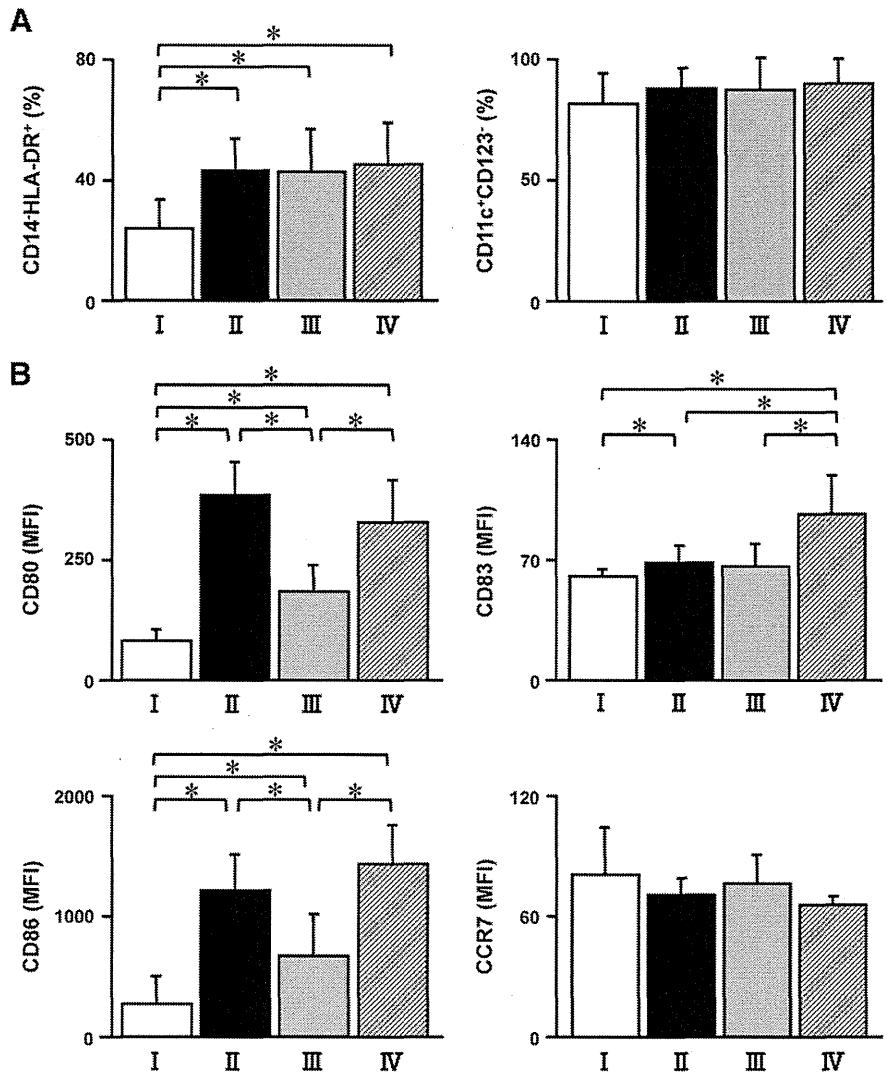

Fig. 4. Comparison of the yield and phenotype of DCs induced from PBMCs of HCC patients using 4 kinds of methods. (A) The yields of $CD14^{-}HLA\text{-}DR^{+}$ and $CD11c^{+}CD123^{-}$ DCs are shown in left and right panels, respectively. (B) The surface markers consisting of CD80, CD83, CD86 and CCR7 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

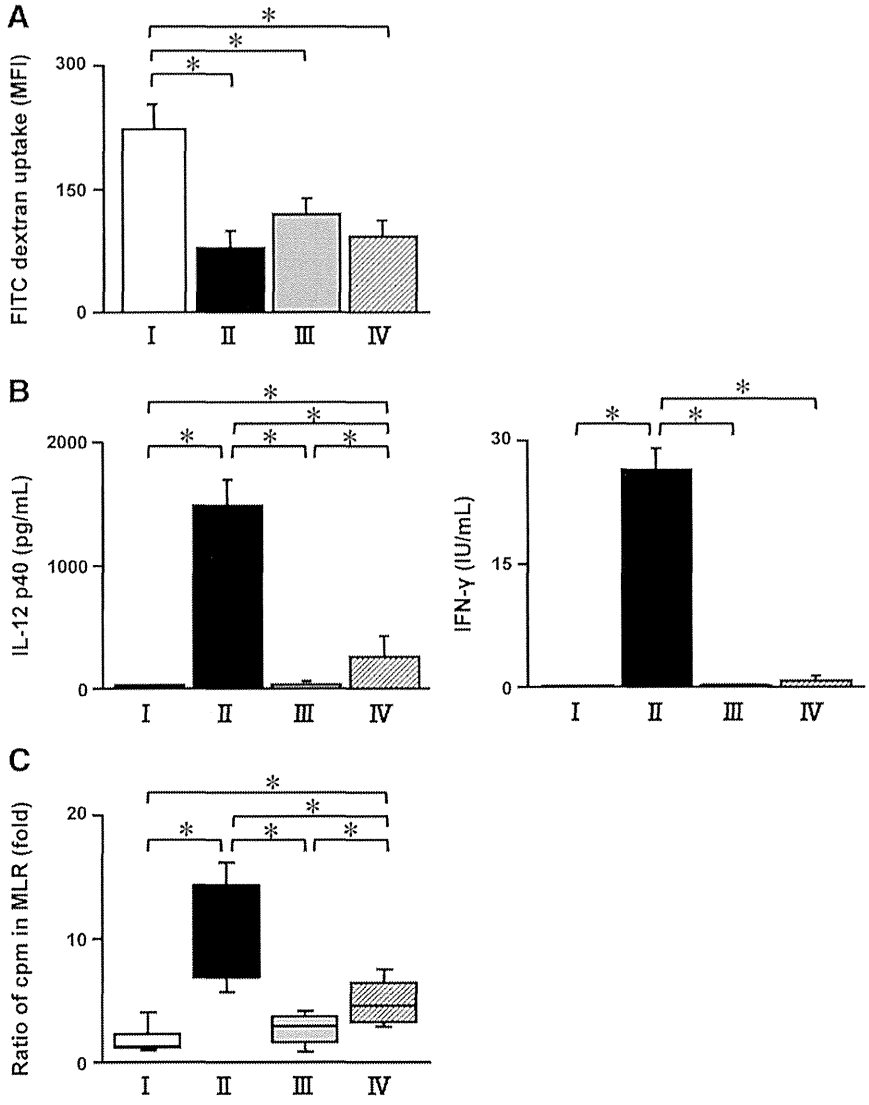

Fig. 5. Comparison of the functions of DCs induced from PBMCs of HCC patients using 4 kinds of methods. (A) The phagocytic capacity of DCs was evaluated by the uptake of FITC dextran using flow cytometry. (B) The capacity of cytokine production of DCs was measured by ELISA. (C) T-cell stimulatory capacity 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

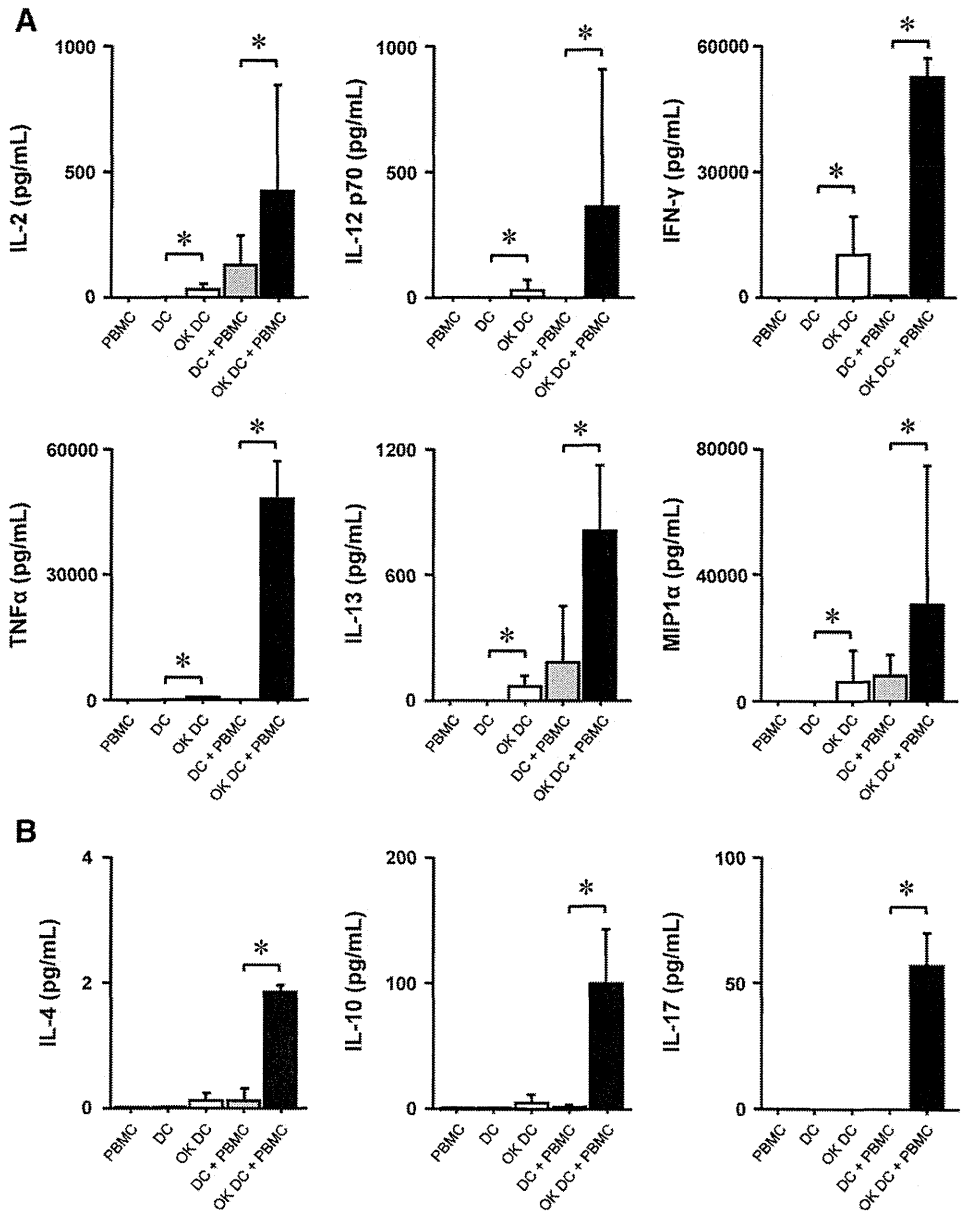

Fig. 6. Cytokine production of DCs induced from PBMCs of HCC patients with and without OK-432 stimulation in MLR. The cytokine levels of culture supernatants in allogeneic MLR were assessed by Bioplex assay. As controls, supernatants of medium cultured with PBMCs alone and DCs alone with and without 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].

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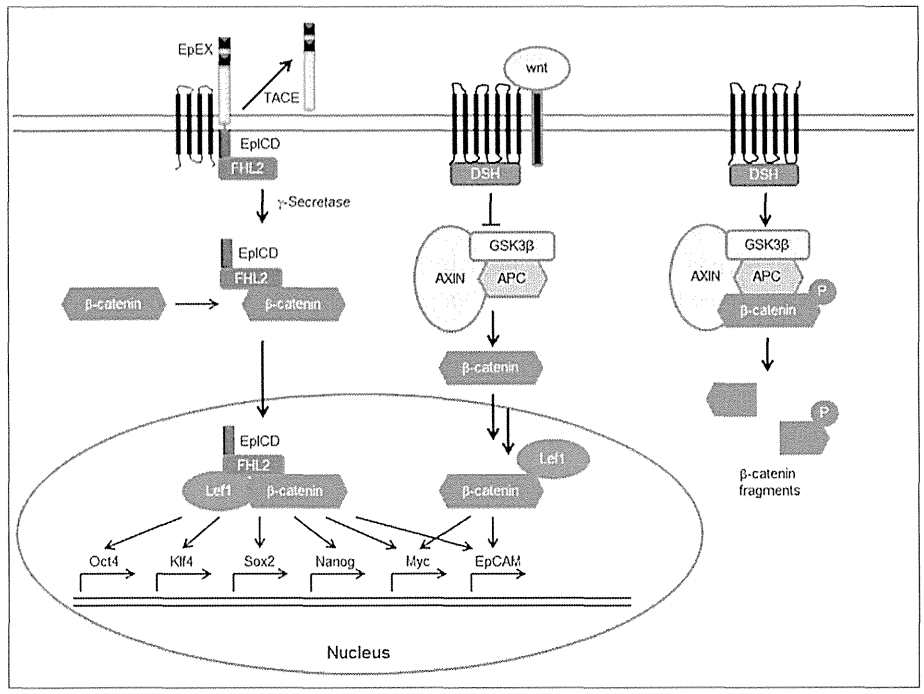

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Fig. 1. Overview of cross-talk between EpCAM signaling and the Wnt pathway. Following cleavage by TACE/PS-2, EpICD translocates to the nucleus in a multiprotein complex. This nuclear complex binds the promoters of genes involved in cell cycle regulation and stemness. EpCAM regulates Nanog, Oct4, Klf4, Sox2, and Myc.

EpCAM Signaling Pathway

EpCAM is a type 1 transmembrane glycoprotein consisting of a large extracellular (EpEX), a single transmembrane and a short intracellular (EpICD) domain. Three independent glycosylation sites in the EpEX part dictate the stability of the whole protein at the cell surface. Liver CSC markers such as EpCAM, CD44, and CD133 share a number of entities and represent the most frequently used markers for the enrichment of tumor-initiating cells from primary human cancer. As is the case for many cell adhesion molecules, EpCAM has dual properties in that it can mediate cell-to-cell contact as well as transmit signals from the plasma membrane to the nucleus in order to regulate gene transcription [8]. In addition, EpCAM is not solely expressed in epithelial cells, but is also strongly expressed in various tissue stem cells, precursors, and in embryonic stem cells of murine and human origin [9]. Its mode of signaling proceeds via regulated intramembrane proteolysis and is activated by regulated intramembrane proteolysis (RIP) and the shedding of its EpEX [8] Sequential cleavage of EpCAM by tumor necrosis-factor alpha converting enzyme (TACE/ADAM17) and a gamma-secretase complex containing presenilin 2 (PS-2) results in the release of EpEX into the culture medium, and the release of the EpICD into the cytoplasm (fig. 1). EpICD then becomes part of a large nuclear complex containing transcriptional regulators β-catenin and Lef, which are both components of Wnt/β-catenin signaling. Four and one-half LIM domain protein 2 (FHL2) is essential for signal transduction by EpCAM, and FHL2 further regulates the localization and activity of TACE and PS-2. Through its function as a co-activator of β-catenin, FHL2 links EpICD with specific DNA sequences and gene regulation. FHL2 also has the potential to serve as a scaffolding protein for various signaling proteins used by EpCAM [10].

Wnt/β-CateninSignaling Pathway

The Wnt/β-catenin pathway is evolutionarily well-conserved and is essential for normal cellular processes such as development, growth, survival, regeneration, and self-renewal [11]. Disruption of Wnt/β-catenin signaling results from both genetic and epigenetic changes and is associated with a range of diseases including many cancers, especially colonic cancer and HCC. Disrupted Wnt/β-catenin signaling by mutational and non-mutational events is observed in around one third of all HCCs, emphasizing the importance of this pathway in hepatocarcinogenesis [12]. The Wnt pathway diversifies into two main branches, canonical (β-catenin-dependent) and non-canonical (β-catenin-independent), which play critical roles in specifying cellular fates and movements, respectively, during both embryonic development and adult tissue regeneration [13].

Wnt ligands signal through binding to seven transmembrane Frizzled (Fzd) receptors and single transmembrane lipoprotein receptor-related protein (LRP) 5 or 6 co-receptors. Canonical signaling mediated by ligands such as Wnt3a inhibits a multiprotein degradation complex consisting minimally of axin, adenomatous polyposis coli, and glycogen synthase kinase 3β. This inhibition culminates in the nuclear translocation of β-catenin, enabling it to interact with T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors to regulate gene expression. The resulting accumulation of β-catenin in the cytoplasm allows for its transfer into the nucleus, where it interacts with transcription factors of the LEF1 family. This functional complex induces the transcription of prominent targets like CD44 [14], cyclin D1 [15], and c-myc [16], which is also a major target of EpCAM signaling [17]. Moreover, c-myc can trigger the induction of a stem-like transcriptional profile in normal and cancer cells and represents the central switch from adult to embryonic stem cells [18].

Thus far, it remains unknown at which point in the signaling cascades of EpCAM and Wnt/Frizzled cross-talk occurs. However, EpICD does not only interact with β-catenin and Lef-1, it also binds to Lef-1 consensus sites in the promoter of Wnt target genes such as cyclin D1. EpICD appears to be essential for the formation of one of the two major nuclear protein/DNA complexes formed at Lef-1 consensus sites in EpCAM-positive carcinoma cells [10]. This suggests that EpICD can provide additional levels of regulation to Wnt target genes, which are central in cell cycle regulation, and thus could play important roles in self-renewal. Since Wnt signaling is reportedly instrumental in tumor-initiating cells (TICs), and because TICs rely on Wnt pathway-inducing signals from their microenvironment for the maintenance of their phenotype [19], it is tempting to speculate that EpCAM overexpression and signaling are also instrumental in this.

In addition to c-myc, other key factors such as Nanog, Klf4, Sox2, and Oct4, which are central to the conversion of somatic cells into induced pluripotent stem cells (iPS), have also been described as direct targets of EpCAM in human embryonic stem cells [20]. EpCAM possesses a crucial role in the induction and/or maintenance of the phenotype of tissue precursors, stem cells, iPS cells, and TICs. This function most likely relates primarily to the proliferation and the maintenance of an undifferentiated state. In the liver, EpCAM expression and Wnt signaling are both associated with a tissue stem cell phenotype and regenerative capacity of cells [21]. It is important to note that EpCAM expression is only detected in regenerating cells such as hepatobiliary stem cells and progenitor cells [21]. The interrelationship of EpCAM and Wnt in HCCs has been further substantiated upon by the finding that the EpCAM gene becomes transcriptionally activated by Tcf-4, a member of the Lef family of transcription factors. EpCAM is a marker for TICs with a stem/progenitor phenotype in HCC [22].

Non-canonical signaling, which is much less defined, is mediated by ligands such as Wnt11 that use the same Fzd receptors. The Wnt-Fzd-G protein complex can also stimulate p38 kinase and activate phosphodiesterase 6, which hydrolyzes cyclic GMP and results in the inactivation of protein kinase G and an increase in intracellular calcium. Wnt-mediated

increases in intracellular Ca^{2+} activate calcineurin and subsequently the nuclear factor of activated T-cells (NF-AT) family of calcineurin-dependent transcription factors, as well as TAK1-Nemo-like kinase (NLK) kinases. Signaling through the TAK-NLK kinases is proposed to inhibit canonical Wnt signaling, stimulating the Jun NH2-terminal kinase [23], calcium-calmodulin-dependent protein kinase II and protein kinase C pathways. These pathways interact with each other, and, in some cases, non-canonical signaling antagonizes the canonical pathway [24].

SALL4 Signaling Pathway

The human homologue of the Drosophilia spalt homeotic gene, SALL4, encodes a C2H2 zinc-finger transcription factor. It is one of the key factors for maintenance of pluripotency and self-renewal of embryonic stem cells, potentially through the interaction with Oct4, Sox2, and Nanog. SALL4 is known to encode two isoforms, namely SALL4A and SALL4B, and recent studies have suggested the important role of SALL4B on maintaining the stemness of embryonic stem cells [25]. In the liver, SALL4 is expressed at high levels in fetal-liver progenitor cells but not in adult hepatocytes, and it plays a critical role in hepatic cell lineage commitment. Recently, this oncofetal gene was identified as a marker of a subtype of HCC with progenitor-like features and is associated with a poor prognosis [26, 27].

SALL4 affects phosphatase and tensin homologue (PTEN) and phosphatidylinositol 3-kinase (PI3 K)-AKT signaling thorough the interaction with NuRD (nucleosome remodeling and histone deacetylase (HDAC)) complex. Since SALL4 is a known inhibitor of PTEN, the silencing of it reduces pAKT levels and blocks PI3 K survival signaling in HCC cells highly expressing SALL4. Furthermore, SALL4-positive HCC cells tend to show high HDAC activity and chemosensitivity to HDAC inhibitors such as suberic bis-hydroxamic acid and suberoylanilide hydroxamic acid. Consistently, HDAC inhibitors might be useful for the eradication of SALL4-positive HCC cells through their inhibitory effects on histone deacetylation of NuRD.

TGF-β Family

The TGF-β family plays a vital role in the control of proliferation and cellular differentiation in both stem cells and cancer cells. Impaired TGF-β signaling through the activation of interleukin-6 in hepatic stem/progenitor cells can contribute to altered differentiation patterns and HCC development [28]. TGF-β inhibits cell proliferation and promotes tumor cell invasion by inducing epithelial-mesenchymal transition (EMT). Reduced expression of the TGF-β receptor might be a key factor in shifting to the late response to TGF-β. Many studies have reported a reduction of TGF-β receptors in up to 70% of HCCs. Moreover, reduced TGF-β receptor 2 expression in HCC has been correlated with intrahepatic metastasis. TGF-β levels in the serum and urine are increased in HCC patients, while up to 40% of HCCs have increased TGF-β expression based on immunohistochemical analysis. In addition, high TGF-β levels have been correlated with advanced clinical stages of HCC. This dual role of TGF-β signaling in HCC is explained by its effect on the tumor tissue microenvironment and on the selective loss of the TGF-β-induced antiproliferative pathway. Tumor cells that have selectively lost their growth-inhibitory responsiveness to TGF-β, but retain an otherwise functional TGF-β signaling pathway may exhibit enhanced migration and invasive behavior in response to TGF-β stimulation. Recently, loss of the TGF-β adaptor and signaling molecule embryonic liver fodrin in the liver was found to cause cancer through deregulated hepatocyte proliferation and stimulation of angiogenesis. More recently, it was reported that HCC cells positive for signal transducers and activators of transcription-3/Oct4, have dysfunctional TGF-β signaling, and are likely cancer progenitor cells with the potential to give rise to HCC [29].

Other Pathways

The Notch signaling pathway plays an important role in stem cell self-renewal and differentiation.

However, other signaling pathways influence whether Notch functions as a tumor suppressor or oncogene depending on the particular tissue [30]. Notch signaling plays a well-defined role in liver embryogenesis and bile duct formation. In addition, Notch family members are involved in angiogenesis and endothelial sprouting. The increased expression of genes involved in this pathway has been shown in CD133-positive liver cancer cells compared to CD133-negative cells. The activated intracellular form of Notch-3, as well as the Notch ligand Jagged, is highly expressed in HCC. Conversely, Notch-1 has been reported to function as a tumor suppressor and participate in cross-talk with other signaling pathways such as Ras/Raf/Mitogen-activated protein kinase/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) through the regulation of the PTEN tumor suppressor. Recent evidence indicates that activation of Notch-1 signaling increases the expression level of death receptor 5 (DR5) with enhancement of TNF-related apoptosis-inducing ligand induced apoptosis *in vitro* and in vivo [31].

Conserved from Drosophilia to humans, the Hedgehog (HH) pathway has a central role in embryonic development and adult tissue homeostasis by controlling cell fate specification and pattern formation [32]. The functional importance of this pathway is illustrated by the multiple birth defects and malignancies associated with mutations and/or aberrant activation of the pathway. Three HH ligands Sonic, Indian, and Desert have been identified in mammals that can bind interchangeably to two related twelve-pass membrane patched receptors. After ligand stimulation, Gli, like β-catenin, accumulates in the nucleus and induces transcription of genes related to the cell cycle and growth including insulin-like growth factor-2, cyclins, and β-catenin. Sonic is the predominant isoform in the liver. Up to 60% of human HCCs express Sonic, and the concomitant downregulation of Gli-related target genes is observed after the specific blockade of this pathway. Furthermore, tumorigenic activation of Smo can mediate overexpression of c-myc, a gene known to play an important pathogenic role in liver carcinogenesis. Recent studies have also shown that activation of Hedgehog signaling is critically related to CSCs and EMT features in many types of cancers including colonic, gastric, esophageal, hepatic, and others [33, 34].

microRNAs

Micro-ribonucleic acids (miRNAs) play critical roles in many biological processes including cancer by directly interacting with specific messenger RNAs (mRNAs) through base pairing, then inhibiting the expression of target genes through a variety of molecular mechanisms. MiRNAs can undergo aberrant regulation during carcinogenesis, and can act as oncogenes or tumor suppressor genes. Disruption of miRNA expression levels in tumor cells may result from distorted epigenetic regulation of miRNA expression, abnormalities in miRNA processing genes and proteins, and the location of miRNAs at cancer-associated genomic regions. Consequently, abnormal miRNA expression is a ubiquitous feature of solid tumors, including HCC. In liver carcinogenesis, miRNAs have been shown to have both tumor suppressive (miR-122, miR-26, miR-223) and oncogenic (miR-130b, miR-221, miR-222) activity [35–39]. Clearly, miRNAs play a critical role in carcinogenesis and oncogenesis. Emerging evidence suggests that certain abnormal miRNA expression levels cause cancer stem cell dysregulation, resulting in unlimited self-renewal and cancer progression. Therefore, miRNA expression is a vital key to CSC dysregulation. The let-7 miRNA precursor, which binds to the mRNA Lin28 (a marker of human embryonic stem cells), is regulated by the product of the oncogene c-myc. Let-7 family members are downregulated in malignancies, including HCC, and are associated with CSCs. The family members Lin28 and Lin28B

each target and inhibit let-7, and Lin28 and Lin28B are overexpressed in primary human tumors and human cancer cell lines, with an overall frequency of 15%. The mammalian homologs of Lin28 bind to the terminal loop of the precursors of let-7 family miRNAs and block their processing into mature miRNAs. Let-7 suppresses the expression of c-myc, which inhibits the transcription of let-7. Loss of such a negative feedback loop appears to be a common event in cancer cells from advanced-stage tumors such as HCC. MiR-181 regulates the Wnt/β-catenin signaling pathway in a positive feedback loop within stem cells. MiR-181 family members are highly expressed in embryogenic livers and isolated hepatic stem cells.

MiR-181 promotes the stem-cell-like features of HCC cells by targeting mRNAs that encode caudal type homeobox transcription factor 2 (CDX2) and GATA6, which are hepatic transcriptional regulators of differentiation. It also inhibits the mRNA that encodes NLK, an inhibitor of Wnt/β-catenin signaling, and maintains HCC stemness by inhibiting CDX2, GATA6, or NLK. Hepatic transcriptional regulators of differentiation and an inhibitor of Wnt/β-catenin signaling are directly targeted by miR-181. This type of positive feedback loop might be used by cancer cells to continuously self-propagate and contribute to metastasis and drug resistance.

Epigenetic Regulation of Hepatic Stem/Progenitor Cells

Although various genes have been identified as stem cell related, the control of stem cells is likely to arise from an integrated expression pattern of multiple genes involved in proliferation and differentiation rather than decimal gene expression [40]. In the self-renewal process of stem cells, it is important that the gene expression pattern is inherited in daughter cells after cell division. Therefore, chromatin regulation is a newly considered parameter that controls and integrates the expression of multiple genes. Chromatin modifying enzymes regulate the expression of target genes by manifesting structural changes in chromatin. As an epigenetic code, this forms the basis of stem cell identity and determines its responsiveness to extrinsic signals at successive developmental stages. In fact, progression from undifferentiated stem cells toward their differentiated progeny is characterized by alterations in the epigenetic landscapes of regulatory and coding regions of genes. The enzyme complex responsible for histone modification regulates activation and inactivation of transcription through methylation and acetylation of lysine residues in histone H3 and H4 [41]. In particular, histone modifications have been shown to affect polycomb group proteins such as Bmi1 and Ezh2 involved in stem cell regulation.

Recently, the bivalent domains, consisting of active modification H3K4me3 and repressive modification H3K27me3, have been shown to play an important role in the mechanism of action of histone modification proteins in stem cells [42]. Functional analyses of these molecules during liver development have advanced the understanding of several complex chromatin-modifying enzymes involved in cell lineage commitment [43]. In addition, it is reported that the expression of liver-specific transcription factors is changed by the administration of histone deacetylase inhibitors *in vitro* [44]. Special attention is being paid to their role in controlling both the growth and differentiation of stem cells *in vitro*.

Therapeutic Target of Molecular Signaling

The successful eradication of malignancy requires anticancer therapy that affects the differentiated neoplastic cells and the potential CSC population [45–47]. At present, conventional anticancer therapies include chemotherapy, radiation, and immunotherapy that kill

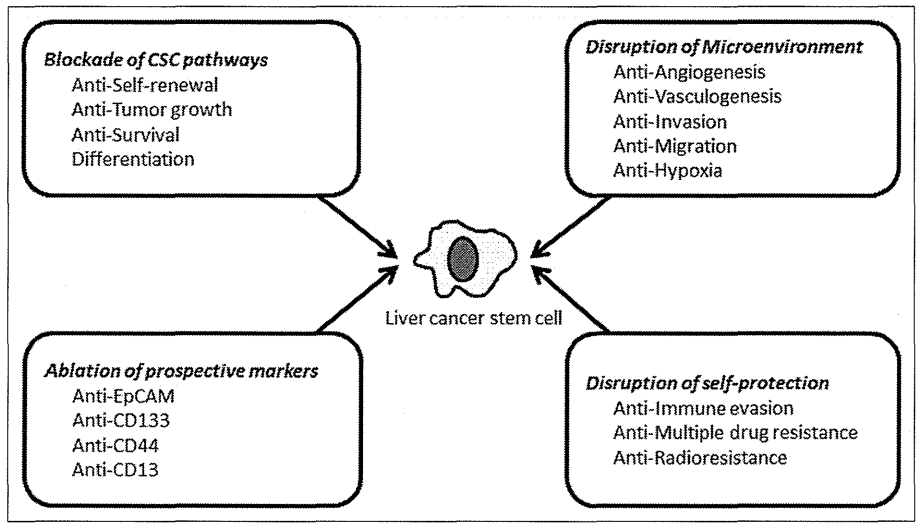

Fig. 2. Strategies to eradicate liver CSCs. CSCs are protected from conventional therapies by changing their microenvironment and self-protection. Specifically targeting any of these areas may lead to the eradication of CSCs.

rapidly growing differentiated tumor cells, thus reducing tumor mass, but potentially leaving behind cancer-initiating cells. Therapies that exclusively address the pool of differentiated cancer cells but fail to eradicate the CSC compartment might ultimately result in relapse and the proliferation of therapy-resistant and more aggressive tumor cells. An ideal drug regime would kill differentiated cancer cells and, at the same time, specifically, selectively, and rapidly target and kill CSCs to avoid toxic side effects in other cell types and to disrupt the self-protection potential of CSCs. 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 a limited benefit. Combinations of therapies may be needed to overcome the complex network of signaling pathways, and ultimately inhibit the signaling that controls tumor growth and survival. In addition to the factors possessed by CSCs themselves, their microenvironment is also important for their maintenance, such as angiogenesis, vasculogenesis, and hypoxia. Many new therapeutic strategies targeting CSCs at various stages of differentiation or targeting the microenvironment have been attempted, as will be discussed below (fig. 2).

Liver Stem/Progenitor Cell Markers

The identification of CSC markers and their exploitation in targeted chemotherapy is an important research goal. It has been shown that CSCs in HCC can be identified on the basis of several cell surface antigens (CD133, CD90, CD44, OV6, and EpCAM), or the presence of side population (SP) cells with Hoechst dye-staining. Given the phenotypic similarities between CSCs and normal stem cells, it is reasonable to infer that the surface phenotype of CSCs resembles that of normal hepatic stem cells.

EpCAM as a Target in Cancer Therapy

EpCAM is potentially a promising target as it is highly expressed in most cancer cells as well as on CSCs. In normal tissue, EpCAM is arranged in a complex with CD9, CD44, and Claudin-7, and is localized to basolateral membranes. Thus, the accessibility for EpCAM-binding antibodies is lower in normal cells than for cancer cells. In cancer cells, EpCAM is strongly overexpressed and so it might be partly unbound and more accessible for targeting antibod-