immunoglobulins in the tissue. Sections were incubated for 30 minutes at room temperature with ImmPRESS peroxidase-micropolymer staining kits and 3,3′-diaminobenzidine substrate (Vector Laboratories). For double immunostaining, a MACH2 peroxidase- and alkaline phosphatase-polymer detection kit, 3,3′-diaminobenzidine, and Warp Red chromogen kit (Biocare Medical, Concord, CA) were used. Sections were lightly counterstained with hematoxylin.

Xenograft Transplantation. Each transplant consisted of 1×10^6 cells of each of the cell lines stably expressing short hairpin RNA (shRNA) against *SALL4* or *luciferase* suspended in 200 μL Dulbecco's modified Eagle medium (DMEM) and Matrigel (1:1). The cells were transplanted into nonobese diabetic, severe combined immunodeficient (NOD/SCID) mice (6-week-old, male) under anesthesia. Control and SALL4-knockdown cells were implanted into the subcutaneous space on the right and left sides of the backs of recipient mice, respectively. For 8 weeks the mice were examined for tumor formation.

SALL4 Profiling Analyses in HCCs. SALL4 expression data were derived from cDNA microarray analysis of 139 HCC specimens as described.[38] The microarray data, with NCI's Human Array-Ready Oligo Set microarray platform (GPL1528), are publicly available at the Gene Expression Omnibus (GEO;http;://www.ncbi.nlm.nih.gov/geo) with accession numbers GSE1898 and GSE4024. High and low SALL4 groups were dichotomized according to the median SALL4 expression in tumors. Kaplan-Meier survival analysis was used to compare patient survival based on dichotomized SALL4 expression using GraphPad Prism software 5.0 (GraphPad Software, San Diego, CA) with statistical *P* values generated by the Cox-Mantel log-rank test. Survival data linking to this cohort were kindly provided by Dr. Snorri Thorgeirsson at NCI.

Other Materials and Methods can be found in the online Supporting Information.

Results

***SALL4 Expression in Human Normal Liver and Biliary Tree Tissues* In Situ *and* In Vitro.** We have previously reported that SALL4 is expressed in murine hepatoblasts (mHBs) but not adult murine hepatocytes and plays a critical role in their differentiation.[39] In these studies, we analyzed SALL4 expression in normal human liver tissues. Immunohistochemical analyses showed that SALL4 is diffusely expressed in the nuclei of liver cells from both fetuses and neonates. Neonatal hepatocytes were more weakly positive for SALL4 than parenchymal cells in fetal livers and some had lost SALL4 expression altogether. In contrast, SALL4 expression was not detected in mature hepatocytes and cholangiocytes in adult livers (Fig. 1A-C). Double immunostaining of EpCAM and cytokeratin19 (CK19) show clearly that EpCAM and CK19 strongly costain the cytoplasm of ductal plate cells, now recognized to comprise hHpSCs, and human hepatoblasts (hHBs) in fetal and neonatal livers. It is found also in hBTSCs within peribiliary glands (PBGs), the stem cell niches of the biliary tree, in neonatal livers (Supporting Fig. S1A), and in adult livers.[40] We found that SALL4 coexpressed with EpCAM+/CK19+ ductal plate cells, known to comprise hHpSCs (arrows), and the adjacent hHBs (arrowheads). It also was found in multiple subpopulations of hBTSCs within PBGs located within livers or biliary tree tissue from all donor ages and included cellular subpopulations that are EpCAM-/CK19+, EpCAM-/CK19-, EpCAM+/CK19-, and EpCAM+/CK19+ cells. Shown are ones from fetal or neonatal livers (Fig. 1D; Supporting Fig. S1A). We also found that SALL4, NCAM, and EpCAM coexpressed in colonies of hHpSCs and in colonies of hBTSCs (Fig. 1E,F; Supporting Fig. S1B). These results suggest that SALL4 is found only in early lineage stage parenchymal cells, such as hHpSCs, hBTSCs, hHBs, and to a less extent in committed progenitors, but not in later lineage stages of parenchymal cells of either liver or biliary tree.

SALL4 Expression in Human Liver Cancers. We analyzed SALL4 expression in surgical specimens of noncancerous liver tissue and in liver cancers. SALL4 was not detected in chronic hepatitis but faintly detected in bile ductules and in hepatocytes at the interface of parenchymal and stromal cells in liver cirrhosis (Supporting Fig. S2A,B). Seventeen of 20 HCC specimens were positive for SALL4 in the nuclei of the tumor cells, whereas three specimens showed no SALL4 expression. In some cases, biliary epithelial cells, presumptive hBTSCs, around the tumors expressed SALL4 (Fig. 2A-C; Supporting Fig. S2C,D). Four of five CC specimens expressed SALL4. We found that SALL4 is expressed in combined hepatocellular and cholangiocarcinoma (HC-CC) and in a transplantable human tumor line derived from a FL-HCC (Fig. 2D-F). Double immunostaining showed that SALL4+/EpCAM+/CK19+ cancer cells were observed in CC, which strongly expressed EpCAM and CK19 in serial sections (Supporting Fig. S2E,F). These results suggest that SALL4 expression indicates selection for stem cells as a minor cell population in normal tissue and cirrhotic tissues and as a dominant cell population in liver cancers.

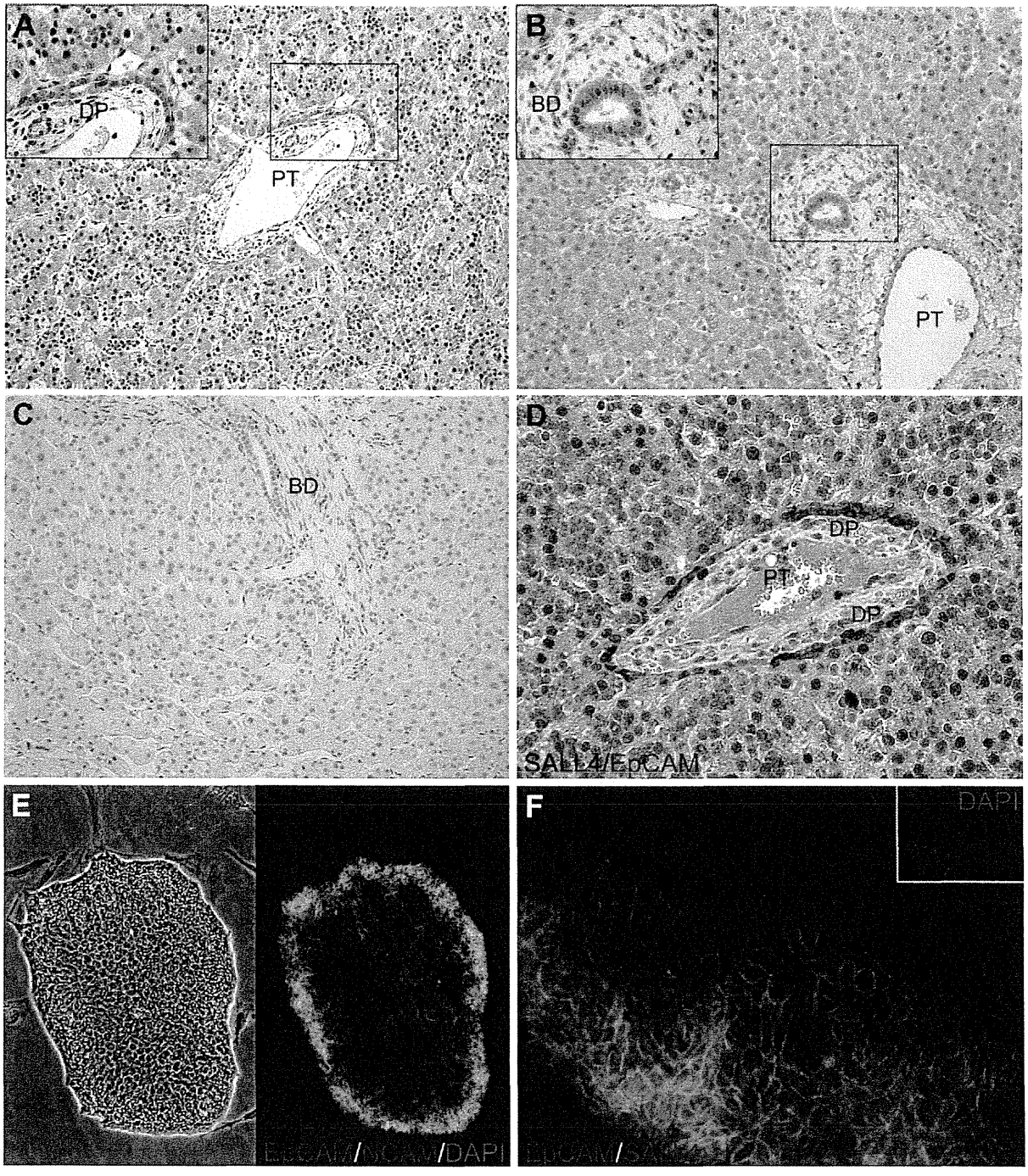

Fig. 1. Representative immunostaining of SALL4 and EpCAM expression in human normal livers and in a colony of hHpSCs. (A-D) Immunostaining of SALL4 expression during liver development. Fetal weeks gestation (A; 19 weeks, D; 16 weeks), neonatal (B; 4 months), and adult liver (C; 68 years) tissues. Sections were stained with an anti-SALL4 antibody (A-C) or antibodies against SALL4 and EpCAM (D). (E,F) A colony of hHpSCs. The colony was stained with antibodies against EpCAM and NCAM (E) or antibodies against EpCAM and SALL4 (F). Magnification ×200 (A-C), ×400 (D, F), ×100 (E). BD, bile duct; DP, ductal plate; PT, portal tract.

SALL4 Expression in Human Liver Cancer Cell Lines. To investigate the functions of SALL4 in liver cancers, we used liver cancer cell lines, Huh7 and PLC/PRF/5 cells. The quantitative real-time polymerase chain reaction (qRT-PCR) analyses showed that both cell lines expressed SALL4A messenger RNA (mRNA). SALL4 protein was also detected using immunocytochemistry (Fig. 3A).

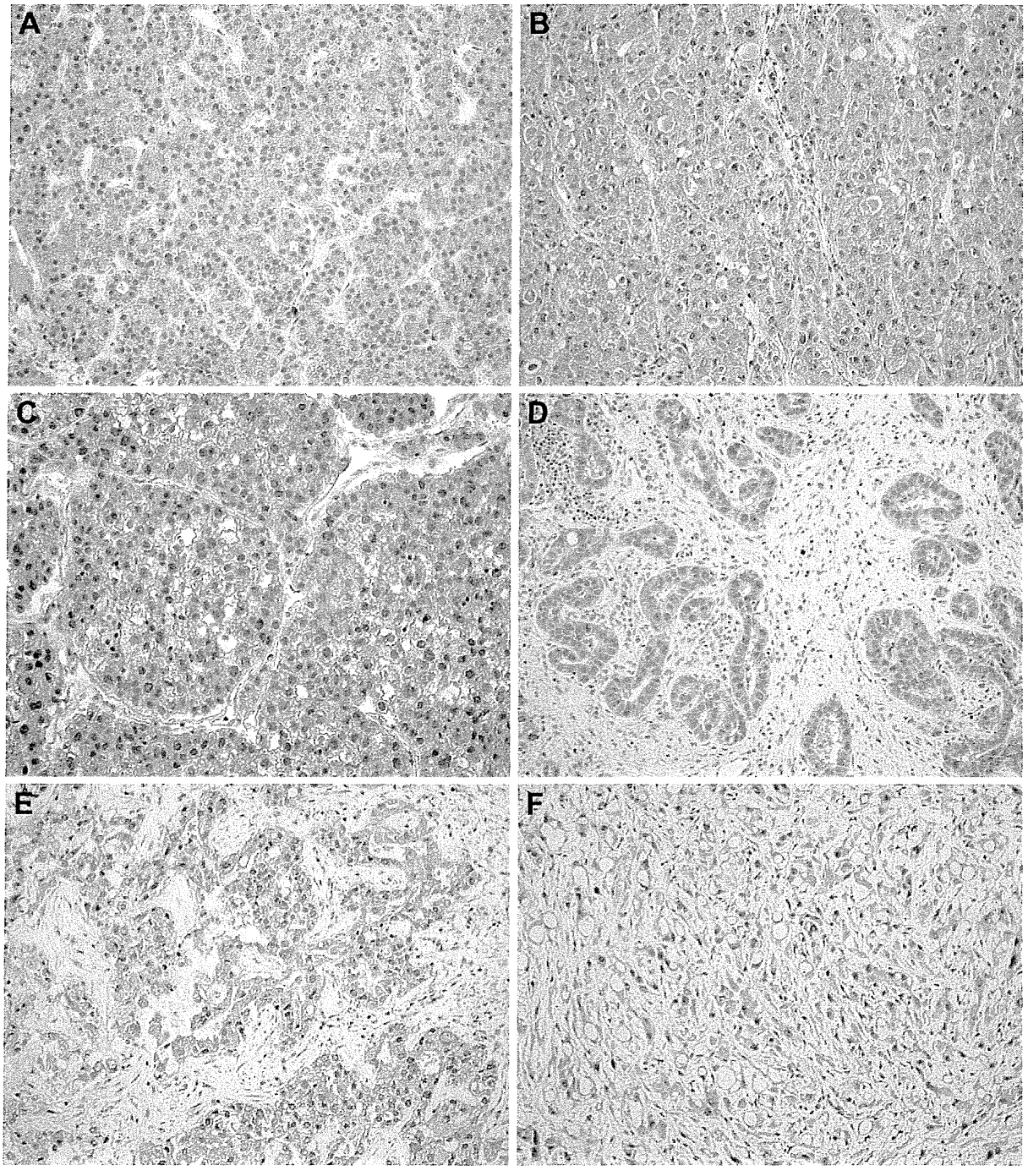

Fig. 2. Representative immunostaining of SALL4 in surgical specimens of liver cancers and transplantable tumor lines of FL-HCC. HCC (A; T41, well differentiated, B; T37, moderately differentiated, C; T49, poorly differentiated). CC (D; T5, poorly differentiated). HC-CC (E; T45, moderately differentiated). FL-HCC (F; poorly differentiated). Magnification ×200.

Regulation of Cell Proliferation by SALL4. To examine whether SALL4 regulates tumor growth of liver cancer cell lines, we used a SALL4A-overexpressing retroviral vector.[28] Overexpression of SALL4A was verified using qRT-PCR. Transduction of SALL4A into the cells significantly increased SALL4A mRNA and also protein levels by western blots and immunocytochemistry (Fig. 3B; Supporting Fig. S3). SALL4A-overexpressing liver cancer cells had enhanced cell proliferation (Fig. 3C).

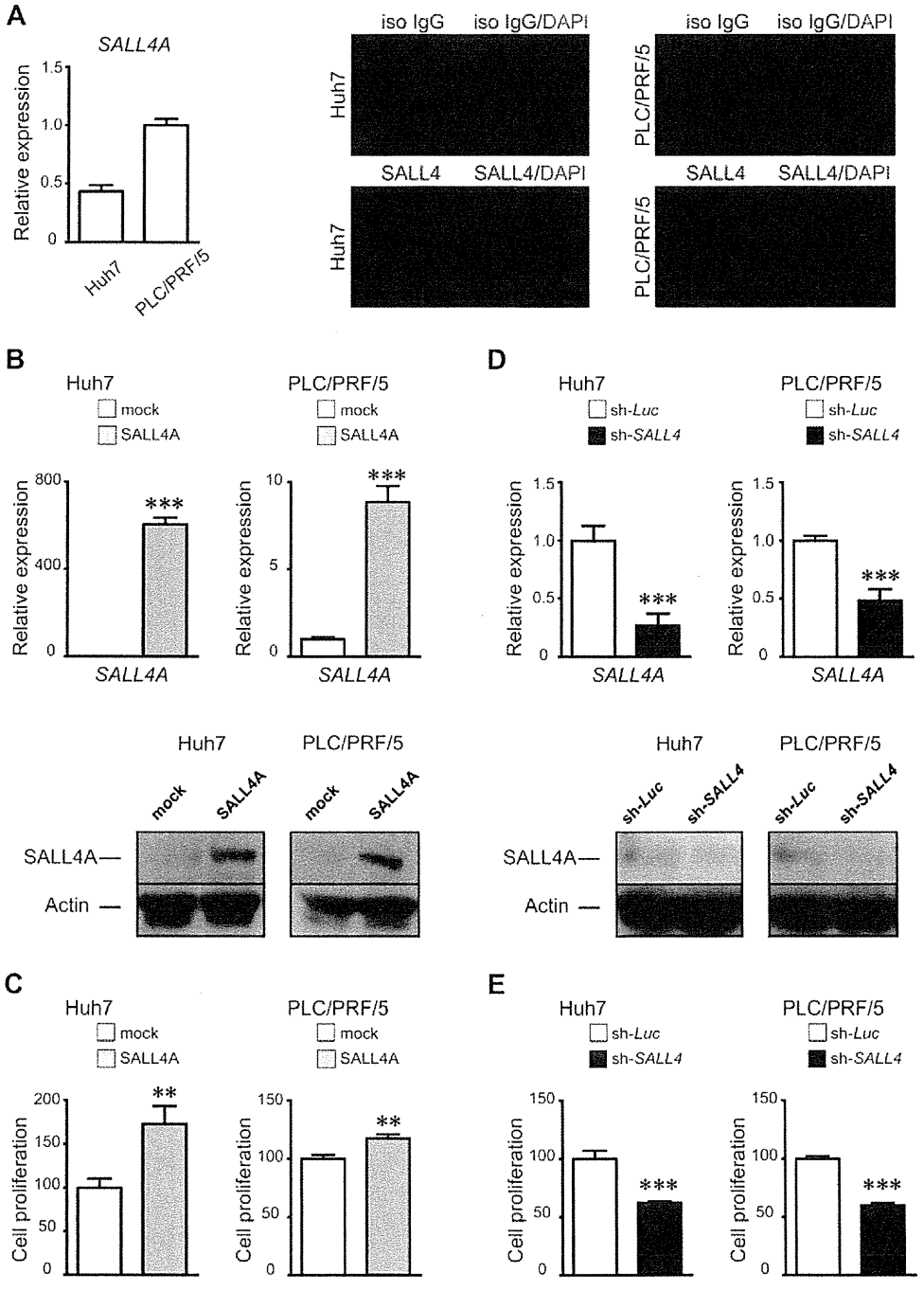

Fig. 3. SALL4 expression and the effects of SALL4 overexpression or knockdown on cell proliferation of liver cancer cells. (A) SALL4A mRNA and protein expression in liver cancer cells. (B,D) Expression of SALL4 mRNA and proteins in cultures derived from SALL4-overexpressing or SALL4-knockdown liver cancer cells. Cells infected with mock- or SALL4-expressing retroviruses, with shRNA against *luciferase* or *SALL4*-expressing lentiviruses were cultured for 3 days. (C,E) Cell proliferation assays of cells transduced by a SALL4-overexpressing retroviral vector or a SALL4-knockdown lentiviral vector were cultured for 7 days. Data are expressed as mean ± SD (triplicate samples, $***P < 0.001$, $**P < 0.01$).

Next, we conducted SALL4 expression knockdown studies using a lentiviral vector expressing-shRNA.[32,39] Transduction efficiency was estimated using FACS revealing that the percentage of cells infected with lentiviruses expressing-shRNA against *luciferase* or *SALL4* was more than 90% (Supporting Fig. S4).

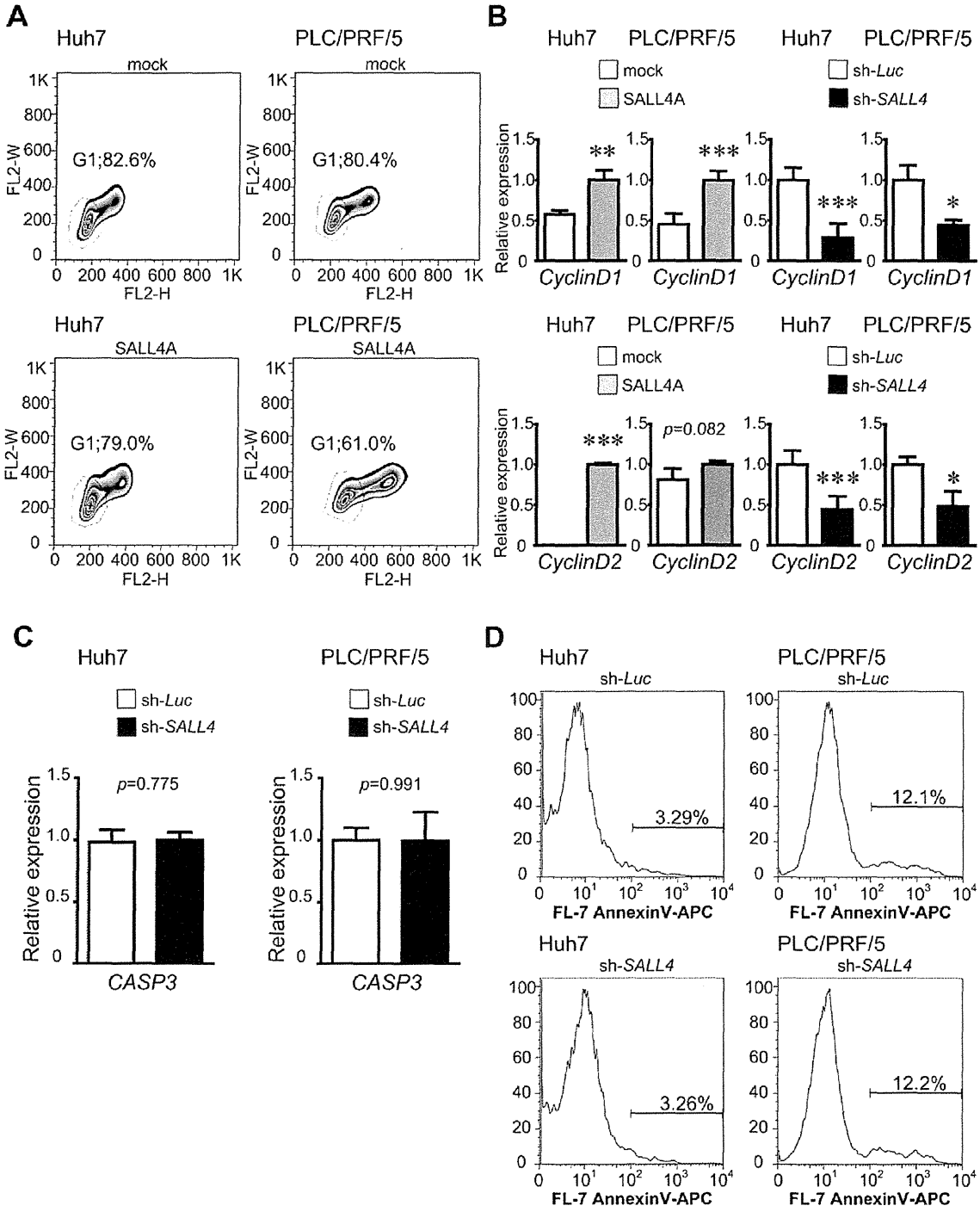

Fig. 4. Correlates of SALL4 overexpression or knockdown with respect to cell proliferation of liver cancer cells. (A) Cell-cycle analysis in SALL4-overexpressing liver cancer cells was estimated by flow cytometry. (B,C) Expression of Cyclin D1, Cyclin D2, and CASP3 in SALL4-overexpressing or SALL4-knockdown liver cancer cells. Cells transduced by a retroviral or lentiviral vector were cultured for 3 days. Cyclin D1, Cyclin D2, and CASP3 mRNA expression was detected using qRT-PCR. Data are expressed as mean ± SD (triplicate samples, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$). (D) Apoptosis in SALL4-knockdown liver cancer cells was estimated by flow cytometry. Cells were cultured for 3 days and stained with allophycocyanin (APC)-conjugated anti-Annexin-V antibody.

Transduction of shRNA into the cells significantly decreased both mRNA and protein production of SALL4 (Fig. 3D). We observed growth inhibition in SALL4-knockdown liver cancer cells in culture (Fig. 3E; Supporting Fig. S5). Therefore, SALL4 regulates the proliferative potential of liver cancer cell lines *in vitro*.

SALL4 Regulates Cell Proliferation Through Cyclin D1 and D2 Expressions. To analyze molecular mechanisms regulating SALL4-induced proliferation of liver cancer cell lines, cell-cycle analyses were examined. Cell-cycle analyses using flow cytometry showed that overexpression of SALL4 induced the decrease of the G_1 phase in liver cancer cells (Fig. 4A). Next, Cyclin D1 and D2 mRNA expressions were examined using qRT-PCR. Consistent with the flow cytometry analysis, Cyclin D1 and D2 levels were induced by SALL4A overexpression. In contrast, their levels were decreased by SALL4 knockdown (Fig. 4B), implicating a correlation of Cyclin levels to those of cell proliferation. Although we also analyzed expression of cyclin inhibitors, significant changes were not observed (data not shown).

To exclude the possibility that shRNA-knockdown of SALL4 expression inhibited cell proliferation by means of an induction of apoptosis, we analyzed the effect of viral infection on apoptosis of the liver cancer cell lines. The qRT-PCR analyses showed that caspase-3 (CASP3) expression, an early stage marker of apoptosis, did not change in SALL4-knockdown liver cancer cells (Fig. 4C). Apoptosis was also evaluated using flow cytometric analyses. The number of Annexin-V+ cells did not change by SALL4 knockdown, suggesting that inhibition of cell proliferation was not due to apoptosis (Fig. 4D).

SALL4 Expression Is Inversely Correlated with Differentiation Markers. Given that hepatocytic maturation was suppressed by SALL4 overexpression in mHBs,[39] we hypothesized that SALL4 could affect the differentiation of liver cancer cell lines. To explore this, we analyzed mRNA expression for hepatocytic differentiation marker genes using qRT-PCR. Expression of albumin (ALB), transthyretin (TTR), and UDP-glucuronosyltransferase-2B7 (UGT2B7) were suppressed by SALL4 overexpression. In contrast, their levels were significantly enhanced in SALL4-knockdown liver cancer cells (Fig. 5A; Supporting Figs. S6A, S7). These results suggested that SALL4 inhibits hepatocytic differentiation in mHBs and also human liver cancer cell lines. Hepatocyte nuclear factor 4-alpha (HNF4α), a key transcriptional factor regulating differentiation of HBs into hepatocytes with acquisition of mature liver functions, did not decrease in SALL4-overexpressing liver cancer cells, indicating that SALL4 inhibits hepatocytic differentiation through a pathway independent of HNF4α (Supporting Fig. S6A). As shown above, CK19 and EpCAM are expressed in normal hHpSCs, hHBs, and cholangiocytes in livers of all donor ages but not adult hepatocytes, and EpCAM is also a TIC marker for liver cancer. Overexpression of SALL4 in liver cancer cells induced expression of CK19 and EpCAM (encoded by TACSTD1 gene), indicating a correlation between SALL4 and CK19. Down-regulation of SALL4 suppressed the expression of CK19 but not EpCAM in liver cancer cells. SALL4-overexpressing PLC/PRF/5 cells had up-regulated POU5F1 (OCT3/4) and CD90 (Fig. 5B; Supporting Figs. S6B, S7). Similarly, ABCG2, a multidrug resistance gene found in normal hHpSCs as well as in CSCs and responsible for chemoresistance, was significantly increased in SALL4-overexpressing Huh7 cells. In contrast, SALL4 knockdown of liver cancer cells resulted in lowered ABCG2 levels (Fig. 5B). These results suggest that SALL4 either plays a role controlling maintenance of stemness and TIC marker genes or is a biomarker for stem cell phenotypic traits.

SALL4 Increases Expression of EMT Genes but Does Not Influence Cell Invasion. Epithelial-mesenchymal transition (EMT) phenomena occurs in invasion and metastasis of cancer cells and is also associated with the acquisition of stem cell-like characteristics. To investigate whether SALL4 regulates EMT, we analyzed its effects on EMT-related genes in liver cancer cell lines. The mRNA expression of CXCR4 and TWIST1, a direct transcriptional target of EMT inducers, was up-regulated by SALL4 overexpression. In contrast, another important EMT phenomenon, down-regulation of E-cadherin (encoded by the CDH1 gene) was not observed in SALL4-overexpressing liver cancer cells (Fig. 6A), nor were there significant changes in cell migration assays with the liver cancer cells (Fig. 6B). These data suggest that cell migration and invasion of liver cancer cells are not directly affected by SALL4 even though some EMT-related genes are up-regulated.

SALL4 Expression Is Correlated with Chemosensitivity. We previously reported that the oncostatin M (OSM) induced maturation of fetal hepatic cells.[41] OSM induced hepatocytic differentiation of EpCAM+ liver CSCs into EpCAM-negative cells and increased chemosensitivity to 5-FU.[42] As shown above, we have shown that overexpression of SALL4 suppressed hepatocytic differentiation and induced stem cell-like phenotype in liver cancer cells. We thus analyzed whether overexpression of SALL4 affects chemosensitivity of liver cancer cell lines. 5-FU treatment decreased cell proliferation in both lines. Cell survival and proliferation of liver cancer cells were induced by SALL4-overexpression with or without 5-FU. Interestingly, overexpression of SALL4 increased cell proliferation (5-FU/PBS) in liver cancer cells (Fig. 7A,B). These results

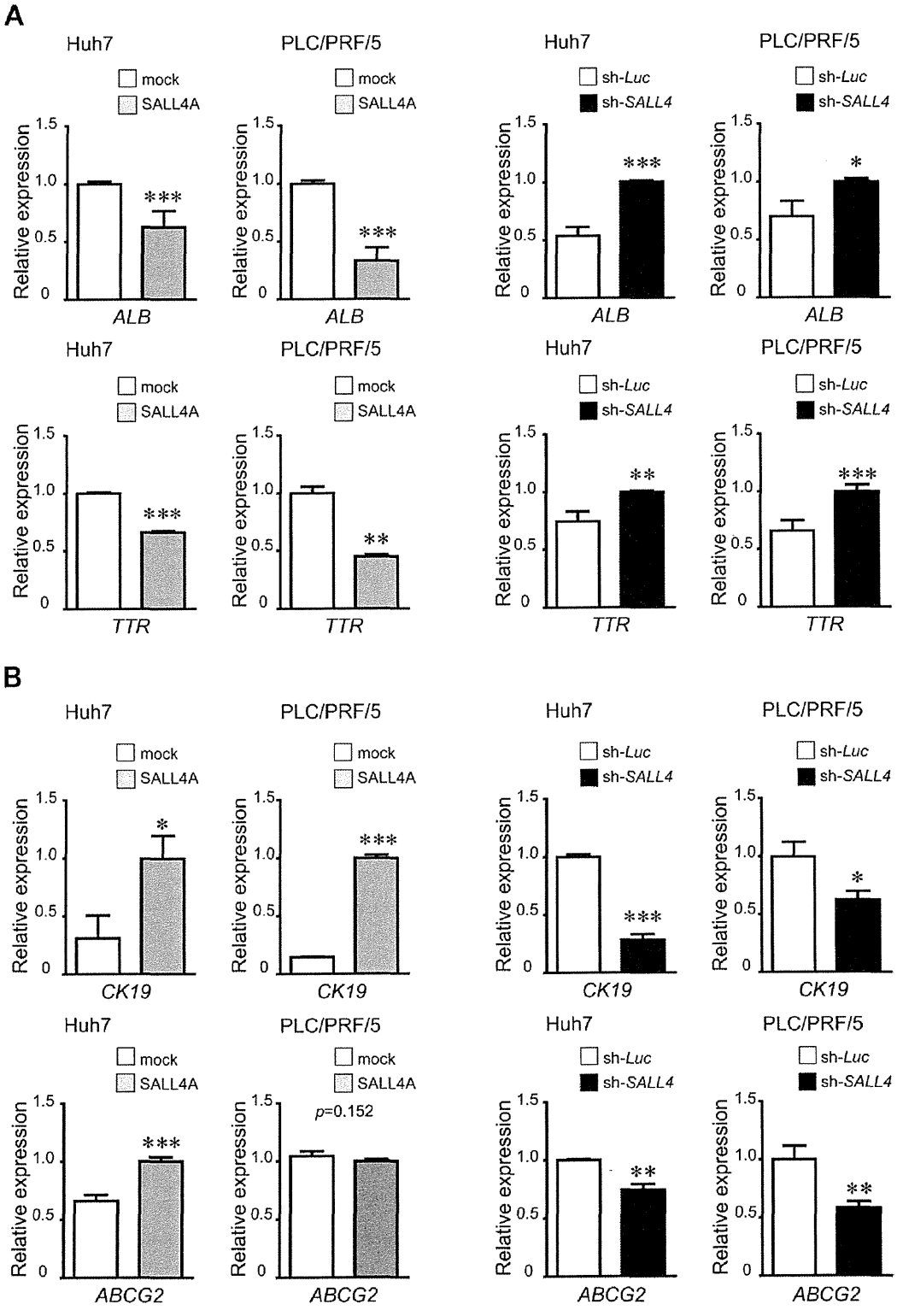

Fig. 5. Expression of hepatocytic differentiation (A) and stemness (B) genes in SALL4-overexpressing or SALL4-knockdown liver cancer cells. Cells transduced by a retroviral or lentiviral vector were cultured for 3 days. ALB, TTR, CK19, and ABCG2 mRNA expression was detected using qRT-PCR. Data are expressed as mean ± SD (triplicate samples, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$).

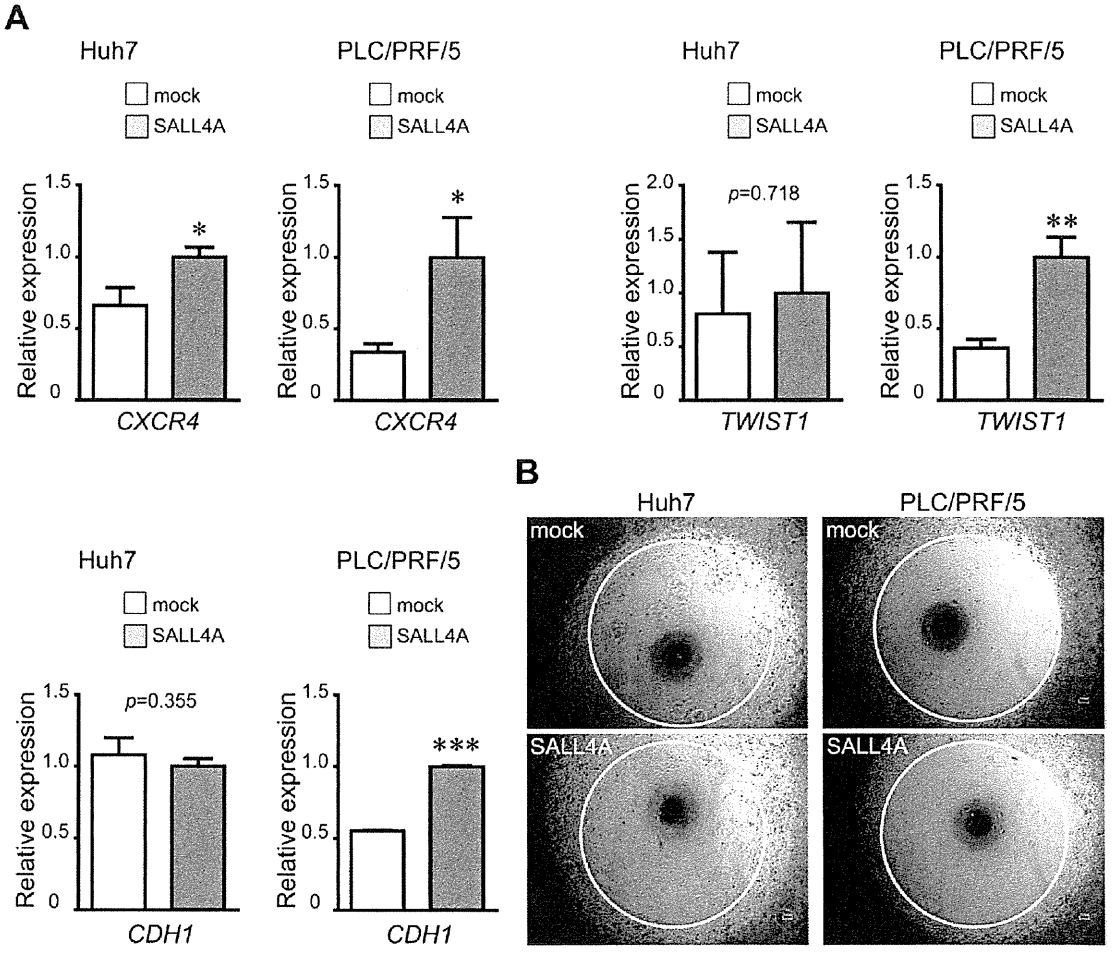

Fig. 6. Expression of EMT-related genes and migration assays in SALL4-overexpressing liver cancer cells. (A) Cells transduced by an overexpressing retroviral vector were cultured for 3 days. CXCR4, TWIST1, and CDH1 mRNA expression was detected using qRT-PCR. Data are expressed as mean ± SD (triplicate samples, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$). (B) Migration assay in SALL4-overexpressing liver cancer cells.

suggest that SALL4 expression results in selection of cells that are chemoresistant.

Down-regulation of SALL4 Inhibits Tumor Growth in Xenograft Transplantation. To determine whether SALL4 affects tumorigenicity of liver cancer cell lines, we generated stable liver cancer cells expressing-shRNA against *luciferase* or *SALL4*, and cells were transplanted into the subcutaneous space on the right versus left sides of immunodeficient mice, respectively. After 8 weeks, both control Huh7 and PLC/PRF/5 cells gave rise to subcutaneous tumors. In contrast, tumors derived from SALL4-knockdown liver cancer cells were significantly smaller than those of control cells (Fig. 8A-C). The tumor weights were also smaller than those from control cells (Fig. 8D). These results suggest that down-regulating SALL4 expression also inhibited growth of the tumors from liver cancer cell lines *in vivo*.

SALL4 Expression in HCC Clinical Specimens Is Prognostic of Patient Survival (Bioinformatics Analyses). We examined SALL4 expression in 139 HCC cases in a microarray dataset published by Lee et al.[38] A total of 110 cases with available expression and overall survival data were selected for survival analysis. We found that HCC patients with high SALL4 expression is significantly associated with shorter survival during the first 3 years of follow-up ($P = 0.038$) (Fig. 8E).

Discussion

Gene expression profiles and signaling pathways associated with self-renewal and differentiation are shared in normal stem cells and in CSCs.[3] Accordingly, fully understanding these common molecular mechanisms that regulate self-renewal and differentiation is a necessary step towards novel therapeutic

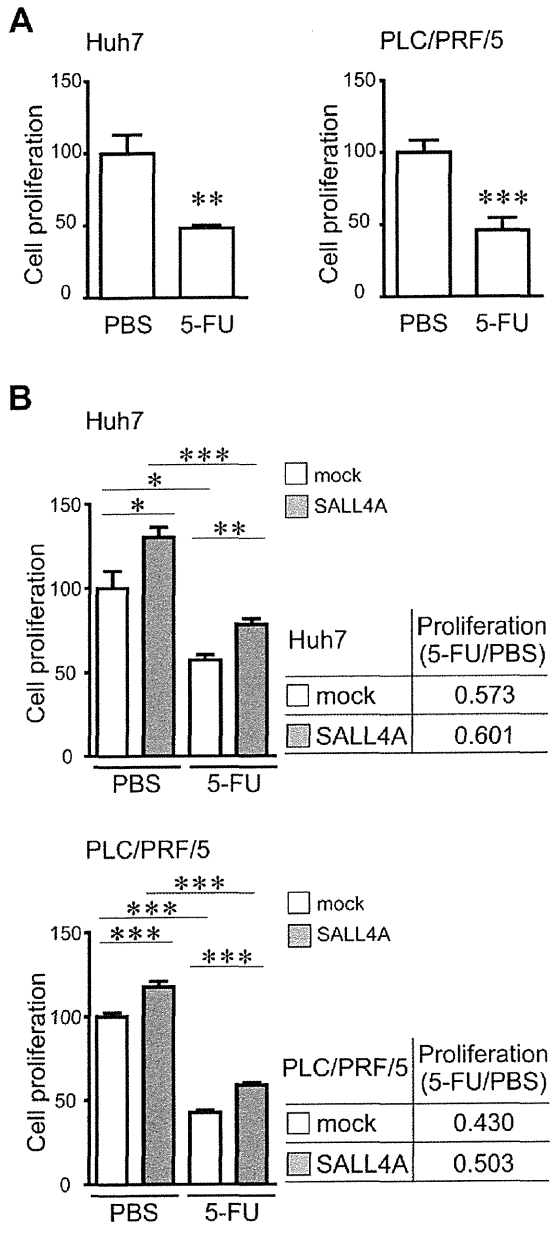

Fig. 7. Chemoresistance assays for SALL4-overexpressing liver cancer cells. Cells were transduced by a retroviral vector. Nontransduced (A) or transduced cells (B) were cultured in the presence or absence of 5-FU (2 μg/mL) for 7 days. The relative cell proliferation between PBS- and 5-FU-treated liver cancer cells is shown. Data are expressed as mean ± SD (triplicate samples, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$).

modalities for cancer. The only curative treatments for liver cancers are surgical resection and liver transplantation for early-stage patients. However, most patients are diagnosed at advanced stages by which time extant therapies are ineffective. For the treatment of advanced HCC patients with unresectable tumors, transcatheter arterial chemoembolization and systemic chemotherapy, including sorafenib, are one of the options, but the effects are limited.[14,17] Therefore, the identification of novel molecules that can become targets for future therapies is urgently needed.

SALL4 is required for cell proliferation and maintenance of pluripotency in several types of stem cells (e.g., ESCs) and in malignantly transformed stem cells (e.g., leukemia and breast cancer).[21-26] In addition, our prior investigations with mHBs revealed that inhibition of SALL4 contributes to cell differentiation.[39] Hence, it seemed likely that SALL4 expression could be a factor in liver cancers in which the CSCs might have a shared gene profile to normal hHpSCs and/or to normal hBTSCs. This hypothesis became plausible when we found SALL4 expression in normal hHpSCs, hHBs, and with weaker expression in committed progenitors in human fetal and neonatal liver tissues, in stem cells in PBGs, the stem cell niches of human biliary tree tissue, and in various liver cancers (Figs. 1, 2). In recent publications it was reported that SALL4 is expressed in hepatoid gastric carcinoma but not in other liver cancer.[36,37] We hereby report that SALL4 expression in liver cancers (and cancers of the biliary tree) can be detected by using EDTA buffers, rather than citrate buffers, for antigen retrieval. The mechanisms of antigen retrieval are poorly understood. It has been reported that antigen retrieval is needed for disruption of methylene-bridges during fixation, which crosslink proteins and therefore mask antigenic sites. Indeed, we were not able to obtain clearly positive SALL4 staining in liver cancer tissues when we used citrate buffer (pH 6.0), the most popular buffer for antigen retrieval. Therefore, we decided to use EDTA buffer (pH 8.0), because it has been reported that the pH of antigen retrieval solution remarkably affects the intensity of immunostaining.[43] SALL4-positive cells were observed by using EDTA/pH8.0 rather than citrate buffer (Supporting Fig. S8). This indicates that the pH of the retrieval buffer and the presence of EDTA, the chelating agent, are important factors for masking the epitopes available for binding either by eliminating masking molecules and/or proper refolding of SALL4-specific epitopes to bind with antibody.

One of the main regulators of G_1-S phase transition in the cell cycle, Cyclin D1, has been shown to have capabilities of carcinogenesis and progression in cancer through controlling cell proliferation.[44] Moreover, the strong relationship of tumorigenesis and self-renewal by Ras-Cyclin D2 activation has been elucidated in spermatogonial stem cells.[45] With respect to SALL4's effects on growth, recent studies revealed that Cyclin D1 has been shown to bind to SALL4 and works

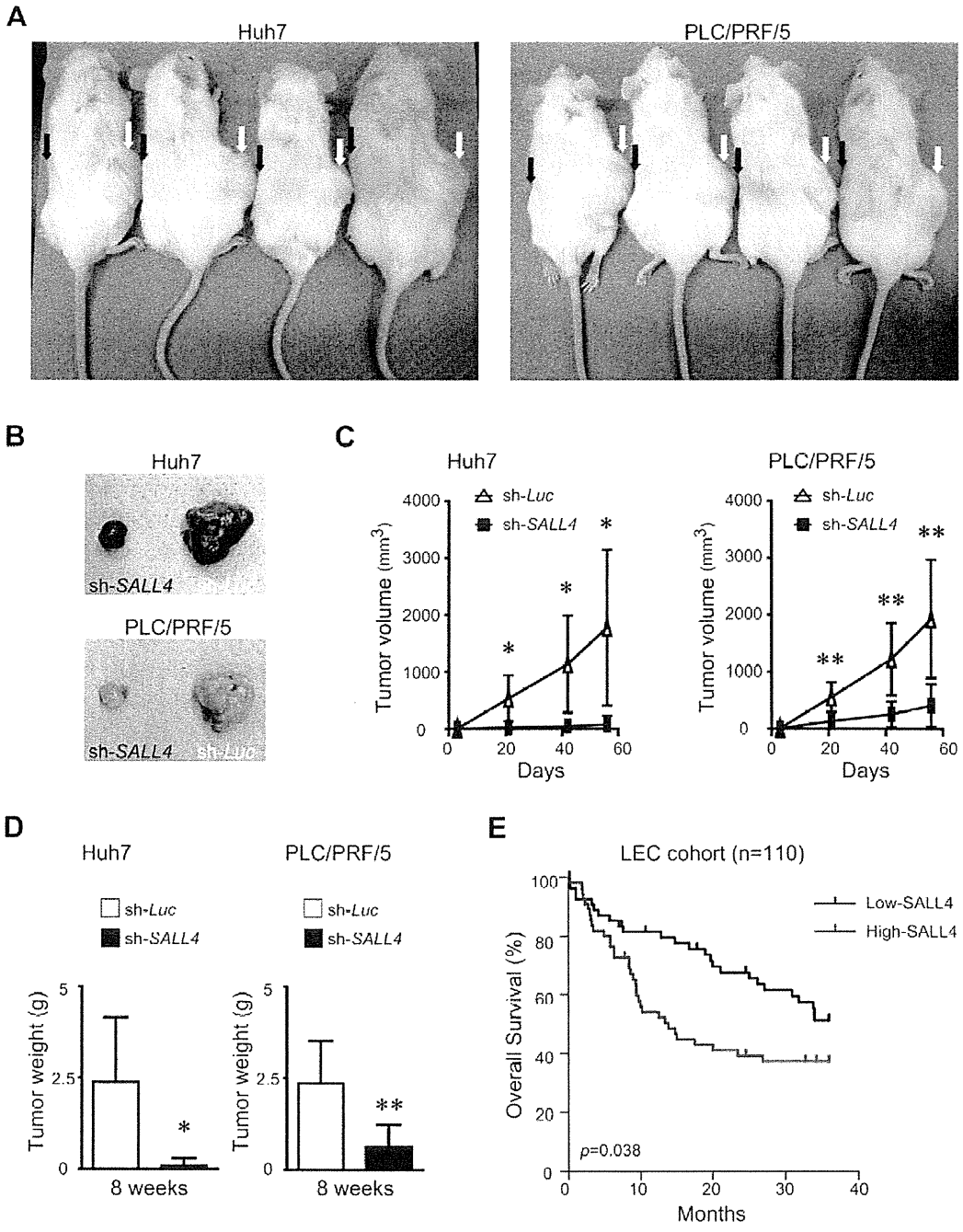

Fig. 8. Effect of SALL4 knockdown on xenograft tumor growth *in vivo*. (A) Control cells and SALL4-knockdown cells were implanted into recipient mice, respectively. White arrows show tumors derived from control cells and black arrows show tumors derived from SALL4-knockdown cells (Huh7 n = 5, PLC/PRF/5 n = 8). (B) Representative tumors derived from control versus SALL4-knockdown liver cancer cells at 8 weeks are shown. (C) The tumor growth curve over 8 weeks is shown. (D) The weight of the tumor at 8 weeks is shown. Data are expressed as mean ± SD (**$P < 0.01$, *$P < 0.05$). (E) Kaplan-Meier survival plot according to the relative level of SALL4 expression in HCC tumor samples, as determined by microarray analyses and with the use of the log-rank test. The median expression level was used to dichotomize low and high SALL4-expressing HCC tumors.

synergistically in transcriptional repression; Cyclin D1 is a downstream target of SALL4 in malignant cells and in ESCs.[25,31,46] We found overexpressing SALL4 induced a shorter G_1 phase, and there was a positive correlation between expression of SALL4 and Cyclin D1 and D2 in liver cancer cell lines. This suggests

that SALL4 regulates cell proliferation either by selection of early lineage stage cells or by controlling G_1-S transition through regulating expression of Cyclin D1 and D2 directly. Although SALL4 has been proposed to play a role in survival and apoptosis in leukemic cells,[32] we did not observe any difference in apoptosis between control and SALL4-knockdown liver cancer cell lines (Fig. 4), indicating that downstream targets for SALL4 may be different in liver cancer cells and leukemic cells.

Analyses of functions using models of liver cancer cell lines indicated that SALL4 overexpression leads to cells with enhanced phenotypic traits such as ABCG2 and CK19 expression, ones highly expressed in stem cells. SALL4 is associated also with CD90 (Thy-1), known to be highly expressed in mesenchymal cells tightly associated with the stem cell. In contrast, SALL4 knockdown provided evidence of slowed growth and more parenchymal cell differentiation. In summary, SALL4 expression is a marker of stem cells and early lineage descendants from those stem cells, implicating it as a marker of TICs. Its expression correlates with cell proliferation, survival, and a minimally differentiated status in normal and in malignantly transformed cells.

Findings reported recently corroborate our own in that OSM induction or HNF4α gene transfer into liver cancer cells resulted in more differentiated cells with reduced tumor-initiating ability and enhancement of sensitivity to 5-FU.[42,47] High levels of SALL4 correlate with growth and stemness features, and SALL4 suppression results in inhibition of growth, increased hepatocytic differentiation of cells, and reduced tumorigenicity (Figs. 3-8).

SALL4 has been found in normal hHpSCs and hHBs, stem/progenitor cell populations found intrahepatically and associated with canals of Hering[6,48]; both of these are positive for EpCAM and CK19, and the hHBs are positive also for AFP and for ALB. Interestingly, it is found strongly expressed in all of the subpopulations of hBTSCs, ones located with PBGs throughout the biliary tree and that comprise the most primitive stem cells identified (LGR5+/NCAM+/SOX17+/PDX1+/CK19+/EpCAM-/AFP-/ALB-); others with phenotypic traits identical to or similar to that of hHpSCs (LGR5+/NCAM+/EpCAM+/SOX17+/PDX1-/CK19+/AFP-/ALB-); and yet others with traits overlapping with those of hHBs (LGR5-/EpCAM+/SOX17-/PDX1-/ICAM-1+/CK19+/AFP++/ALB+-).[7,8,40] It is also found in stem/progenitor cells of human fetal but not adult pancreas (Oikawa, Wauthier, and Reid, unpublished data).

SALL4 has also been identified as a novel molecule in reprogramming of somatic cells to become iPSCs.[27,28] This background makes interpretable published bioinformatics analyses[49] in which there is no significant correlations between the expression of SALL4, EpCAM, AFP, or ALB in liver cancers. Rather, we found that it correlates with HCC patient's prognosis because an increased SALL4 expression is associated with shorter survival in HCC patients (Fig. 8). It should be noted that we have not yet done bioinformatics analyses relating SALL4 expression in survival of patients with CC; however, we hypothesize that it will be relevant to survival for patients with CC, given that SALL4 expression is strong in all the subpopulations of normal hBTSCs. We interpret this to mean that high SALL4 expression indicates tumors enriched for CSCs, whether or not they express EpCAM, AFP, or ALB. Thus, SALL4 is a reliable indicator of stem cell populations, whether normal or malignantly transformed, and its levels quantitatively indicate the proportion of the tissue comprised of those stem cells. Therefore, our findings corroborate those of others suggesting that SALL4 is indicative of aggressiveness and poor prognosis in liver cancers.[9,38,50]

Taken together, SALL4 is an excellent target for identifying treatments for liver cancers. Suppression of SALL4 expression may contribute to inhibition of tumor growth by (1) attenuation of cell cycle progression by way of Cyclin D1 and D2; (2) reduction in stem cell traits and, thereby, allowing a more differentiated state; and (3) reduction in multidrug resistance genes with increased sensitivity to chemotherapies. Further analyses on SALL4-mediated mechanisms may provide a novel future therapeutic strategy against liver cancers.

Acknowledgment: We thank Prof. Yamanaka and Prof. Takahashi (Kyoto University, Kyoto, Japan) for the hSALL4 expression vector. We thank Dr. Derek Y. Chiang (UNC School of Medicine, Chapel Hill, NC) for preliminary bioinformatics analyses on SALL4, EpCAM, albumin, and alpha-fetoprotein, findings that indicated no significant relationships. Discussions with Dr. Oswaldo A. Lozoya (Duke University Medical Center, Durham, NC) made us aware of the fact that some published bioinformatics databases consist of data from tumors normalized to that from the so-called normal margins of the tumors; other studies of our own had revealed that the so-called "normal margins," in fact, are not normal both with respect to the epithelial and mesenchymal cellular components. Dr. Lozoya's counseling resulted in our searching for alternative ways to assess the relevance of SALL4 to liver tumors using bioinformatics analyses. With respect to the investigations within the United States, we thank

Ms. Lucendia English for glassware washing and laboratory management. Various core services provided essential support including several of the histology cores, confocal microscopy core, and tissue culture core. No author has equity or a position in Vertex Pharmaceuticals or in Vesta Therapeutics, and none are paid consultants.

Author Contributions

The project was originally conceived and experimentally designed by T. Oikawa, A. Kamiya, and H. Nakauchi. T. Oikawa and A. Kamiya did the collection and assembly of data, data analyses, and interpretation of the data, especially that on mechanistic studies with respect to SALL4. H. Chikada, Y. Yamaszaki, and A.D. Hyuck helped with collection and assembly of data. E. Wauthier and L.M. Reid established the bank of normal fetal, neonatal, pediatric, and adult human livers, biliary tree tissue, and pancreatic tissues and that of surgical specimens of hepatocellular carcinomas (HCCs), cholangiocarcinomas (CCs) the combined hepatocellular and cholangiocarcinoma (HC-CC), and the fibrolamellar hepatocellular carcinoma (FL-HCC). They also established cultures and transplantable tumor lines of some of the HCCs, CCs, and the FL-HCC. The management and funding of these studies on human tissues were done by L.M. Reid. L.M. Reid and T. Oikawa designed the experiments; T. Oikawa collected the data; and T. Oikawa and L.M. Reid together did data analyses and interpretation of data. X.W. Wang and L.D. Miller performed the bioinformatics analyses correlating SALL4 expression in liver cancers with patient survival. The article was drafted and edited by T. Oikawa, A. Kamiya, L.M. Reid, and they handled responses to reviewers. M. Zeniya, H. Tajiri helped with editing of the article. H. Nakauchi also did management and interpretation of the data and helped with writing and editing of the article.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74-108.
2. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105-111.
3. Lobo NA, Shimono Y, Qian D, Clarke MF. The biology of cancer stem cells. Annu Rev Cell Dev Biol 2007;23:675-699.
4. Chiba T, Kita K, Zheng YW, Yokosuka O, Saisho H, Iwama A, et al. Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. HEPATOLOGY 2006;44:240-251.
5. Zen Y, Fujii T, Yoshikawa S, Takamura H, Tani T, Ohta T, et al. Histological and culture studies with respect to ABCG2 expression support the existence of a cancer cell hierarchy in human hepatocellular carcinoma. Am J Pathol 2007;170:1750-1762.
6. Schmelzer E, Zhang L, Bruce A, Wauthier E, Ludlow J, Yao HL, et al. Human hepatic stem cells from fetal and postnatal donors. J Exp Med 2007;204:1973-1987.
7. Cardinale V, Wang Y, Carpino G, Cui CB, Gatto M, Rossi M, et al. Multipotent stem/progenitor cells in human biliary tree give rise to hepatocytes, cholangiocytes, and pancreatic islets. HEPATOLOGY 2011;54: 2159-2172.
8. Cardinale V, Wang Y, Carpino G, Mendel G, Alpini G, Gaudio E, et al. The biliary tree:a reservoir of multipotent stem cells. Nat Rev Gastroenterol Hepatol 2012;9:231-240.
9. Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY, et al. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. Gastroenterology 2009;136:1012-1024.
10. Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007;132:2542-2556.
11. Yang ZF, Ho DW, Ng MN, Lau CK, Yu WC, Ngai P, et al. Significance of CD90+ cancer stem cells in human liver cancer. Cancer Cell 2008;13:153-166.
12. Haraguchi N, Ishii H, Mimori K, Tanaka F, Ohkuma M, Kim HM, et al. CD13 is a therapeutic target in human liver cancer stem cells. J Clin Invest 2010;120:3326-3339.
13. Wang Y, Yao HL, Cui CB, Wauthier E, Barbier C, Costello MJ, et al. Paracrine signals from mesenchymal cell populations govern the expansion and differentiation of human hepatic stem cells to adult liver fates. HEPATOLOGY 2010;52:1443-1454.
14. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002;31:339-346.
15. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer 2006;6:674-687.
16. Yamashita T, Budhu A, Forgues M, Wang XW. Activation of hepatic stem cell marker EpCAM by Wnt-beta-catenin signaling in hepatocellular carcinoma. Cancer Res 2007;67:10831-10839.
17. Llovet JM, Bruix J. Molecular targeted therapies in hepatocellular carcinoma. HEPATOLOGY 2008;48:1312-1327.
18. Mishra L, Banker T, Murray J, Byers S, Thenappan A, He AR, et al. Liver stem cells and hepatocellular carcinoma. HEPATOLOGY 2009;49:318-329.
19. Marquardt JU, Factor VM, Thorgeirsson SS. Epigenetic regulation of cancer stem cells in liver cancer: current concepts and clinical implications. J Hepatol 2010;53:568-577.
20. Nault JC, Zucman-Rossi J. Genetics of hepatobiliary carcinogenesis. Semin Liver Dis 2011;31:173-187.
21. Sakaki-Yumoto M, Kobayashi C, Sato A, Fujimura S, Matsumoto Y, Takasato M, et al. The murine homolog of SALL4, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with Sall1 in anorectal, heart, brain and kidney development. Development 2006;133:3005-3013.
22. Zhang J, Tam WL, Tong GQ, Wu Q, Chan HY, Soh BS, et al. Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1. Nat Cell Biol 2006;8:1114-1123.
23. Elling U, Klasen C, Eisenberger T, Anlag K, Treier M. Murine inner cell mass-derived lineages depend on Sall4 function. Proc Natl Acad Sci U S A 2006;103:16319-16324.
24. Lim CY, Tam WL, Zhang J, Ang HS, Jia H, Lipovich L, et al. Sall4 regulates distinct transcription circuitries in different blastocyst-derived stem cell lineages. Cell Stem Cell 2008;3:543-554.
25. Yuri S, Fujimura S, Nimura K, Takeda N, Toyooka Y, Fujimura Y, et al. Sall4 is essential for stabilization, but not for pluripotency, of embryonic stem cells by repressing aberrant trophectoderm gene expression. Stem Cells 2009;27:796-805.
26. Yang J, Gao C, Chai L, Ma Y. A novel SALL4/OCT4 transcriptional feedback network for pluripotency of embryonic stem cells. PLoS One 2010;5:e10766.
27. Wong CC, Gaspar-Maia A, Ramalho-Santos M, Reijo Pera RA. High-efficiency stem cell fusion-mediated assay reveals Sall4 as an enhancer of reprogramming. PLoS One 2008;3:e1955.

28. Tsubooka N, Ichisaka T, Okita K, Takahashi K, Nakagawa M, Yamanaka S. Roles of Sall4 in the generation of pluripotent stem cells from blastocysts and fibroblasts. Genes Cells 2009;14:683-694.
29. Kohlhase J, Heinrich M, Schubert L, Liebers M, Kispert A, Laccone F, et al. Okihiro syndrome is caused by SALL4 mutations. Hum Mol Genet 2002;11:2979-2987.
30. Aguila JR, Liao W, Yang J, Avila C, Hagag N, Senzel L, et al. SALL4 is a robust stimulator for the expansion of hematopoietic stem cells. Blood 2011;118:576-585.
31. Ma Y, Cui W, Yang J, Qu J, Di C, Amin HM, et al. SALL4, a novel oncogene, is constitutively expressed in human acute myeloid leukemia (AML) and induces AML in transgenic mice. Blood 2006;108: 2726-2735.
32. Yang J, Chai L, Gao C, Fowles TC, Alipio Z, Dang H, et al. SALL4 is a key regulator of survival and apoptosis in human leukemic cells. Blood 2008;112:805-813.
33. Jeong HW, Cui W, Yang Y, Lu J, He J, Li A, et al. SALL4, a stem cell factor, affects the side population by regulation of the ATP-binding cassette drug transport genes. PLoS One 2011;6:e18372.
34. Cao D, Humphrey PA, Allan RW. SALL4 is a novel sensitive and specific marker for metastatic germ cell tumors, with particular utility in detection of metastatic yolk sac tumors. Cancer 2009;115:2640-2651.
35. Bard JD, Gelebart P, Amin HM, Young LC, Ma Y, Lai R. Signal transducer and activator of transcription3 is a transcriptional factor regulating the gene expression of SALL4. Faseb J 2009;23:1405-1414.
36. Ushiku T, Shinozaki A, Shibahara J, Iwasaki Y, Tateishi Y, Funata N, et al. SALL4 represents fetal gut differentiation of gastric cancer, and is diagnostically useful in distinguishing hepatoid gastric carcinoma from hepatocellular carcinoma. Am J Surg Pathol 2010;34:533-540.
37. Ikeda H, Sato Y, Yoneda N, Harada K, Sasaki M, Kitamura S, et al. alpha-Fetoprotein-producing gastric carcinoma and combined hepatocellular and cholangiocarcinoma show similar morphology but different histogenesis with respect to SALL4 expression. Hum Pathol 2012;43:1955-1963.
38. Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat Med 2006;12:410-416.
39. Oikawa T, Kamiya A, Kakinuma S, Zeniya M, Nishinakamura R, Tajiri H, et al. Sall4 regulates cell fate decision in fetal hepatic stem/progenitor cells. Gastroenterology 2009;136:1000-1011.
40. Wang Y, Carpino G, Cui C, Dominguez-Bendala J, Wauthier E, Cardinale V, et al. Biliary tree stem cells, precursors to pancreatic committed progenitors: evidence for possible life-long pancreatic organogenesis. Stem Cell 2012 (in press).
41. Kamiya A, Kinoshita T, Ito Y, Matsui T, Morikawa Y, Senba E, et al. Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. Embo J 1999;18: 2127-2136.
42. Yamashita T, Honda M, Nio K, Nakamoto Y, Yamashita T, Takamura H, et al. Oncostatin m renders epithelial cell adhesion molecule-positive liver cancer stem cells sensitive to 5-Fluorouracil by inducing hepatocytic differentiation. Cancer Res 2010;70:4687-4697.
43. Shi SR, Imam SA, Young L, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. J Histochem Cytochem 1995;43:193-201.
44. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. Nat Rev Cancer 2011;11: 558-572.
45. Lee J, Kanatsu-Shinohara M, Morimoto H, Kazuki Y, Takashima S, Oshimura M, et al. Genetic reconstruction of mouse spermatogonial stem cell self-renewal in vitro by Ras-cyclin D2 activation. Cell Stem Cell 2009;5:76-86.
46. Bohm J, Kaiser FJ, Borozdin W, Depping R, Kohlhase J. Synergistic cooperation of Sall4 and Cyclin D1 in transcriptional repression. Biochem Biophys Res Commun 2007;356:773-779.
47. Yin C, Lin Y, Zhang X, Chen YX, Zeng X, Yue HY, et al. Differentiation therapy of hepatocellular carcinoma in mice with recombinant adenovirus carrying hepatocyte nuclear factor-4alpha gene. HEPATOLOGY 2008;48:1528-1539.
48. Zhang L, Theise N, Chua M, Reid LM. The stem cell niche of human livers: symmetry between development and regeneration. HEPATOLOGY 2008;48:1598-1607.
49. Yamashita T, Forgues M, Wang W, Kim JW, Ye Q, Jia H, et al. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. Cancer Res 2008;68:1451-1461.
50. Yang XR, Xu Y, Yu B, Zhou J, Qiu SJ, Shi GM, et al. High expression levels of putative hepatic stem/progenitor cell biomarkers related to tumour angiogenesis and poor prognosis of hepatocellular carcinoma. Gut 2010;59:953-962.

Clinical & Experimental Immunology
The Journal of Translational Immunology
immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2012.04664.x

Activated natural killer T cells producing interferon-gamma elicit promoting activity to murine dendritic cell-based autoimmune hepatic inflammation

M. Nakano,*† C. Saeki,*† H. Takahashi,* S. Homma,† H. Tajiri* and M. Zeniya‡

*Division of Gastroenterology and Hepatology, Department of Internal Medicine, †Department of Oncology, Institute of DNA Medicine and ‡Gastroenterology, Jikei University School of Medicine, Tokyo, Japan

Accepted for publication 15 August 2012
Correspondence: S. Homma, Department of Oncology, Institute of DNA Medicine, Jikei University School of Medicine, 3-25-8 Nishi-shimbashi, Minato-ku, Tokyo 105-8461, Japan.
E-mail: sahya@jikei.ac.jp

Summary

As natural killer (NK) T cells play an important role in the development of autoimmune diseases, they should have significant roles for the pathogenesis of autoimmune liver disease. Implication of the NK T cells in the generation of autoimmune-related hepatic inflammation was investigated using a novel mouse model. Immunization of mice with dendritic cells (DCs) loaded with hepatocyte-mimicking hepatocellular carcinoma cells (DC/Hepa1-6) induces cytotoxic T lymphocytes (CTL) capable of killing hepatocytes. Subsequent administration of interleukin (IL)-12, a potent interferon-gamma (IFN-γ) inducer, to the immunized mice generates autoimmune hepatic inflammation (AHI), as reported previously. Upon onset of the AHI response, the number of intrahepatic $CD3^+NK1{\cdot}1^+$ NK T cells increased markedly, along with a decrease in the number of splenic NK T cells, augmented expression of CXCR6 on intrahepatic NK T cells and CXCL16 in hepatic tissue, suggesting that NK T cells were recruited into the inflamed liver. The NK T cells were strongly positive for CD69 and produced IFN-γ, but not IL-4. AHI activity was attenuated markedly in $CD1d^{-/-}$ NK T cell-deficient mice, indicating that NK T cells play a pivotal role in the development of AHI. Mice treated with DC/Hepa1-6 and alpha-galactosylceramide, a potent NK T cell activator, also exhibited similar hepatic inflammation, in which activated NK T cells producing IFN-γ and $CD8^+$ T cells cytotoxic to hepatocytes were induced in liver-infiltrating mononuclear cells. Activated NK T cells producing IFN-γ potentiate DC-based AHI in the mouse model.

Keywords: α-GalCer, autoimmune hepatic inflammation, cytotoxic T lymphocyte, interferon-gamma, natural killer T cell

Introduction

Autoimmune hepatitis (AIH) is an immune-mediated chronic inflammatory liver disease that predominantly affects women genetically predisposed to its development [1]. Although the pathogenic mechanism of AIH and aetiological agents involved are not known, it has been postulated that autoreactive T cells targeting at hepatocytes play a central role in the development of AIH [2]. Until recently, autoreactive $CD4^+$ T cells were considered to be critical for disease development [3], but increasing evidence has shown that $CD8^+$ T cells also play significant roles [4]. $CD8^+$ T cells are observed mainly in areas of interface hepatitis of AIH, and $CD4^+$ T cells are found in the central part of the portal tract [5]. Peripheral $CD8^+CD25^+$ lymphocytes are more prevalent in patients with acute-onset AIH than those with chronic AIH. After treatment with immunosuppressive therapies, the number of $CD8^+CD25^+$ T lymphocytes in the blood decrease in parallel with the serum aminotransferase level [6]. Although hepatic tissue in AIH exhibits abundant apoptosis of hepatocytes [7], and highly activated infiltrating T lymphocytes [8] may play an important role in the induction of hepatocyte apoptosis, the mechanism of establishment and progression of AIH remains unclear.

Natural killer (NK) T cells are innate immune cells that were described originally as expressing both T cell and NK cell phenotypes [9]. NK T cells are activated in a CD1d-dependent manner in response to glycolipid antigens such as α-galactosylceramide (α-GalCer) [10,11], and rapidly produce large amounts of T helper type 1 (Th1) cytokine,

interferon-gamma (IFN-γ) and Th2 cytokine, interleukin (IL)-4 [12]. The implication of NK T cells in autoimmune diseases such as multiple sclerosis [13–15] and diabetes [16–18] has been shown using well-established animal models. In mice, NK T cells represent up to 30% of T cells in the liver, where they reside within the sinusoids and appear to provide intravascular immune surveillance [19,20], and may also be associated with the development of liver injury in the setting of hepatitis [21,22]. In humans, it has not been clarified whether NK T cells are beneficial or harmful in the setting of liver disease [23]. The importance of NK T cells in the pathogenesis of autoimmune liver disease also remains unknown.

We have reported previously a mouse model of autoimmune hepatic inflammation (AHI) generated by immunization of C57BL/6 mice with dendritic cells (DC) loaded with well-differentiated hepatocellular carcinoma cells (Hepa1-6), followed by IL-12 administration [24,25]. In this model, liver specific inflammation is mediated by hepatocyte-responsive autoreactive T cells. Our findings indicate that two independent steps are necessary for the development of autoimmune-mediated liver damage: one step concerns the induction of autoreactive T cells responsive to hepatocytes. Because of similar phenotypic expression between normal hepatocytes and Hepa1-6 cells, cytotoxic T lymphocytes (CTLs) recognizing shared antigen between them are induced by immunization of mice with DCs loaded with Hepa1-6 [24]. The other step is the modulation of the hepatic microenvironment to promote recruitment of autoreactive T cells into the liver and CTL response to hepatocytes, because the sole induction of autoreactive T cells cannot generate autoimmune hepatic injury *in vivo*. The key cytokine for this response is IFN-γ induced by IL-12, which provides the enhanced expression of major histocompatibility complex (MHC) class I, adhesion molecules and chemokines on hepatocytes [24]. In fact, treatment of the immunized mice with anti-IFN-γ monoclonal antibody or immunization of IFN-γ knock-out mice abolishes AHI activity [24]. Although, unlike human AIH, histological features of AHI are characterized by an acute inflammatory response located mainly in hepatic parenchyma, this model could contribute to analysis of the mechanism of the liver-specific autoimmune response [25].

In this study we show, using our mouse model, that intrahepatic NK T cells play a pivotal role in promoting the CTL-mediated autoimmune hepatic inflammation.

Materials and methods

Animals

Eight-week-old female C57BL/6 wild-type (WT) mice were purchased from Sankyo Labo Service Co., Ltd (Tokyo, Japan). CD1d$^{-/-}$ mice (C57BL/6 genetic background) were kindly provided by Dr Shinsuke Taki (University of Shinshu, Japan). All animals were maintained in our facilities and received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences (NIH publication 86-23, revised 1985). Mice were used at the age of 8–10 weeks and were matched for sex and age.

Cell lines, cytokines and antibodies

Hepa1-6, a well-differentiated murine hepatocellular carcinoma cell line, was obtained from the American Type Culture Collection (Manassas, VA, USA). Recombinant murine granulocyte–macrophage colony-stimulating factor (GM-CSF) and recombinant IL-2, IL-4 and IL-12 were purchased from PeproTech (Rocky Hill, NJ, USA). Anti-CD8a monoclonal antibody (mAb) (clone 53-6·7) conjugated with fluorescein isothiocyanate (FITC) was purchased from BD Biosciences (San Diego, CA, USA). Anti-CD69 mAb (clone H1·2F3) conjugated with allophycocyanin (APC) and anti-CD62L mAb (clone MEL-14) conjugated with phycoerythrin (PE) were purchased from BioLegend (San Diego, CA, USA). Anti-CXCR6 mAb (clone 221002) conjugated with PE was purchased from R&D Systems, Inc. (Minneaoplis, MN, USA). Anti-IFN-γ (clone XMG1·2), anti-IL-4 (clone 11B11) conjugated with APC for intracellular cytokine staining were purchased from eBioscience (San Diego, CA, USA).

Treatment of mice

AHI was induced in mice as described previously [24,25]. Briefly, bone marrow-derived DCs loaded with Hepa1-6 cells (DC/Hepa1-6) were generated by quick treatment of a mixture of the DCs and Hepa1-6 cells with 50% polyethylene glycol (Peg solution; Sigma-Aldrich, Inc., St Louis, MO, USA). DC/Hepa1-6 cells were injected subcutaneously into 8-week-old female C57BL/6 WT mice or CD1d$^{-/-}$ mice on days 1 and 14. Then, IL-12 (500 ng/mouse) was injected intraperitoneally on days 15, 17 and 19. The mice were sacrificed on day 21. To analyse the role of activated NK T cell in this mouse model, α-GalCer (KRN7000; Funakoshi Co., Ltd, Tokyo, Japan) was dissolved in 0·1 ml phosphate-buffered saline (PBS) and injected intraperitoneally (0·5 μg/mouse) instead of IL-12 to DC/Hepa1-6 pretreated and untreated WT mice on day 15. The mice were sacrificed 48 h after α-GalCer administration.

Assay for serum transaminase levels

Serum alanine aminotransferase (ALT) levels were measured using the DriChem system (L3500V; Fuji Film Medical Co., Ltd, Tokyo, Japan), according to the manufacturer's instructions.

Histology

Liver tissue was fixed in 10% formalin for at least 24 h and paraffin-embedded. Sections of 2 μm thickness were stained with haematoxylin and eosin (H&E) to determine morphological changes. The numbers of inflammatory foci were determined as described previously [24].

Preparation of liver mononuclear cells

Hepatic mononuclear cells (MNCs) were isolated from murine liver, as described previously [24].

Quantitative reverse transcription–polymerase chain reaction (qRT–PCR)

Liver RNA extraction and messenger RNA (mRNA) quantification by real-time qRT–PCR were performed as described previously [25]. The expression levels of CXCL16 were normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Flow cytometry and intracellular cytokine staining

For cell-surface staining, after blocking with anti-FcR (clone 93; eBioscience), cells were incubated with various mAbs in darkness at 4°C for 30 min and examined by flow cytometry [fluorescence activated celll sorter (FACS)Calibur, BD Biosciences Immunocytometry Systems, San Jose, CA, USA]. For intracellular cytokine staining, isolated intrahepatic MNCs were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml; Sigma-Aldrich, Inc.) and ionomycin (1μg/ml; Sigma-Aldrich, Inc.) in the presence of Brefeldin A (10 μg/ml; Sigma-Aldrich, Inc.) for 5 h. After labelling the cell-surface antigens, cells were fixed and permeabilized using a Cytofix/Cytoperm plus kit (BD Biosciences) and then stained with anti-IFN-γ or IL-4 conjugated with APC. The stained cells were analysed by flow cytometry. The data were analysed using CellQuest Pro version 5·2 software (BD Biosciences Immunocytometry Systems, San Jose, CA, USA).

Isolation of hepatocytes and cytotoxicity assay

Hepatocytes were isolated as described previously [24]. A cytotoxicity assay against primary murine cultured hepatocytes was performed as described previously [24]. Briefly, isolated hepatocytes were seeded at 1×10^4 cells/well into 96-well collagen-coated plates (Iwaki, Asahi Techno Glass, Chiba, Japan). After overnight incubation, 4×10^5 effector, intrahepatic whole MNCs or $CD8^+$ T cells or non-$CD8^+$ T cells, which were isolated using a magnetic cell sorting system ($CD8^+$ T Cell Isolation kit II; Miltenyi Biotec, Bergisch Gladbach, Germany), were co-cultured for 24 h. Aspartate aminotransferase (AST) activity of the culture supernatant was determined and percentage of cytotoxic activity was calculated as [(experimental AST release – spontaneous AST release)/(total AST release – spontaneous AST release) × 100], according to the formula described previously [24].

Statistical analysis

The significance of difference among the groups was analysed with Tukey's test for multiple group comparisons. Unpaired Student's *t*-test was used for comparison of means in two groups. Differences were considered to be significant at a *P*-value less than 0·05.

Results

Accumulation of activated NK T cells producing IFN-γ in the AHI liver

As shown in Fig. 1a,i, the number of NK T cells increased significantly in the AHI liver generated by treatment of mice with DC/Hepa1-6 and IL-12. The number of NK T cells was higher in combined treatment with DC/Hepa1-6 and IL-12 than treatment with DC/Hepa1-6 or IL-12 alone (Supplementary Fig. S1A-a). The absolute number of intrahepatic $CD69^+$ activated NK T cells was high in AHI (Fig. 1a,ii). Conversely, the number of NK T cells in the spleen was lower than in the untreated control at maximum hepatic inflammation (Fig. 1a,iii). Expression levels of CXCL16, a ligand of CXCR6, in hepatic tissue and the population of $CXCR6^+$ intrahepatic NK T cells were elevated in AHI liver (Fig. 1b,i–iii). Although the population of IFN-γ-producing intrahepatic NK T cells increased at maximum hepatic inflammation (Fig. 1c,i,iii), the population of IL-4-producing intrahepatic NK T cells was not affected (Fig. 1c,ii,iii). These results suggest that activated NK T cells which show a Th1 phenotype might have been accumulated into the liver of AHI. Because of a vigorous increase in the number of intrahepatic T cells by generation of AHI, frequency of NK T cells in the intrahepatic MHCs was decreased in spite of an increase in absolute number of NK T cells (Supplementary Fig. S2).

The activity of AHI was suppressed in the livers of $CD1d^{-/-}$ mice

When AHI was generated in NK T cell-deficient $CD1d^{-/-}$ mice, the inflammatory activity was markedly attenuated (Fig. 2a). The level of serum ALT, number of clusters of MHCs (inflammatory foci, IF) in hepatic tissue and absolute number of MNCs in the liver (Fig. 2b–d) were significantly lower in the AHI livers of $CD1d^{-/-}$ mice compared with those in AHI of WT mice. Although $CD8^+$T cells were found in $CD1d^{-/-}$ mice, they were significantly fewer than in

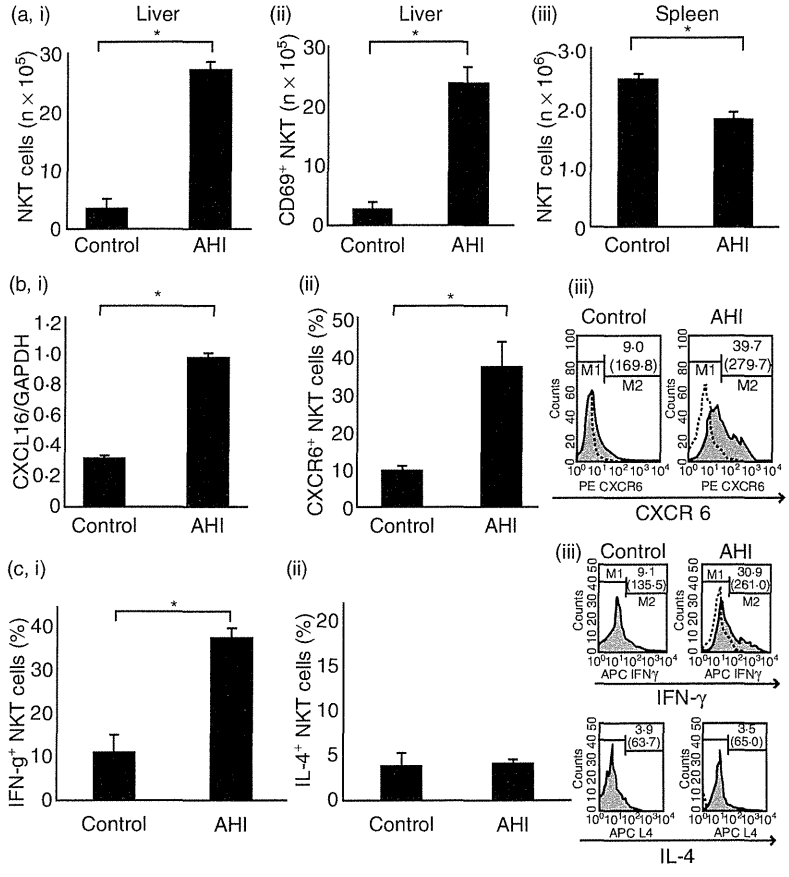

Fig. 1. The dynamic statistics of natural killer (NK) T cells in the autoimmune hepatic inflammation (AHI) liver and spleen. (a) Absolute number of total NK T cells (i) and CD69$^+$NK T cells (ii) in the liver and NK T cells in the spleen (iii) in AHI. The number was determined as [total number of mononuclear cells (MNCs) in the liver or spleen] × [the frequency of CD3$^+$NK1·1$^+$ cells or CD3$^+$NK1·1$^+$ CD69$^+$cells] in each group [$n = 5$, mean ± standard deviation (s.d.), $^*P < 0{\cdot}001$]. (b) (i) Expression of CXCL16 in hepatic tissue. Levels of CXCL16 mRNA in each group were determined by quantitative reverse transcription–polymerase chain reaction (qRT–PCR). Bars indicate mean ± s.d., $^*P < 0{\cdot}001$. (ii) Population of intrahepatic CXCR6$^+$NK T cells ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (iii) Representative flow cytometry of intrahepatic CXCR6$^+$NK T cells in control and AHI. Grey-filled histograms; CXCR6$^+$cells, dotted histogram; isotype control. The value shows the frequency of CXCR6$^+$ cells and the numbers in parentheses shows mean fluorescence intensity (MFI) of CXCR6. (c) (i) Frequency of interferon (IFN)-γ$^+$ NK T cells in each group ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (ii) Frequency of interleukin (IL)-4$^+$ NK T cells in control and AHI. (iii) Representative flow cytometry of IFN-γ$^+$ or IL-4$^+$ intrahepatic NK T cells in each group. Grey-filled histograms; IFN-γ$^+$ or IL-4$^+$ cells, dotted histogram; isotype control. The value shows the frequency of IFN-γ- or IL-4-producing cells and the numbers in parentheses shows MFI of IFN-γ or IL-4. All experiments were repeated at least three times.

AHI liver of WT mice (Fig. 2e). These results demonstrate that NK T cells play a pivotal role in the establishment of AHI.

α-GalCer treatment of DC/Hepa1-6 immunized mice produced severe hepatic inflammation

Hepatic inflammation was induced when mice immunized with DC/Hepa1-6 were treated with α-GalCer instead of IL-12 (Fig. 3a,i). This was characterized by the emergence of abundant IF in hepatic lobules, similar to the AHI generated by treatment with DC/Hepa1-6 and IL-12. Importantly, the hepatic inflammatory activity generated by treatment with DC/Hepa1-6 and α-GalCer was greater than that induced by treatment with α-GalCer alone (Fig. 3a,i,ii,b,c). The population of IFN-γ-producing NK T cells were enhanced but the population of IL-4-producing NK T cells was not affected in hepatic inflammation induced by treatment with α-GalCer alone or DC/Hepa1-6 and α-GalCer (Fig. 3d,i,ii).

Autoreactive CD8$^+$ CTLs cytocidal to hepatocytes were induced by treatment of mice with DC/Hepa1-6 and α-GalCer

When DC/Hepa1-6 immunized mice were treated with α-GalCer and hepatic inflammation was generated, the population of CD8$^+$ T cells in intrahepatic MNCs was increased (Fig. 4a,i). Among these CD8$^+$ T cells, the percentage of CD62L$^-$ active cells was increased (Fig. 4a,ii,iii).

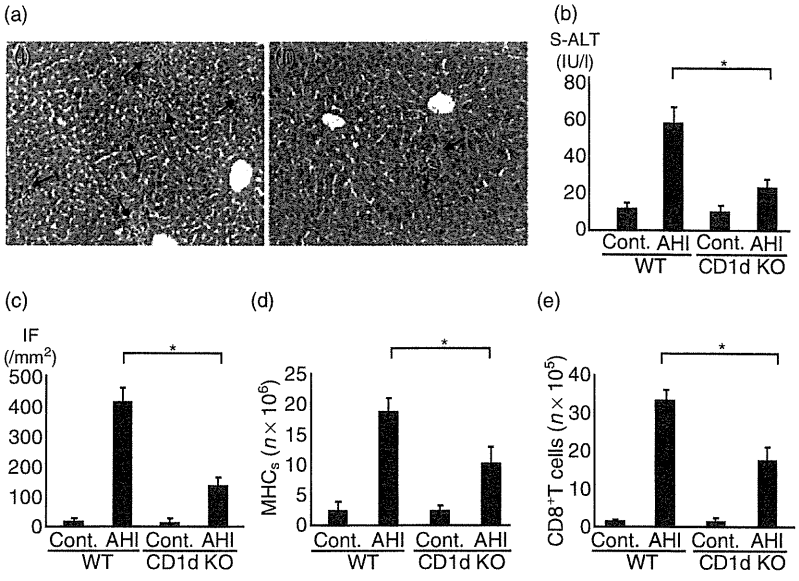

Fig. 2. Autoimmune hepatic inflammation (AHI) activity was attenuated in natural killer (NK) T cell-deficient CD1d$^{-/-}$ mice. AHI was generated in wild-type (WT) or CD1d$^{-/-}$ mice. (a) Histological change of AHI liver (haematoxylin and eosin staining, × 200). Arrows indicate inflammatory foci (IF). (i) Representative hepatic tissue of AHI in WT mice. (ii) Representative hepatic tissue of AHI in CD1d$^{-/-}$ mice. (b) Serum alanine aminotransferase (ALT) levels in WT and CD1d$^{-/-}$ mice ($n = 5$, mean ± standard deviation (s.d.), $^*P < 0{\cdot}001$). (c) Number of IF of AHI in WT and CD1d$^{-/-}$ mice ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (d) Absolute number of intrahepatic mononuclear cells (MNCs) in WT and CD1d$^{-/-}$ mice ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$) (E) Absolute number of intrahepatic CD8$^+$T cells in WT and CD1d$^{-/-}$ mice ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). All experiments were repeated at least three times.

Notably, CD8$^+$ T cells from the livers of mice treated with DC/Hepa1-6 and α-GalCer showed high cytotoxic activity against hepatocytes, while the cytotoxic activity of intrahepatic CD8$^+$ T cells of mice treated with α-GalCer alone was lower (Fig. 4b). These results indicate that treatment of mice with DC/Hepa1-6 and α-GalCer could induce CTLs cytocidal to autologous hepatocytes, as for the treatment of mice with DC/Hepa1-6 and IL-12.

Discussion

AHI is generated in mice by induction of hepatocyte-responsive CTLs by immunization with DC/Hepa1-6 and accumulation of these autoreactive CTLs to the liver by enhanced expression of adhesion molecules, chemokine ligands and MHC in hepatic tissue by IFN-γ induced by IL-12 [24]. As IFN-γ makes hepatocyte vulnerable to CD8$^+$CTL attack by up-regulation of MHC class I expression [3], IFN-γ is a key cytokine to generate AHI in this mouse model [24]. It is probable that activated NK T cells, accumulated into the liver upon onset of AHI, may contribute to the generation of the AHI as producers of IFN-γ. Because IL-12 is a potent IFN-γ inducer [26], it may stimulate various immune cell types to produce IFN-γ. Thus, the involvement of NK T cells as IFN-γ producers in the generation of AHI has not been clear. When CD1d$^{-/-}$ mice, which show NK T cell deficiency, were immunized with DC/Hepa1-6 and treated with IL-12, the resultant AHI was suppressed significantly, indicating that IFN-γ produced by NK T cells has an important role in development of AHI. Although the number of CD8$^+$ T cells in the AHI liver was decreased significantly in CD1d$^{-/-}$ mice, CD8$^+$ T cells existed in infiltrating cells of the AHI liver of CD1d$^{-/-}$ mice. This result suggests that IFN-γ produced by IL-12-activated immune cells other than NK T cells might have stimulated the CD8$^+$T cell response.

The involvement of NK T cells in the generation of AHI was studied further by treatment of DC/Hepa1-6-immunized mice with α-GalCer. Because treatment with α-GalCer itself causes hepatic injury [27], the minimum dose of α-GalCer that induced hepatic injury (0·5 μg/mouse) was used to analyse the combined effect with DC/Hepa1-6 immunization and α-GalCer on generation of hepatic inflammation. The activity of hepatic inflammation was significantly higher in mice treated with DC/Hepa1-6 and α-GalCer than α-GalCer alone. CD8$^+$ T cells from mice treated with DC/Hepa1-6 plus α-GalCer showed higher percentage of CD62$^-$ cells and significant cytotoxicity against primary cultured hepatocytes, but CD8$^+$ T cells from mice treated with α-GalCer alone showed low cytotoxicity. These results suggest strongly that antigen-specific activated T cells capable of killing hepatocytes, which were induced by immunization with DC/Hepa1-6, had accumulated in the liver following modulation of the hepatic microenvironment by IFN-γ secreted from α-GalCer-activated NK T cells. Although infiltration of CD8$^+$ T cells was seen in the liver of mice treated with DC/Hepa1-6 alone, inflammatory activity of the liver in such mice was somewhat low, as reported previously [24]. Accordingly, because of few numbers of intrahepatic CD8$^+$ T cells of mice treated with DC/Hepa1-6 alone, it was extremely hard to collect them and examine their cytotoxic activity. Cytotoxic activity of CD8$^+$ T cells in the AHI liver of CD1d$^{-/-}$ mice is important for interpretation of the role of NK T cells. According to our previous data, splenic CD8$^+$ T cells from sole DC/Hepa1-6 immunized mice without IL-12 treatment could elicit significant cytotoxic activity to autologous hepatocytes *in vitro*. However, without the effect

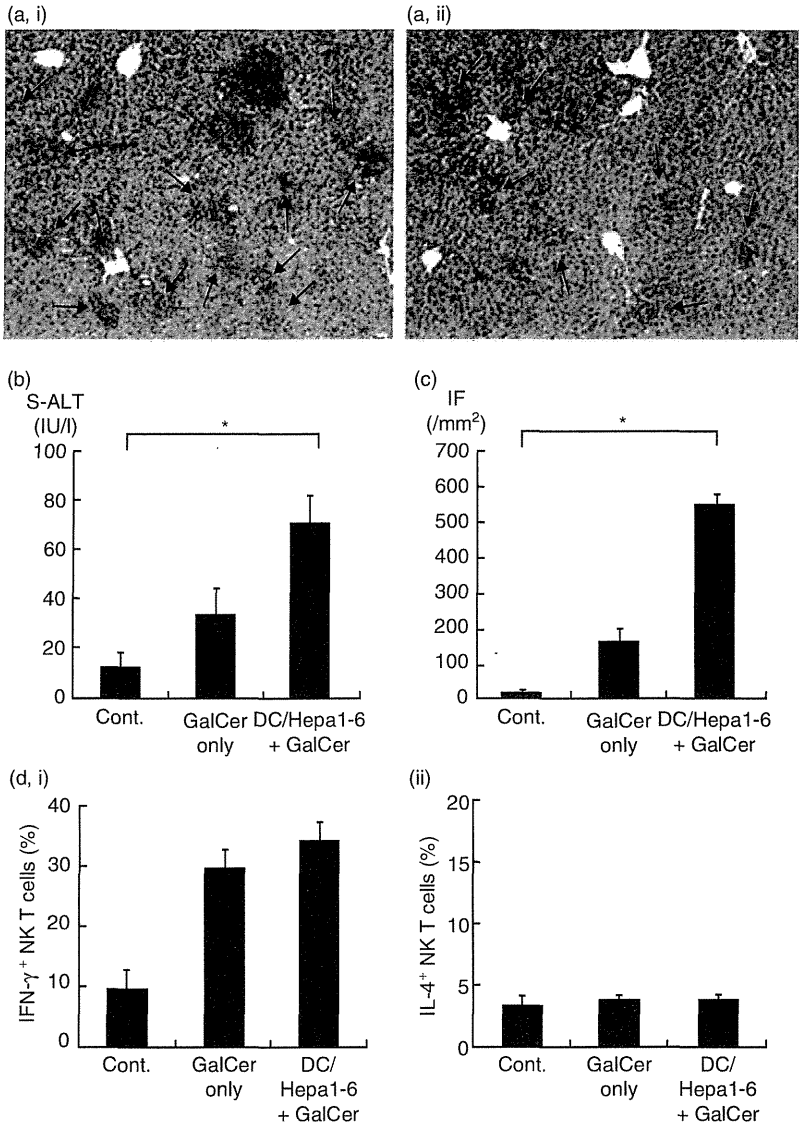

Fig. 3. Treatment of dendritic cell (DC)/Hepa1-6 pre-immunized mice with α-galactosylceramide (α-GalCer) generated marked hepatic inflammation with induction of interferon (IFN)-γ-producing natural killer (NK) T cells. (a) Histological changes in the liver (haematoxylin and eosin staining, × 100). Arrows indicate inflammatory foci (IF). (i) Hepatic tissue of DC/Hepa1-6 pre-immunized mice treated with α-GalCer. (ii) Hepatic tissue of mice treated with α-GalCer alone. (b) Serum alanine aminotransferase (ALT) levels of untreated control mice, mice treated with α-GalCer alone and DC/Hepa1-6 pre-immunized mice treated with α-GalCer [$n = 5$, mean ± standard deviation (s.d.), $^*P < 0{\cdot}001$]. (c) Numbers of IF in each group ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (d) (i) Frequency of IFN-γ^+ intrahepatic NK T cells in each group ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (ii) Frequency of IL-4^+ intrahepatic NK T cells in each group. All experiments were repeated at least three times.

of IFN-γ, which was provided by systemic IL-12 treatment or activation of intrahepatic NK T cells, to increase the expression of MHC class I or several adhesion molecules in hepatic tissue, such $CD8^+$ cytotoxic T cells could not recruit into the liver. Thus, it might be probable that $CD8^+$ T cells in the AHI liver of $CD1d^{-/-}$ mice could show cytotoxic activity to hepatocytes, but because of lack of IFN-γ effect provided by activated NK T cells and less infiltration of $CD8^+$CTLs into the liver, hepatic inflammatory activity was reduced in $CD1d^{-/-}$ mice.

Non-$CD8^+$ T cells among the hepatic MHCs showed considerable cytotoxic activity to hepatocytes, although the activity was lower than that of $CD8^+$ T cells. Under Th1 conditions rich in IFN-γ, activated intrahepatic bystander cells such as macrophages and NK or NK T cells might elicit non-specific cytotoxic activity to hepatocytes [28]. As intrahepatic NK T cells express FasL [29], it cannot be excluded that activated NK T cells might elicit direct hepatocyte injury, possibly through Fas-FasL interaction, and this point should be studied further.

Concanavalin A (ConA) hepatitis is used widely as a mouse model of immune-related hepatitis, although the specific autoimmune response to hepatocytes has not been defined. Several studies have shown convincingly the involvement of NK T cells in the pathology of ConA hepatitis. Depletion of hepatic NK T cells in ConA-administered mice, or treatment of NK T cell-deficient mice with ConA, reduced the activity of ConA hepatitis significantly [30]. Moreover, in the interaction between $CD8^+$ T cells responsive to ovalbumin in a MHC class I-restricted manner (OTI T cells) and hepatocytes expressing transferrin-membrane-bound ovalbumin (Tf-mO transferrin-mOVA) in Tf-mOVA mice, specific effector function to antigen was stimulated by co-activation of Vα14 NK T cells using α-GalCer [31]. Also,

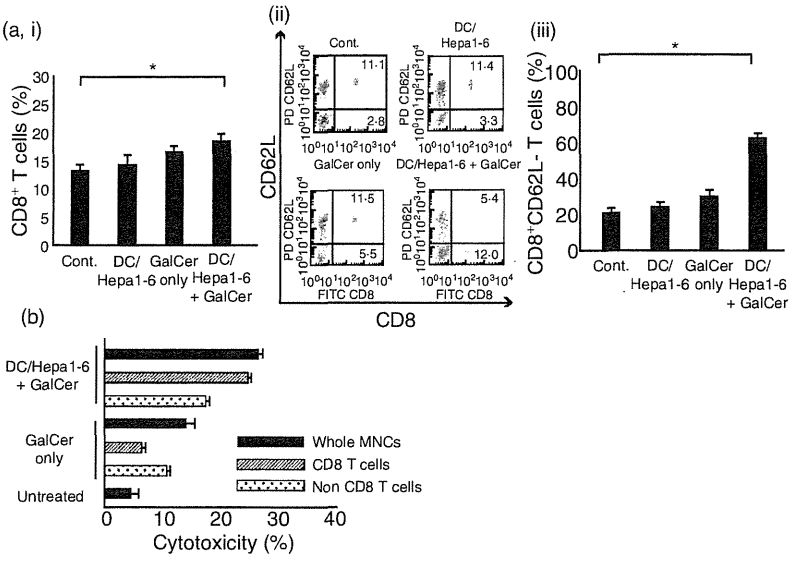

Fig. 4. Treatment of dendritic cell (DC)/Hepa1-6 pre-immunized mice with α-galactosylceramide (α-GalCer) generated active $CD8^+$ T cells which were cytocidal to hepatocytes. (a) (i) Population of intrahepatic $CD8^+$ T cells in each group. The mice were sacrificed 48 h after α-GalCer administration (17 days after the first DC/Hepa1-6 treatment). Intrahepatic mononuclear cells (MNCs) were isolated and the percentage of $CD8^+$ T cells was determined by flow cytometry [$n = 5$, mean ± standard deviation (s.d.), $^*P < 0{\cdot}001$]. (ii) Representative flow cytometry of each group. Intrahepatic MNCs were isolated and analysed by flow cytometry using anti-CD8 and anti-CD62L monoclonal antibodies (mAbs). Upper left; control, upper right; DC/Hepa1-6, lower left; α-GalCer only, lower right; DC/Hepa1-6 + α-GalCer. Values in the upper and lower right indicate the frequency of cells in each area. (iii) Frequency of $CD8^+$ $CD62L^-$ activated T cells in total intrahepatic $CD8^+$ T cells of each group ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (b) Cytotoxic activity of intrahepatic whole MNCs, $CD8^+$ T cells and non-$CD8^+$ T cells to autologous hepatocytes. Cytotoxic activity to primary cultured autologous hepatocytes was examined using aspartate aminotransferase (AST) release assay. Effector : target ratio was 40:1. All experiments were repeated at least three times.

in an animal model of primary biliary cirrhosis, in which infection of mice with *Novosphingobium aromaticivorans* induces antibody against mitochondrial component and T cell-mediated autoimmunity, disease induction requires NK T cells [32]. These results suggest that NK T cells play pivotal roles for development of immune-related liver disease.

Our AHI model seems to be an artificial model generated by extreme treatment of mice. However, we demonstrated that in order to generate liver-specific autoimmune response, two independent factors, induction of hepatocyte-responsive $CD8^+$ T cells by immunization with DC/Hepa1-6 and recruitment of such $CD8^+$ T cells into the liver by modulation of hepatic environment with IFN-γ, were required. In humans, the similar mechanism for development of autoimmune hepatitis might be considered. Prior to onset of human autoimmune hepatitis, infection of hepatitis virus or drug-induced liver injury might contribute to induction of hepatocyte-responsive T lymphocytes just as does immunization with DC/Hepa1-6. Some promoting factors such as massive cytokine production from NK T cells for activation of these autoreactive T cells might be associated with onset of autoimmune hepatitis.

Although this study showed an important role for activated NK T cells in the generation of AHI, the implication of NK T cell activation in patients with autoimmune hepatitis remains obscure. Several potential physiological ligands for NK T cells have been reported [33,34], but the specific endogenous ligand for NK T cell activation remains unknown [35]. Recently, several reports suggested the involvement of Toll-like receptors (TLRs) in the pathogenesis of autoimmune diabetes, inflammatory bowel diseases, multiple sclerosis and systemic lupus erythematosus [36,37]. It may be possible that the inflammatory activity in human AIH is affected by activation of IFN-γ-producing NK T cells in the liver through TLR stimulation by intestinal microbial components. If so, the regulation of intrahepatic NK T cell activity might lead to the establishment of a new modality for controlling disease activity in human AIH.

Acknowledgements

This work was supported by Health Labor Science Research on Measures for intractable disease (Intractable Hepato-Biliary Disease Study Group) founded by the Ministry of Health, Labor and welfare of Japan.

Disclosure

The authors declare that there are no conflicts of interest.