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Sal-Like Protein 4 (SALL4), a Stem Cell Biomarker in Liver Cancers

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Liver cancers, including hepatocellular carcinomas (HCCs), cholangiocarcinomas (CCs), and fibrolamellar HCCs (FL-HCCs) are among the most common cancers worldwide and are associated with a poor prognosis. Investigations of genes important in liver cancers have focused on Sal-like protein 4 (SALL4), a member of a family of zinc finger transcription factors. It is a regulator of embryogenesis, organogenesis, pluripotency, can elicit reprogramming of somatic cells, and is a marker of stem cells. We found it expressed in normal murine hepatoblasts, normal human hepatic stem cells, hepatoblasts and biliary tree stem cells, but not in mature parenchymal cells of liver or biliary tree. It was strongly expressed in surgical specimens of human HCCs, CCs, a combined hepatocellular and cholangiocarcinoma, a FL-HCC, and in derivative, transplantable tumor lines in immune-compromised hosts. Bioinformatics analyses indicated that elevated expression of SALL4 in tumors is associated with poor survival of HCC patients. Experimental manipulation of SALL4's expression results in changes in proliferation versus differentiation in human HCC cell lines *in vitro* and *in vivo* in immune-compromised hosts. Virus-mediated gene transfer of SALL4 was used for gain- and loss-of-function analyses in the cell lines. Significant growth inhibition *in vitro* and *in vivo*, accompanied by an increase in differentiation occurred with down-regulation of SALL4. Overexpression of SALL4 resulted in increased cell proliferation *in vitro*, correlating with an increase in expression of cytokeratin19 (CK19), epithelial cell adhesion molecules (EpCAM), and adenosine triphosphate (ATP)-binding cassette-G2 (ABCG2). *Conclusion:* SALL4's expression is an indicator of stem cells, a prognostic marker in liver cancers, correlates with cell and tumor growth, with resistance to 5-FU, and its suppression results in differentiation and slowed tumor growth. SALL4 is a novel therapeutic target for liver cancers. (HEPATOLOGY 2013;57:1469-1483)

Liver cancers, comprised primarily of hepatocellular carcinomas (HCCs), cholangiocarcinomas (CCs), and fibrolamellar HCCs (FL-HCCs), are the fifth most common cancer and the third leading cause of cancer mortality in the world.[1] Cancers have a subpopulation of cancer stem cells (CSCs) or tumor-initiating cells (TICs), which have properties shared with normal stem cells.[2,3] CSCs and TICs have highly

Abbreviations: ABCG2, ATP-binding Cassette-G2; AFP, alpha-fetoprotein; ALB, albumin; BD, bile duct; CASP3, caspase-3; CC, cholangiocarcinoma; CK19, cytokeratin19; CSCs, cancer stem cells; DAPI, 4',6-diamidino-2-phenylindole; DP, ductal plate; EMT, epithelial-mesenchymal transition; EpCAM, epithelial cell adhesion molecules; FACS, fluorescent-activated cell sorter; FL-HCC, fibrolamellar hepatocellular carcinoma; 5-FU, 5-fluorouracil; hBTSCs, human biliary tree stem cells; HCC, hepatocellular carcinoma; HC-CC, combined hepatocellular and cholangiocarcinoma; hHBs, human hepatoblasts; hHpSCs, human hepatic stem cells; HNF4α, hepatocyte nuclear factor 4-alpha; PBGs, peribiliary glands; PT, portal tract; qRT-PCR, quantitative real-time polymerase chain reaction; SALL4, Sal-like protein 4; shRNA, short hairpin RNA; TICs, tumor-initiating cells; TTR, transthyretin; UGT2B7, UDP-glucuronosyltransferase-2B7.

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aggressive phenotypes in oncogenesis and are resistant to chemotherapies and radiation therapies. Expression of membrane pumps, adenosine triphosphate (ATP)-binding cassette-G2 (ABCG2), account for the resistance to chemotherapies and are responsible for elimination of DNA-binding dyes causing the cells to be displayed as a side fraction, a "side population (SP)."[4,5] Epithelial cell adhesion molecule (EpCAM), a key factor in the Wnt signaling pathway, was reported as a specific cell surface markers of human hepatic stem cells (hHpSCs), of some, but not all, subpopulations of human biliary tree stem cells (hBTSCs)[6-8] and liver TICs.[9] CD133 (prominin), CD90 (Thy-1), CD44 (hyaluronan receptor), and CD13 (alanine aminopeptidase) have also been found in liver TICs.[10-12] In parallel, CD133 and CD90 have been found on angioblasts or other mesenchymal cells tightly associated with hHpSCs,[13] and so some data discussing CD90 or CD133 may actually be interpreted as relevant to the mesenchymal cell components of the tumors. Several lines of evidence implicate genetic alternations during hepatocarcinogenesis, particularly the Wnt signaling pathway, p53 and alterations in matrix-degrading enzyme secretion.[14-20]

Sal-like Protein 4 (SALL4), a homolog of the *Drosophila* homeotic gene spalt, is a zinc finger transcription factor required for proliferation and maintenance of pluripotency through interactions with OCT3/4, SOX2, and NANOG. It is found at high levels in embryonic stem cells (ESCs),[21-26] and is one of the genes capable of eliciting reprogramming of somatic cells to become induced pluripotent stem cells (iPSCs).[27,28] Mutations in SALL4 cause Okihiro syndrome, known as an autosomal dominant disorder and characterized by multiple organ defects.[29] Recent studies have demonstrated that SALL4 is constitutively expressed in hematopoietic stem cells and a potent regulator of their expansion.[30,31] SALL4 transgenic mice exhibit symptoms like myelodysplastic syndrome (MDS) and subsequently develop acute myeloid leukemia (AML). Primary AML and MDS patients have higher SALL4 expression levels than that in controls, indicating that SALL4 plays a major role in leukemogenesis. Furthermore, SALL4 contributed to the maintenance of SP cells and chemosensitivity in leukemia by regulating the ABC drug transporter genes.[31-33] Solid tumors, such as germ cell tumors, breast, and alpha-fetoprotein (AFP)-producing gastric cancers also express SALL4.[34-37] Taken together, these data suggest that SALL4 is a novel stem cell marker, a gene involved in embryogenesis and organogenesis and a putative stem cell gene associated with CSCs. We now report that SALL4 expression occurs in diverse liver cancers including HCCs, CCs, and FL-HCCs, and that SALL4 increases growth and blocks differentiation in liver cancer cell lines.

Materials and Methods

Cell Proliferation and Chemoresistance Assays. Liver cancer cell lines were infected with a retroviruses or lentivirus at a multiplicity of infection of 40 in the presence of 10 μg/mL protamine sulfate. After infection, cells were cultured for 3 days. Cells then were collected and isolated using a MoFlo fluorescence-activated cell sorter (FACS) (DAKO, Glostrup, Denmark). Then 2×10^3 cells were seeded into 96-well plates and cultured in the presence or absence of 2 μg/mL 5-fluorouracil (5-FU) for 3 to 7 days. Cell proliferation was evaluated in triplicate using the Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan). After incubation at 37°C for 2 hours, the absorbance at 450 nm was measured.

Immunohistochemistry. The tissues were embedded in paraffin and cut into 5-μm sections. After deparaffinization, antigen retrieval was performed with sodium citrate buffer for EpCAM, CK19, or ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) for SALL4 in a steamer for 20 minutes. Endogenous peroxidases were blocked by incubation for 30 minutes in 0.3% H_2O_2. After blocking, primary antibodies (Supporting Table 3) were applied at 4°C overnight. The M.O.M immunodetection kit (Vector Laboratories, Burlingame, CA) was used for detecting primary mouse anti-human SALL4 antibody on mouse xenotransplant FL-HCC tumor to avoid the inability of the antimouse secondary antibody to endogenous mouse

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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.

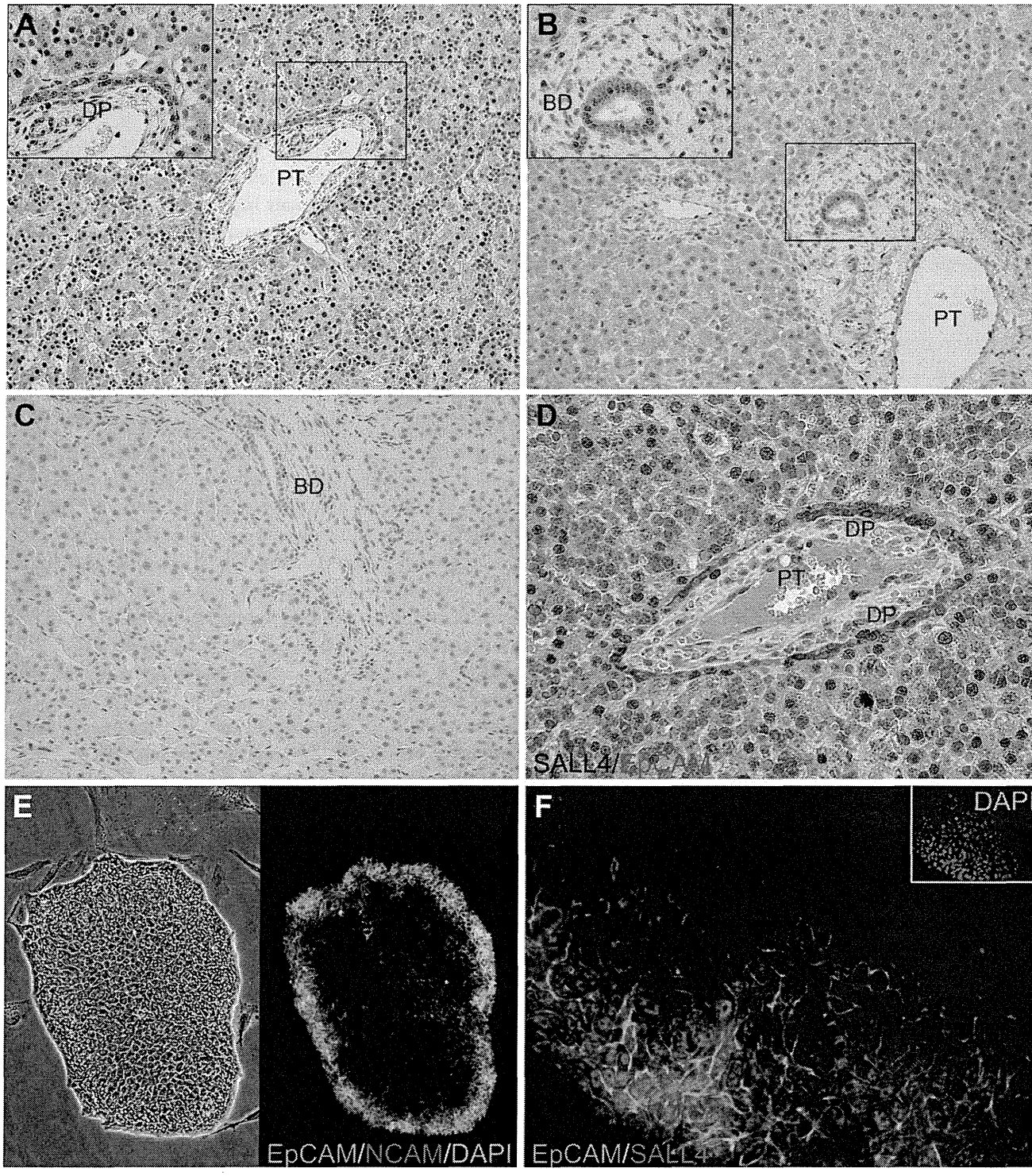

Fig. 1. Representative immunostaining of SALL4 and EpCAM expression in human normal livers and in a colony of hHpSCs. (A-D) Immunostaining of SALL4 expression during liver development. Fetal weeks gestation (A; 19 weeks, D; 16 weeks), neonatal (B; 4 months), and adult liver (C; 68 years) tissues. Sections 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).

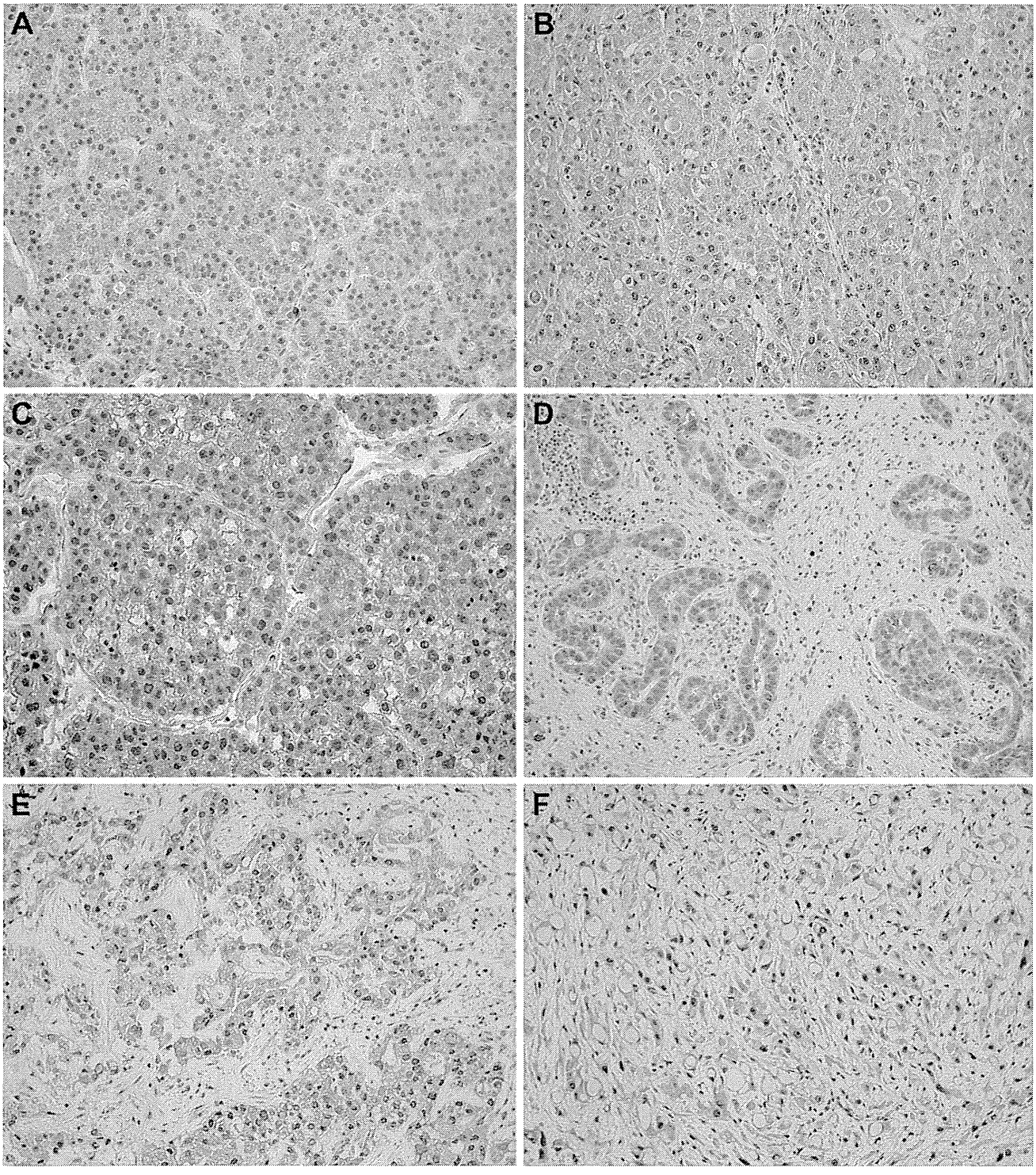

Fig. 2. Representative immunostaining of SALL4 in surgical specimens of liver cancers and transplantable tumor lines of FL-HCC. HCC (A; T41, well differentiated, B; T37, moderately differentiated, C; T49, poorly differentiated). CC (D; T5, poorly differentiated). HC-CC (E; T45, moderately differentiated).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

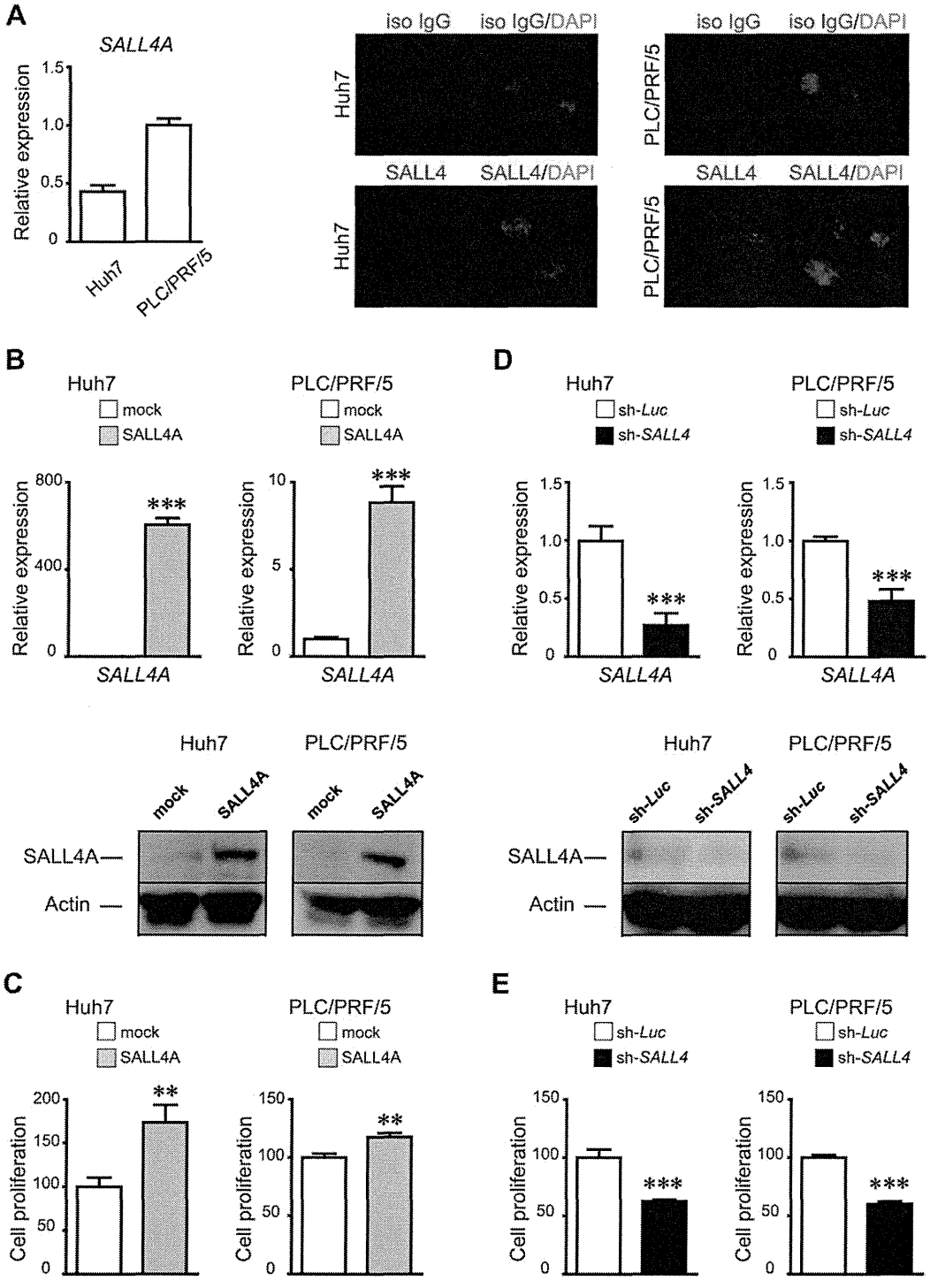

Fig. 3. SALL4 expression and the effects of SALL4 overexpression or knockdown on cell proliferation of liver cancer cells. (A) SALL4A mRNA and protein expression in liver cancer cells. (B,D) Expression of SALL4 mRNA and proteins in cultures derived from SALL4-overexpressing or SALL4-knockdown liver cancer 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).

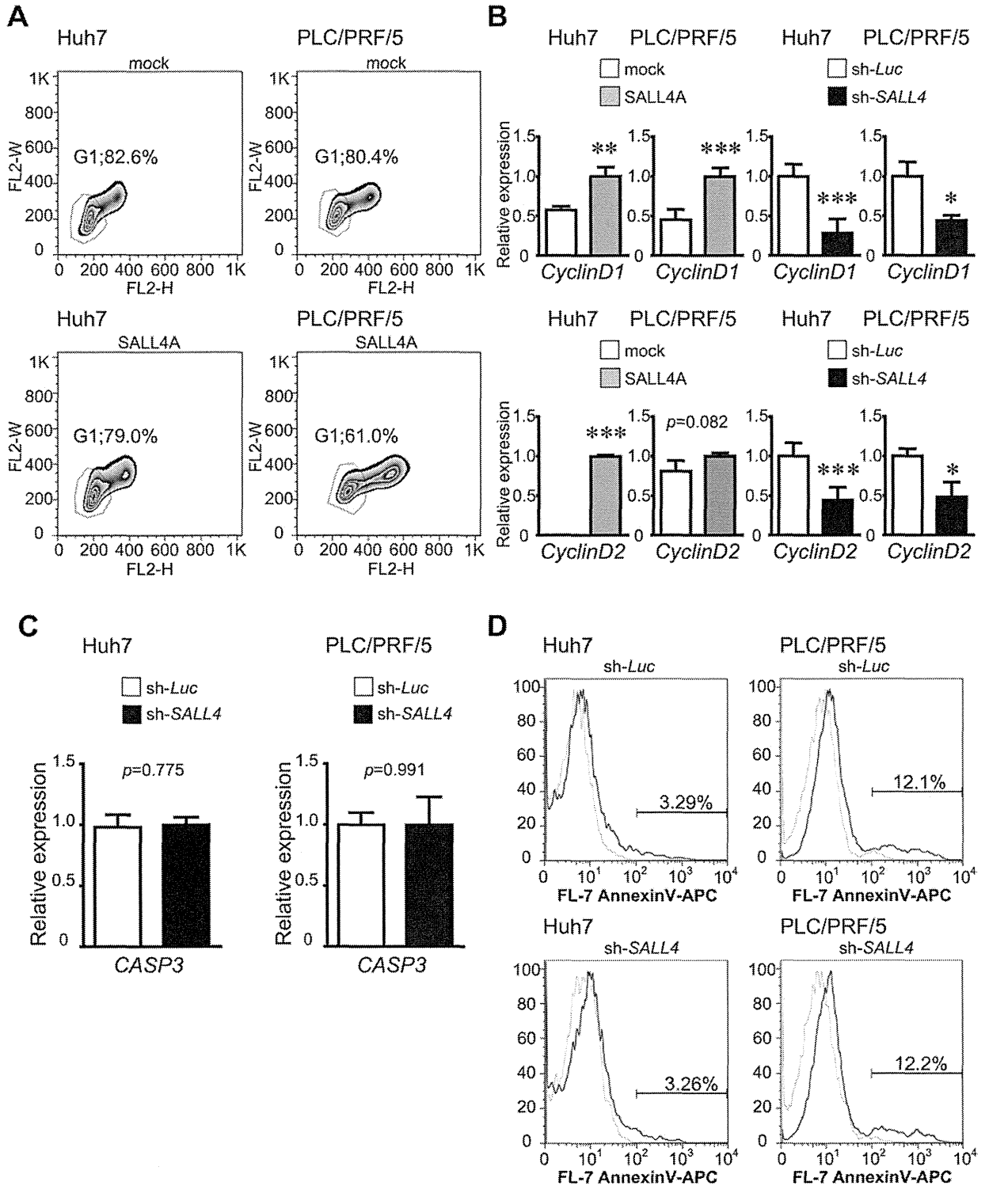

Fig. 4. Correlates of SALL4 overexpression or knockdown with respect to cell proliferation of liver cancer cells. (A) Cell-cycle analysis in SALL4-overexpressing liver cancer cells was estimated by flow cytometry. (B,C) Expression of Cyclin D1, Cyclin D2, and CASP3 in SALL4-overexpressing or SALL4-knockdown 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

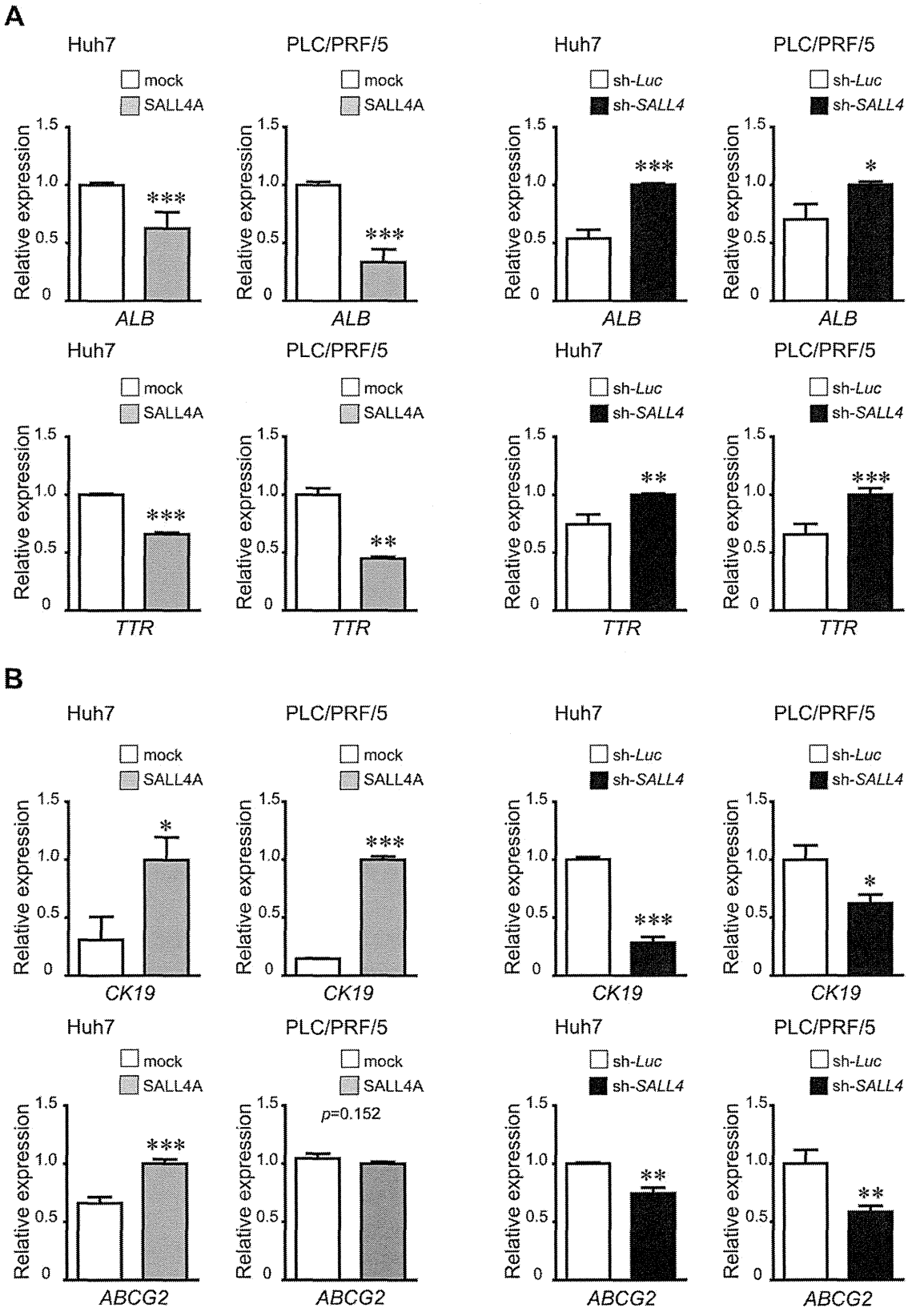

Fig. 5. Expression of hepatocytic differentiation (A) and stemness (B) genes in SALL4-overexpressing or SALL4-knockdown liver cancer cells. Cells transduced by a retroviral or lentiviral vector were cultured for 3 days. ALB, TTR, CK19, and ABCG2 mRNA expression was detected using qRT-PCR. Data are expressed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

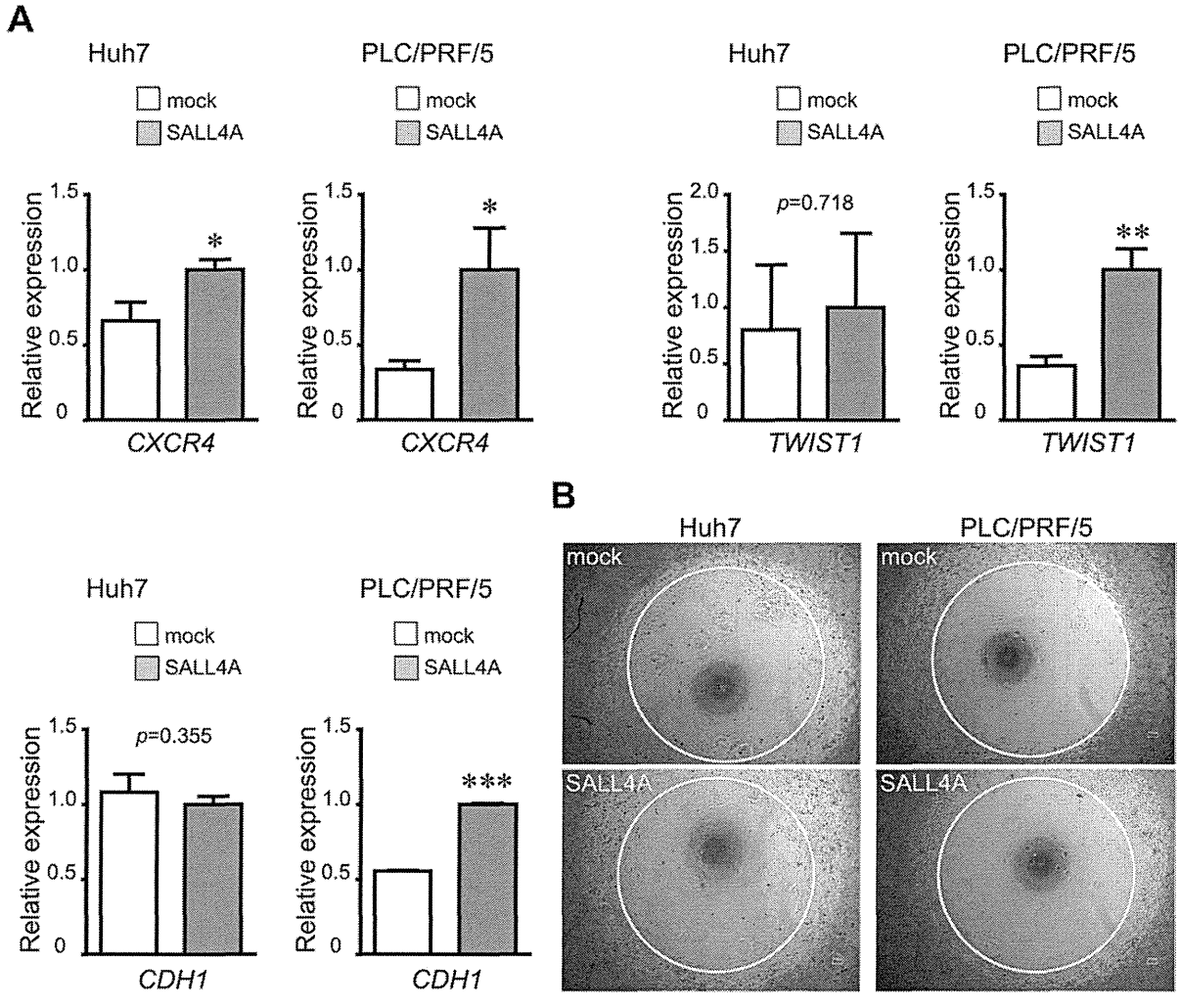

Fig. 6. Expression of EMT-related genes and migration assays in SALL4-overexpressing liver cancer cells. (A) Cells transduced by an overexpressing retroviral vector were cultured for 3 days. CXCR4, TWIST1, and CDH1 mRNA expression was detected using qRT-PCR. Data are expressed as mean ± SD (triplicate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

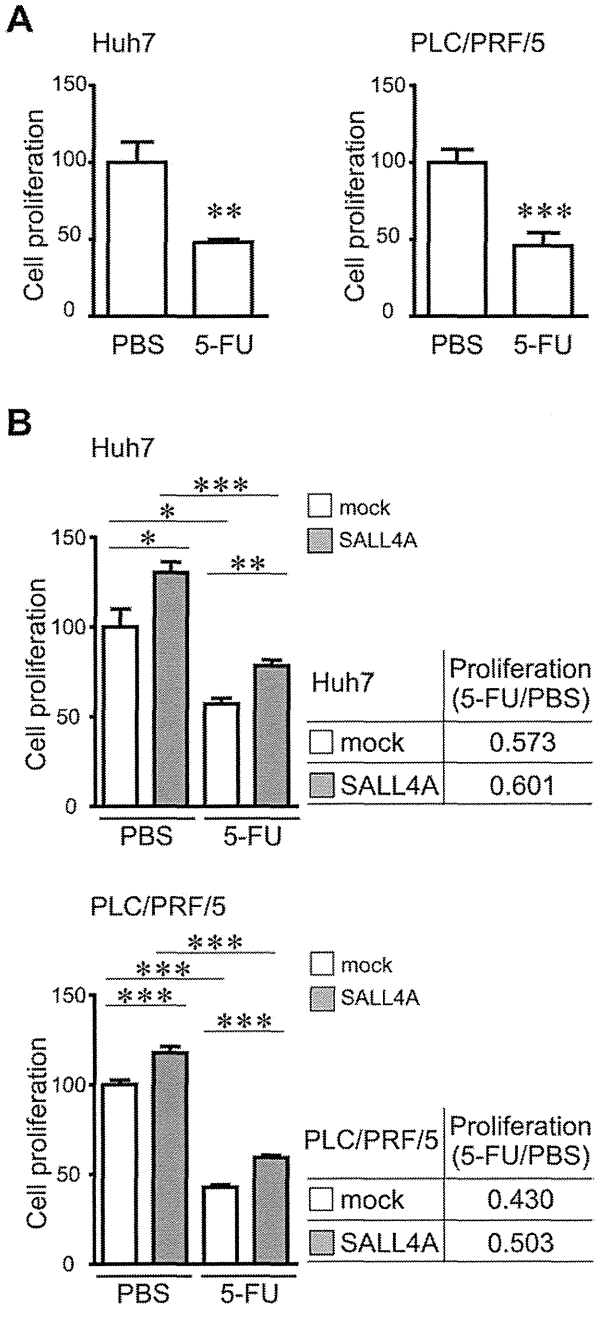

Fig. 7. Chemoresistance assays for SALL4-overexpressing liver cancer cells. Cells were transduced by a retroviral vector. Nontransduced (A) or transduced cells (B) were cultured in the presence or absence of 5-FU (2 μg/mL) for 7 days. The relative cell proliferation between PBS- and 5-FU-treated liver 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

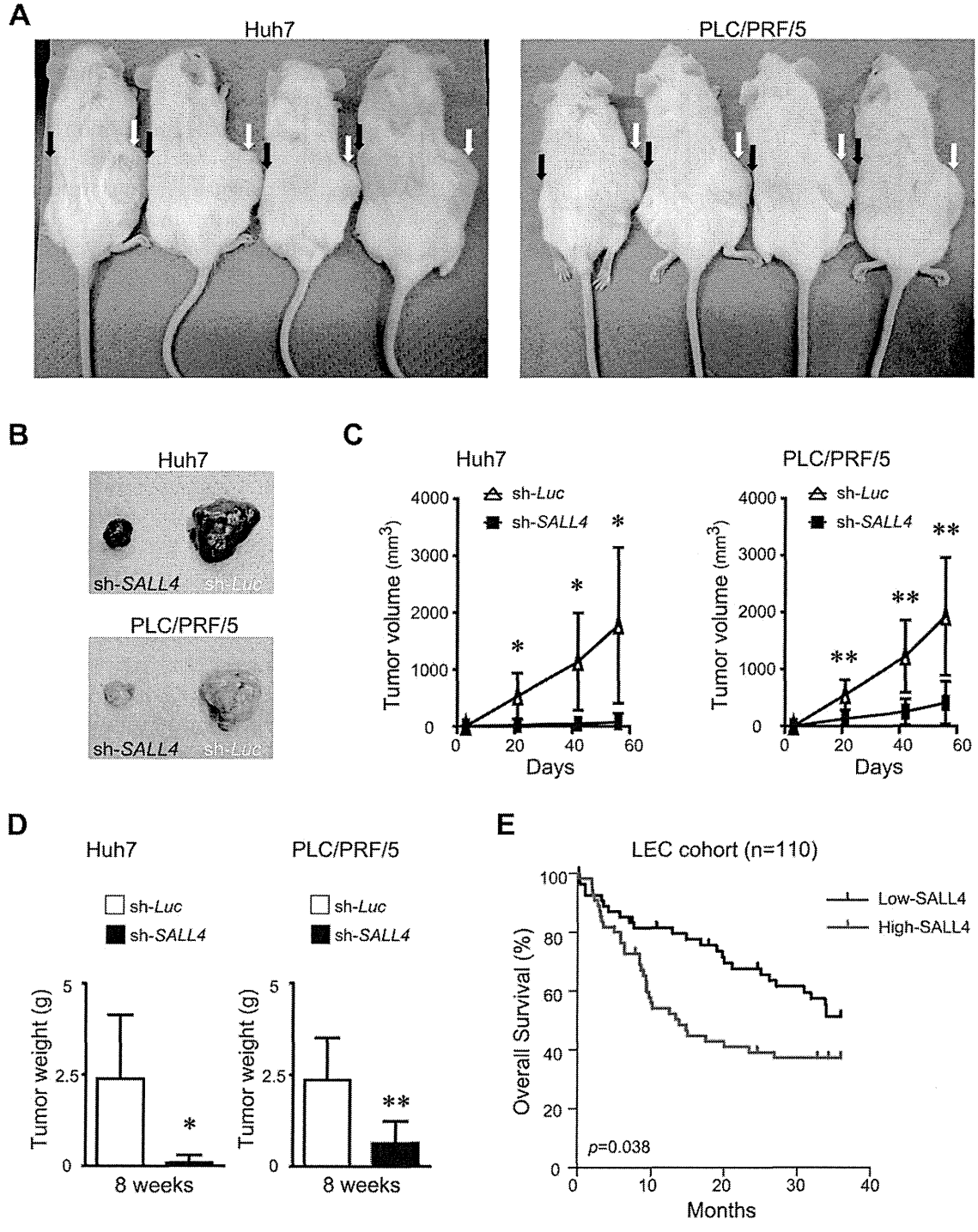

Fig. 8. Effect of SALL4 knockdown on xenograft tumor growth *in vivo*. (A) Control cells and SALL4-knockdown cells were implanted into recipient mice, respectively. White arrows show tumors derived from control cells and black arrows show tumors derived from SALL4-knockdown cells (Huh7 n = 5, PLC/PRF/5 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.

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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.

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発生過程・成体における肝幹・前駆細胞の分化誘導の分子メカニズム

紙谷聡英*

索引用語：肝幹・前駆細胞，肝発生，肝細胞分化，胆管分化

1 序論

肝臓は固形臓器としては特徴的な高い再生能力を持つ．他の再生臓器(血液，皮膚，毛髪など)では，各臓器に存在する幹・前駆細胞からの細胞供給・機能細胞への分化により再生を行う．肝臓ではこれらの臓器と異なり，通常の再生時には機能細胞である成熟肝細胞が一時的に増殖し元の臓器の機能・大きさを回復する．再生過程における関与が少ないために肝臓における幹・前駆細胞の研究は他の再生臓器に比較し遅れていたが，近年，強い肝障害などにより成熟肝細胞の増殖が阻害される条件下で肝幹・前駆細胞の増殖や再生への寄与が報告されてきた．われわれを含むさまざまなグループの研究成果により，障害肝臓や正常な成体肝臓内に高増殖能と多分化能を示す肝幹・前駆細胞が存在することが明らかになってきた[1,2]．胎仔期では，腸管の一部が心臓・横中隔からのシグナルを受けて肝芽へと分化し肝発生がスタートする．この肝芽に存在する胎生肝幹・前駆細胞が幼弱細胞を経て，成体肝臓の機能細胞である成熟肝細胞・胆管細胞へと分化する[3]．

本稿では，胎仔期および成体肝臓に存在する肝幹・前駆細胞の性状を成熟細胞への分化過程を中心に解説する(図1)．

2 胎生肝幹・前駆細胞の分化・成熟の分子メカニズム

肝発生中期の肝臓(マウスでは胎生13日前後)は，代謝器官である成熟肝臓と異なりほとんど代謝酵素を発現していない．この時期の肝臓には多数の血液細胞が存在しており，造血幹細胞の活発な増殖や赤血球系細胞への分化を行う造血器官として機能している．また，成熟肝細胞・胆管細胞の元になる胎生肝幹・前駆細胞(肝芽細胞)が増殖・分化する場である．肝発生中期の肝芽細胞の研究から，Dlk, CD13, CD133, Liv2などの表面抗原陽性

Akihide KAMIYA : Molecular mechanism regulating differentiation of hepatic stem/progenitor cells during liver development
*東海大学・創造科学技術研究機構・医学部門［〒259-1292 神奈川県平塚市北金目 4-1-1］

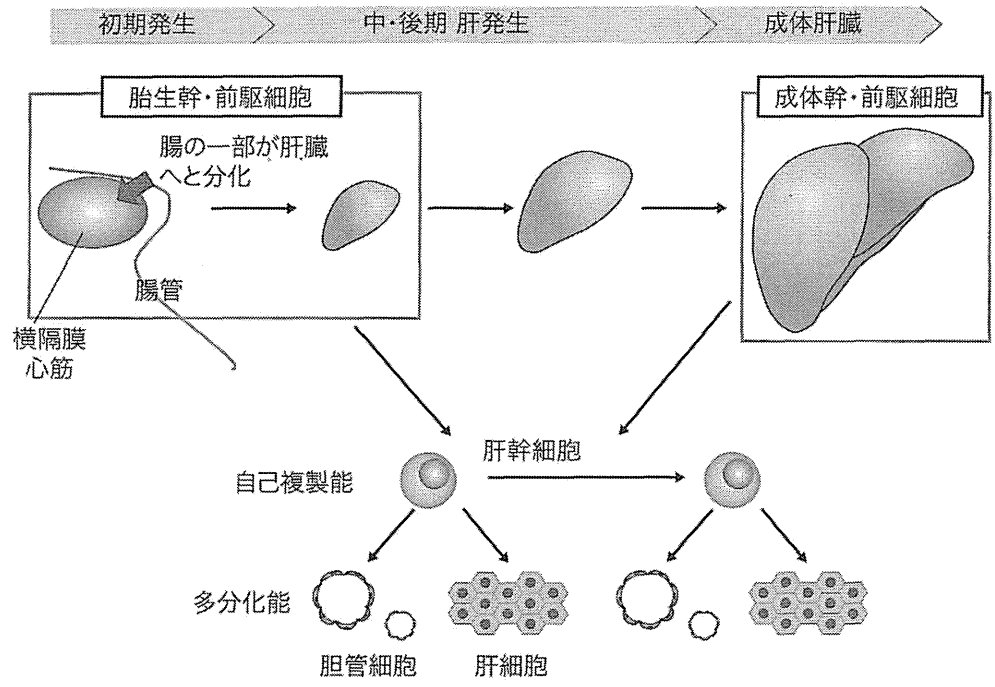

図1　肝発生過程における幹細胞システム
肝臓は発生初期に腸管の一部が肝芽へと分化することで生じる．この肝芽に含まれる肝幹・前駆細胞(肝芽細胞)から成熟肝細胞および胆管細胞が分化する．一方，成体肝臓にも肝幹・前駆細胞が存在することが知られている．

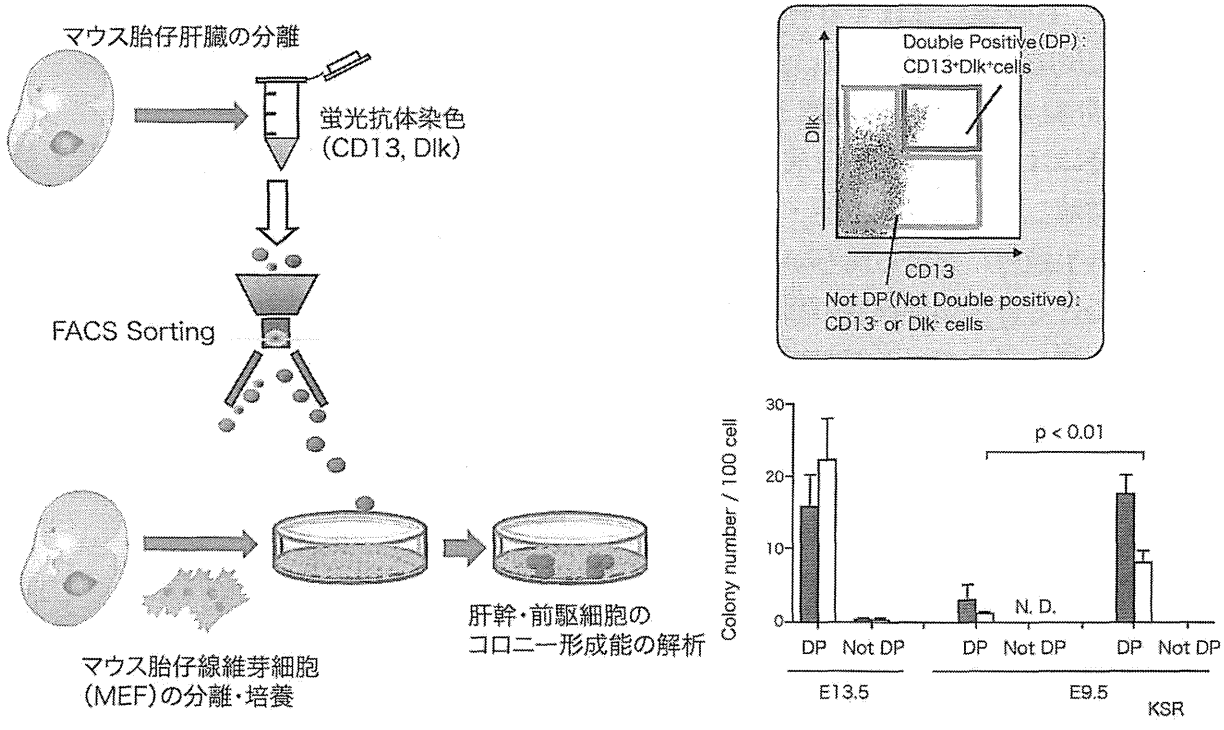

図2　肝発生初期の肝幹・前駆細胞様細胞の分離
マウス胎仔肝臓よりCD13^{+}Dlk^{+}細胞を分離し，MEFとの共培養によるコロニー形成能を観察した．肝発生初期(マウス胎生9.5日)肝臓由来細胞の中でCD13^{+}Dlk^{+}両陽性画分にのみ高いコロニー形成能を持つ高増殖性の肝幹・前駆細胞が存在する．特に細胞をKSR(knockout serum replacement)で培養した際に高いコロニー形成がみられた(文献7より改変して引用)．

の細胞として同定されている[2,4~6]．しかし，肝発生初期のどのような細胞から肝芽細胞へと分化するのかについては不明な点が多かった．われわれは肝発生初期（マウス胎生9.5～10.5日）の胎仔肝臓細胞を酵素的に分散させ，さまざまな表面抗原抗体での染色，スクリーニングを行った[7]．その結果，CD13$^+$Dlk$^+$細胞が肝発生初期から中期の肝臓に継続して存在した．CD13$^+$Dlk$^+$細胞を各発生段階で純化し遺伝子発現を解析したところ，胎生中期のCD13$^+$Dlk$^+$細胞がαフェトプロテイン（AFP）・Albumin陽性，サイトケラチン（CK）19陰性の肝芽細胞のPhenotypeを示すのに対し，肝発生初期のCD13$^+$Dlk$^+$細胞はAFP・Albuminの発現が非常に弱い一方で内胚葉系前駆細胞マーカー（Sox17）などの発現が高いことがわかった．そこで，肝発生初期の内胚葉系前駆細胞様のCD13$^+$Dlk$^+$細胞から肝発生中期の肝芽細胞への分化過程のメカニズム解析の目的で*in vitro*培養系を構築した．マウス胎生9.5日および胎生13日CD13$^+$Dlk$^+$細胞をコラーゲンコート培養皿上で低密度培養したところ，胎生13日細胞は単独培養で大型のコロニーを形成する高増殖能を維持しているのに対して，胎生9.5日細胞は単独培養ではほとんど増殖できなかった．この時期の胎生肝臓は，肝芽が横中核の間葉系組織に侵入しつつ増殖する時期であり，内胚葉系細胞と間葉系組織の相互作用が重要である可能性を考えた．そこで，胎生9.5日CD13$^+$Dlk$^+$細胞とマウス胎仔線維芽細胞（MEF）との共培養系を構築した結果，単独培養ではみられなかった大型のコロニー形成能を持つ細胞が多数得られた（図2）．形成されたSingle Cell由来のコロニーは，肝芽細胞から形成されたコロニーと同様に肝細胞マーカーAlbumin，胆管細胞マーカーCK19の両方を発現しており，2方向の分化能を持つことが示された．以上の結果から，肝発生初期から中期にかけての肝幹・前駆細胞の分化誘導における間葉系細胞との相互作用の重要性を明らかとした．

胎生中期の肝幹・前駆細胞は，肝発生後期にかけて一部は門脈周囲に胆管構造を形成する胆管細胞へと分化誘導し，他のほとんどの細胞は成体肝臓の主要な機能細胞である成熟肝細胞へと分化する．この肝幹・前駆細胞から幼弱肝細胞，さらに成熟肝細胞へと分化する分子メカニズムは，遺伝子欠損マウスや培養系を用いた研究から徐々に明らかとなりつつある．Hepatocyte Growth Factor（HGF）は，肝細胞の増殖を誘導するサイトカインとして同定された．そのノックアウトマウスは胎生中期前後に死亡し，肝臓の委縮，特に実質細胞の欠損がみられ，肝幹・前駆細胞の増殖などを制御することが明らかとなっている[8]．また，肝細胞の機能転写因子であるHNF4αを幼弱肝細胞で特異的に欠損させると，E-cadherinなどの発現制御が障害され成熟肝細胞への分化が抑制される[9]．われわれは，マウス肝芽細胞・幼弱肝細胞の*in vitro*培養系を構築し，その分化・成熟を制御する液性因子の探索を行った．その結果，IL-6ファミリーサイトカインに属するオンコスタチンM（OSM）をグルココルチコイドとともに培養系に添加した際に，胎生期の肝細胞が成熟肝細胞様の形態へと変化しその一部は多核化することを見いだした．さらに，新生仔期および成体肝臓で強く発現する代謝酵素群（グルコース6リン酸ホスファターゼやチロシンアミノトランスフェラーゼ）や多糖類の蓄積が，OSM依存的に誘導されることを見いだした[10]．胎仔肝臓ではOSMは血液細胞に主に発現し，一方OSM受容体は幼弱肝細胞などに発現している．つまり，OSMは血液細

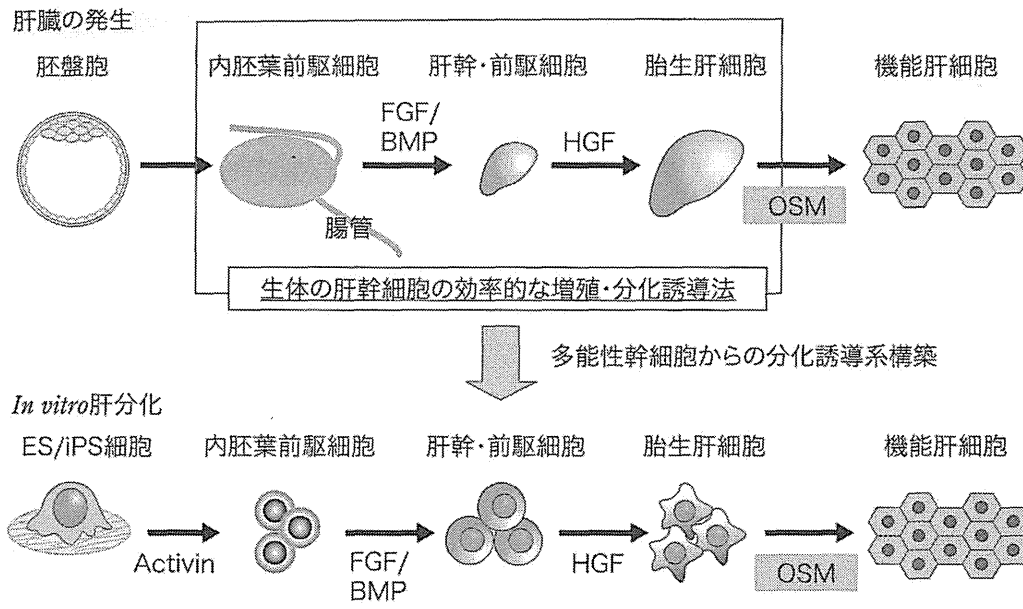

図3　生体および*in vitro*での肝発生

生体肝臓では，腸管から発生した肝芽細胞が，その後幼弱な胎生肝細胞を経て成熟肝細胞へと分化する．この過程ではさまざまな液性因子が寄与している．ES/iPS細胞といった多能性幹細胞からの*in vitro*肝分化系でも同様の因子を添加することで成熟肝細胞への分化が可能である．

胞から供給され肝芽細胞・幼弱肝細胞の成熟肝細胞への分化を誘導するパラクライン的液性因子であることを同定した．胎仔肝発生にはさまざまなサイトカインが関与するが，多能性幹細胞の*in vitro*肝分化誘導に同様のサイトカインが利用されている．マウスおよびヒトES, iPS細胞の成熟肝細胞分化誘導では，アクチビンA添加による内胚葉系細胞への分化誘導，Fibroblast growth factor (FGF)，Bone morphogenetic protein (BMP)，HGFの連続投与による肝芽細胞への誘導，OSM添加による成熟肝細胞への誘導と，*in vivo*肝発生を模倣して行われている(図3)．今後も，発生過程の肝臓で得られた知見が，多能性幹細胞からの肝分化誘導系にも応用できると考えられる．

肝芽細胞からの胆管系への分化では，細胞間相互作用や液性因子の関与，さらには肝芽細胞内の転写因子の発現制御が重要な役割を果たしている[11]．胆管の発生過程は，胎生中期から後期におけるductal plateの門脈周囲での形成から始まる．その後，ductal plateの一部が管腔構造をとりCanals of heringを通じて微小胆管と接続することで肝内胆管網が形成されると考えられる．肝芽細胞と門脈周囲の間葉系細胞との相互作用がductal plate発生や管腔形成に重要である．ヒトの遺伝病解析(アラジール症候群)から，Jagged-Notchシグナル伝達系の胆管細胞分化や管腔形成への関与が指摘され，このシグナル伝達に関わる遺伝子群(Jagged, Notch, HES-1, RBP-Jk)などのノックアウトマウスで，胆管形成障害が報告された．液性因子ではTGFβの濃度勾配やWnt系のシグナルの重要性が指摘されている．また，肝芽細胞に発現する転写因子群によって胆管分化が制御される．われわれは肝芽細胞の肝細胞・胆管細胞分化を制御する遺伝子として，Sall4を同定した[12]．

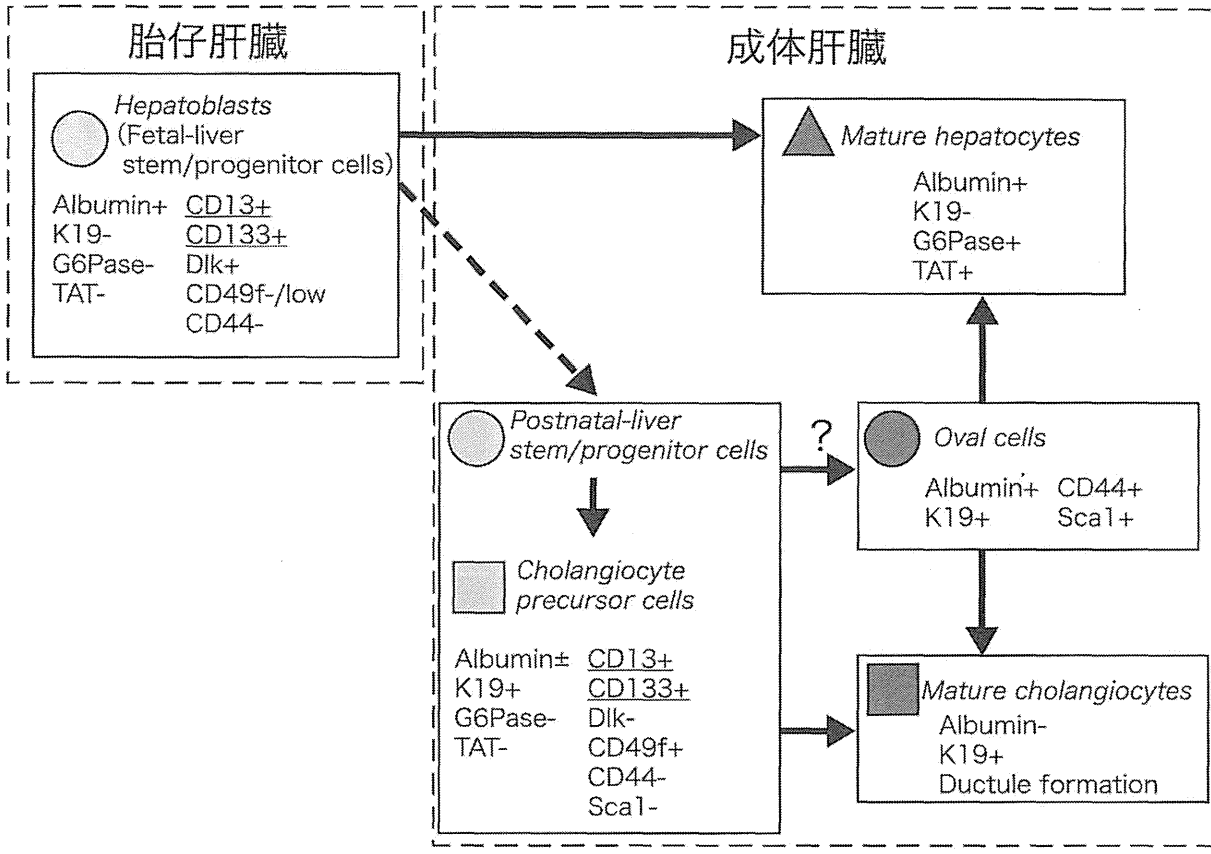

図4　肝発生過程における幹細胞の分化・成熟過程

胎仔肝臓では$Dlk^+CD13^+CD133^+$の肝幹・前駆細胞(肝芽細胞)が存在し，発生過程が進むにつれて成熟肝細胞・胆管細胞へと分化する．一方，成体肝臓では肝芽細胞から分化したと考えられる$CD13^+CD133^+$の成体肝幹・前駆細胞や胆管前駆細胞が存在している(文献2より引用)．

Sall4は胎仔発生や器官形成，ES細胞の未分化性維持や分化に重要な役割を果たす転写因子である．マウス肝発生中期由来の肝芽細胞を分離しSall4を強制発現させた結果，肝芽細胞の成熟肝細胞への分化誘導(機能遺伝子の発現など)を抑制する一方で，胆管系細胞への分化誘導を促進する．逆に，Sall4のノックダウンによって胆管系細胞への分化が抑制された．このSall4による胆管系分化誘導はPI3Kシグナル経路の活性化を介しており，その阻害剤であるLY294002の添加により胆管系への分化が強く抑制される．以上の結果から，Sall4が肝芽細胞の二方向性運命決定に重要な転写因子である可能性を明らかとした．

3　成体肝臓での肝幹・前駆細胞分化の分子メカニズム

成体肝臓は，肝臓の主要な機能細胞(実質細胞)である成熟肝細胞に加えて，さまざまな非実質細胞(胆管細胞，類洞内皮細胞，Kupper細胞，星細胞，間葉系細胞など)が存在している．しかし，成熟肝細胞のTurn Overに関わる幹細胞システムについては不明な点が多かった．近年，Cre-LoxPシステムを利用したLineage Tracing系がさまざまな細胞系譜で応用され，各細胞の発生段階での運命決定を*in vivo*で追跡することが可能となった．Sox9は内胚葉系臓器のさまざまな管腔構造の細胞(胆管系，膵管系，消