

**FIG. 3.** Early fetal liver CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cells could form large colonies in the coculture with MEF. **(A)** Representative view of a colony formed from a single E9.5 CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cell cocultured with MEF. **(B)** KSR induced colony formation by E9.5 CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cells. E13.5 and E9.5 fetal liver cells were sorted and cocultured with MEF for 6 days. Cells were cultured in H-CFU-C medium supplemented with either fetal bovine serum or KSR. Small colonies (*gray bars*) consisting of 50–100 cells and large colonies (*white bars*) consisting of >100 cells were counted. Results are represented as mean colony count ± SD (triplicate samples; \* and \*\* denote  $P < 0.05$  and  $P < 0.01$ ). MEF, mouse embryonic fibroblast; KSR, KnockOut Serum Replacement.

cells, Y-27632 (a Rock inhibitor), PD0325901 (a MEK inhibitor), CHIR99021 (a GSK3 $\beta$  inhibitor), and A-83-01 (a transforming growth factor  $\beta$  type I receptor inhibitor) were added to cell cultures. The morphology of colonies was not changed by the addition of these inhibitors (Fig. 4A). Although PD0325901, CHIR99021, and A-83-01 did not change the number of large colonies formed by E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells, Y-27632 significantly increased the number of large colonies formed by these cells, indicating that inhibition of Rock is important for proliferation of E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells (Fig. 4B). The addition of Y-27632 induced colony formation of E10.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells (Fig. 4C). In contrast, the addition of Y-27632 could not induce proliferation of E13.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells, as previously shown (Fig. 4B) [9].

We used conditioned medium derived from E14.5 liver cells in conventional H-CFU-C culture system for mid-fetal liver hepatoblasts [10,13]. When E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells were cocultured with MEF, however, the addition of fetal liver cell-conditioned medium decreased the number of large and small colonies (data not shown). The number of proliferative cells in individual colonies was not significantly changed (Fig. 5A), suggesting that cell apoptosis might be involved in the inhibition of colony formation by the addition of fetal

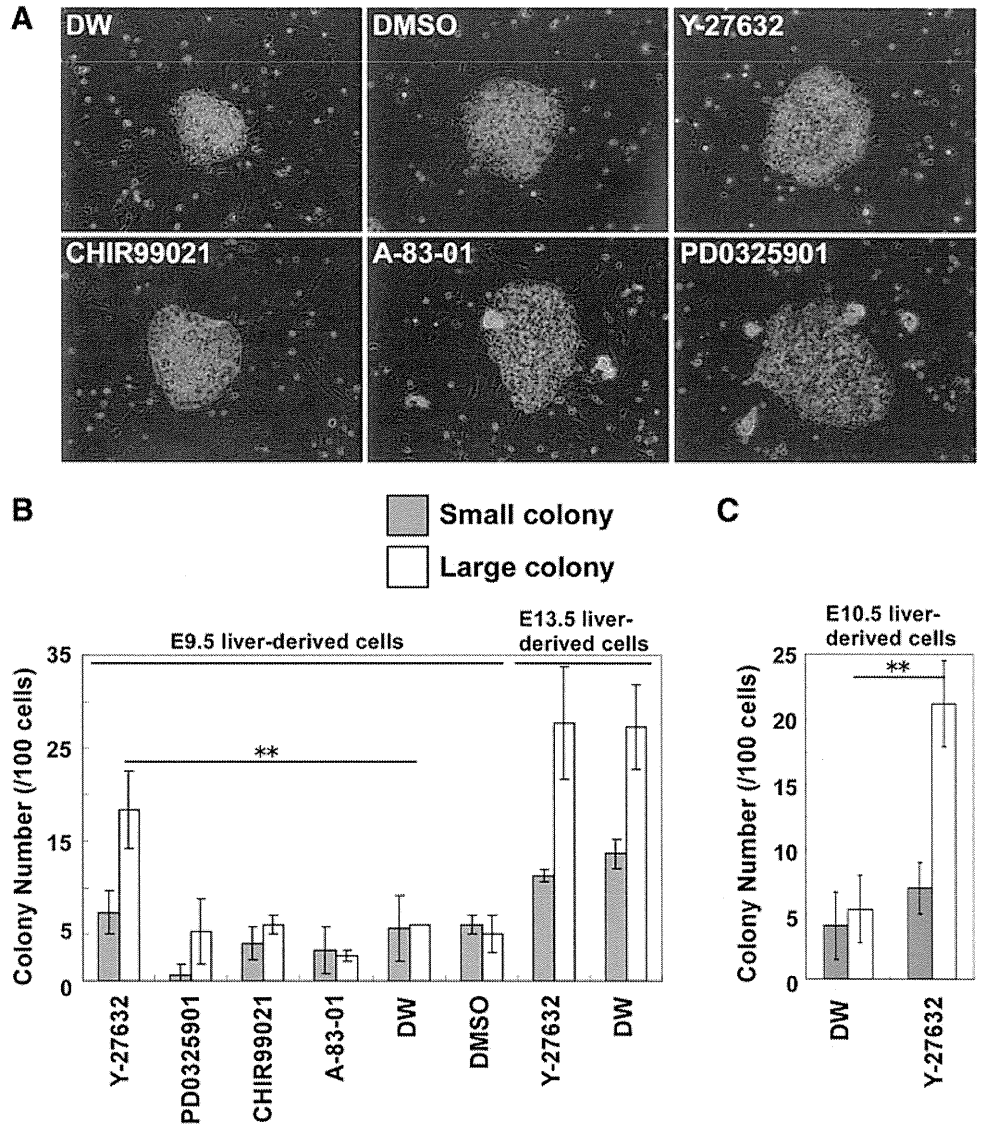
liver cell-conditioned medium. In consequence, we cultured early fetal liver cells in the following experiments without using fetal liver cell-conditioned medium. We also found that these isolation and culture methods could induce proliferation of early fetal HSPCs derived from C3H mice, in addition to C57BL6 mice (Supplementary Fig. S4A, B).

In addition to CD13<sup>+</sup>Dlk<sup>+</sup> cells, the CD13<sup>mid</sup>Dlk<sup>mid</sup> cells (the intermediate fraction) existed in E10.5 and E11.5 livers. We asked whether the intermediate fractions also contain HSPCs. However, significant colony formation by these fractions was not detected compared with the CD13<sup>+</sup>Dlk<sup>+</sup> fractions (Supplementary Fig. S4C, D), indicating that most progenitor cells exist in the CD13<sup>+</sup>Dlk<sup>+</sup> fraction during early- to mid-fetal liver development. These results suggested that the addition of ROCK inhibitor was required for clonal expansion of early fetal CD13<sup>+</sup>Dlk<sup>+</sup> progenitor cells but not of mid-fetal CD13<sup>+</sup>Dlk<sup>+</sup> hepatoblasts.

*Inhibition of the Rock-myosin II pathway induced colony formation of early fetal liver CD13<sup>+</sup>Dlk<sup>+</sup> cells*

We varied length of exposure to Y-27632 in E9.5 fetal liver cell culture. Short-time exposure to Y-27632 (culture days 0–3

**FIG. 4.** Addition of Rock inhibitor is important for colony formation of early fetal liver CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cells. **(A)** Representative view of colonies formed from a single E9.5 CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cell in the presence of signaling inhibitors. E9.5 CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cells were cocultured with MEF for 6 days in the presence of either Y-27632 (a Rock inhibitor), PD0325901 (a MEK inhibitor), CHIR99021 (a GSK3 $\beta$  inhibitor), or A-83-01 (a transforming growth factor  $\beta$  type I receptor inhibitor). DW: distilled water (DW) added (control for Y-27632); DMSO: 0.01% DMSO added (control for PD0325901, CHIR99021, and A-83-01). **(B)** E9.5 and E13.5 CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cells were cocultured with MEF for 6 days in the presence of inhibitors shown in A. The number of colonies derived from E9.5 CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cells was significantly increased by culture in medium containing Y-27632, whereas the number of colonies derived from E13.5 CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cells did not increase with Y-27632 exposure. **(C)** E10.5 CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cells were cocultured for 6 days with MEF in the presence of Y-27632. DW: DW added (control). **(B, C)** Small colonies (*gray bars*) consisting of 50–100 cells and large colonies (*white bars*) consisting of >100 cells were counted. Results are represented as mean colony count  $\pm$  SD (triplicate samples; \* and \*\* denote  $P < 0.05$  and  $P < 0.01$ ). DMSO, dimethyl sulfoxide.

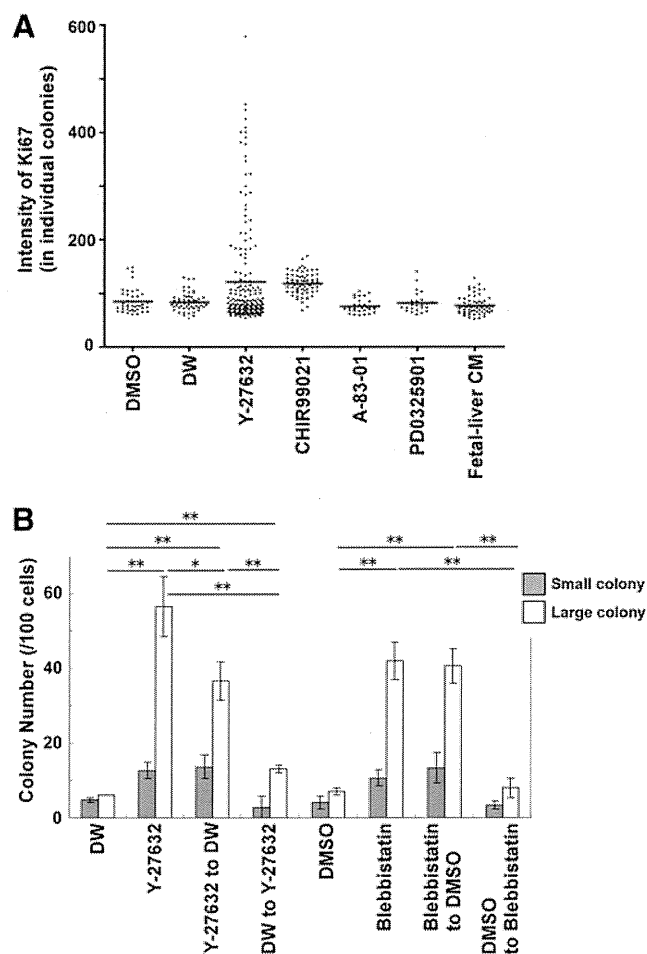


or 3–6) partially induced progression of colony formation. Interestingly, early-stage addition of Y-27632 (days 0–3) significantly induced formation of large colonies compared with late-stage addition of Y-27632 (days 3–6). Thus, inhibition of Rock is particularly important for the early stage of colony formation by E9.5 cells (Fig. 5B). Rock induces phosphorylation of several substrates, leading to various cellular responses [18]. The inactivation of myosin phosphatase target subunit, which is induced by Rock, protects the phosphorylated form of myosin regulatory light chain. This phosphorylation keeps myosin II in its active form. Blebbistatin, which specifically inhibits myosin II, has an effect similar to that of Y-27632. It inhibits apoptosis of single-suspended human embryonic stem cells [19,20]. Blebbistatin, like Y-27632, significantly induced colony formation by E9.5 cells (Fig. 5B). We analyzed proliferation of colonies in the presence of several inhibitors and found that a

number of colonies expressed high levels of Ki67 proliferation marker in the culture stimulated with Y-27632 (Fig. 5A). These results suggest that inhibition of the Rock-myosin II pathway is important in expansion of early fetal HSPCs.

*Soluble factors derived from MEF partly induced proliferation of early fetal CD13<sup>+</sup>Dlk<sup>+</sup> cells*

We then assessed whether soluble factors derived from MEF are involved in expansion of E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells. Confluent MEFs were cultured for 2 days in H-CFU-C medium. This medium (now “MEF conditioned”) was used as medium for various colony formation assays (Fig. 6A). When cells were cocultured with MEF, use of MEF-conditioned medium made no difference in the efficiency of colony formation by E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells. However, when E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells were cultured on collagen type I, small and



**FIG. 5.** A Rock inhibitor, Y-27632, and a myosin II synthetic inhibitor, blebbistatin, induced colony formation by early fetal liver CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cells. **(A)** Proliferation of early fetal HSPCs regulated by ROCK inhibitor. Intensities of Ki67 in individual albumin-positive colonies were analyzed. DW: distilled water (DW) added (control for Y-27632); DMSO: 0.01% DMSO added (control for PD0325901, CHIR99021, and A-83-01); Fetal-liver CM: fetal-liver conditioned medium added. **(B)** CD45<sup>-</sup>Ter119<sup>-</sup>Dlk<sup>+</sup> cells were cocultured for 6 days with MEF in the presence of either Y-27632 or blebbistatin. DW: DW added (control for Y-27632); DMSO: 0.1% DMSO added (control for blebbistatin); Y-27632 to DW: first 3 days of culture with Y-27632 followed by 3 days of culture without Y-27632; DW to Y-27632: first 3 days of culture without Y-27632 followed by 3 days of culture with Y-27632; Blebbistatin to DMSO: first 3 days of culture with blebbistatin followed by 3 days of culture with DMSO; DMSO to blebbistatin: first 3 days of culture with DMSO followed by 3 days of culture with blebbistatin. Small colonies (gray bars) consisting of 50–100 cells and large colonies (white bars) consisting of >100 cells were counted. Results are represented as mean colony count  $\pm$  SD (triplicate samples; \* and \*\* denote  $P < 0.05$  and  $P < 0.01$ ).

large colonies were detected only when MEF-conditioned media were used. Fresh medium not conditioned with MEF did not support colony formation on collagen-coated dishes. These data suggest that expansion of early fetal progenitor cells was partly supported by soluble factors derived from MEF.

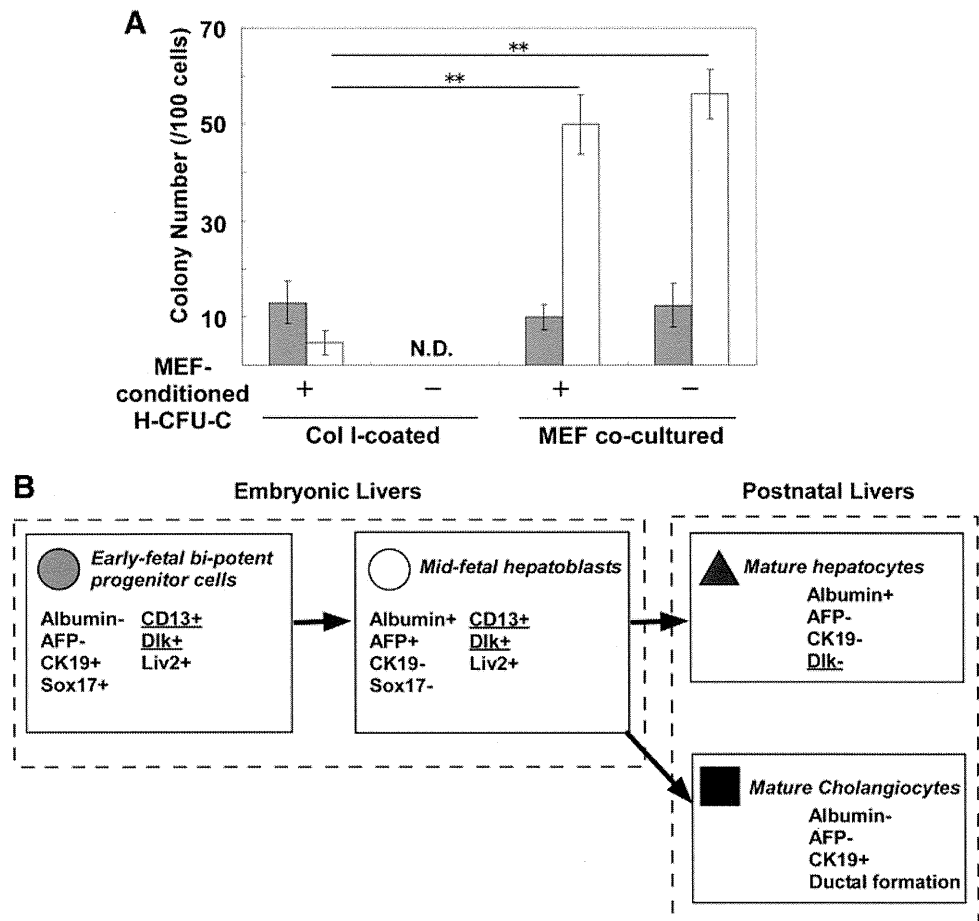
## Discussion

In this report, we showed that early fetal (E9.5 and E10.5) liver-derived CD13<sup>+</sup>Dlk<sup>+</sup> cells have characteristics of hepatic progenitor cells: They have a high proliferative capacity and the ability to differentiate into both albumin-positive hepatocytic cells and CK19-positive cholangiocytic cells. In contrast to mid-fetal hepatoblasts, early fetal HSPCs require interaction with MEF to expand clonally. Hlx is a transcription factor expressed in septum transversum mesenchyme and fetal liver expansion is severely deficient in Hlx-knockout mice [17]. Therefore, at an early fetal liver developmental stage, interaction with Hlx-positive mesenchymal cells is important for proliferation of hepatoblasts in vivo. Under our culture conditions, MEF, which express Hlx (data not shown), supported proliferation of early fetal progenitor cells. MEF-conditioned medium partially supported clonal expansion of E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells, suggesting that soluble factors derived from MEF are at least partly necessary for the survival or growth of E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells. Other cell–cell and cell–matrix interactions also appear important for proliferation of early fetal progenitor cells, because large colonies derived from E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells cocultured with MEF were significantly more numerous than when the same population of cells was cultured on collagen-coated dishes in the presence of MEF-conditioned medium.

Not only coculture with MEF but also inhibition of Rock or myosin II activity remarkably improved clonal expansion of E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells. Rock inhibitor Y-27632 promotes the survival and growth of various other types of cells, including human embryonic stem cells and adult liver-derived progenitor cells [9,21]. The molecular mechanisms by which Rock and myosin II inhibitors promoted the colony forming efficiency of early fetal liver cells in our culture system are unknown and await further investigation. As hepatic progenitor cells differentiated from foregut endoderm in E9.5 embryos into the septum transversum mesenchyme, they start to lose epithelial properties and to acquire mesenchymal properties [22]. An epithelium-specific property is that of polarity, established by the segregation of apical and basolateral domains. Epithelial shape is regulated by apical constriction, a process dependent on activated myosin II [23,24]. Rock also participates in apical constriction [25]. Dissociated early fetal progenitor cells, deprived of cell–cell contact and of traction from adjacent cells, may not be able to tolerate the force generated by apical constriction and thus undergo apoptosis. Inhibition of Rock or myosin II activity might thus rescue these cells from apoptosis through inhibition of excessive apical constriction.

We found that E9.5 to E13.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells in fetal livers can serve as bipotent progenitor cells. However, expression of several hepatic and endodermal genes differed remarkably between early- and mid-fetal CD13<sup>+</sup>Dlk<sup>+</sup> cells. Levels of albumin mRNA, but not CK19 mRNA, were high in E13.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells. In contrast, E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells scarcely expressed mRNA of hepatic genes (AFP, albumin, and c-met) but exhibited high Sox17 and CK19 mRNA levels (Fig. 2A, B). Sox17 is expressed in definitive endodermal progenitor cells and CK19, a cholangiocytic marker gene in mid-fetal livers, is also expressed in primitive gut endoderm [16]. Therefore, E9.5 CD13<sup>+</sup>Dlk<sup>+</sup> cells, which differentiate into mid-fetal hepatoblasts during liver development, seem

**FIG. 6.** Soluble factors and cell–cell/extracellular matrix interactions are important for MEF-induced expansion of early fetal cells. **(A)** E9.5 CD45<sup>-</sup>Ter119<sup>-</sup>CD13<sup>+</sup>Dlk<sup>+</sup> cells were cultured for 6 days with MEF-conditioned medium or fresh medium in the presence of Y-27632. Cells were sorted onto collagen-coated dishes or dishes containing MEF. Small colonies (gray bars) consisting of 50–100 cells and large colonies (white bars) consisting of >100 cells were counted. Results are represented as mean colony count ± SD (triplicate samples; \*\**P* < 0.01). **(B)** Schema of phenotypes of progenitor cells during fetal liver development. CD13 and Dlk are surface markers common to early fetal progenitor cells and mid-fetal hepatoblasts. However, expression of several genes (albumin, AFP, CK19, and Sox17) differed significantly between early fetal progenitor cells and mid-fetal hepatoblasts. N.D., not detected.



to possess the properties of endodermal progenitor cells (Fig. 6B).

In the present study, we showed that CD13<sup>+</sup>Dlk<sup>+</sup> cells derived from early fetal livers have high proliferative capacity and can differentiate into both albumin-positive cells and CK19-positive cells, suggesting that at the single-cell level CD13 and Dlk are markers for bipotent progenitor cells in the early fetal liver developmental stage. These cells show gene expression patterns distinct from those of hepatoblasts in the mid-fetal liver. Signaling pathways regulating the proliferative capacity of CD13<sup>+</sup>Dlk<sup>+</sup> hepatic progenitor cells in vitro also differ between cells derived from early fetal livers and those derived from mid-fetal livers. These findings highlight a biologically important and potentially therapeutic role for mesenchymal cells and for the Rock-myosin II signaling pathway in the differentiation and expansion of hepatic progenitor cells derived from pluripotent stem cells.

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### Author Disclosure Statement

There is no conflict of interest to disclose.

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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.<sup>1</sup> Cancers have a subpopulation of cancer stem cells (CSCs) or tumor-initiating cells (TICs), which have properties shared with normal stem cells.<sup>2,3</sup> 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; HNF4 $\alpha$ , 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).”<sup>4,5</sup> 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)<sup>6-8</sup> and liver TICs.<sup>9</sup> CD133 (prominin), CD90 (Thy-1), CD44 (hyaluronan receptor), and CD13 (alanine aminopeptidase) have also been found in liver TICs.<sup>10-12</sup> In parallel, CD133 and CD90 have been found on angioblasts or other mesenchymal cells tightly associated with hHpSCs,<sup>13</sup> 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.<sup>14-20</sup>

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),<sup>21-26</sup> and is one of the genes capable of eliciting reprogramming of somatic cells to become induced pluripotent stem cells (iPSCs).<sup>27,28</sup> Mutations in SALL4 cause Okhiro syndrome, known as an autosomal dominant disorder and characterized by multiple organ defects.<sup>29</sup> Recent studies have demonstrated that SALL4 is constitutively expressed in hematopoietic stem cells and a potent regulator of their expansion.<sup>30,31</sup> 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. Further-

more, SALL4 contributed to the maintenance of SP cells and chemosensitivity in leukemia by regulating the ABC drug transporter genes.<sup>31-33</sup> Solid tumors, such as germ cell tumors, breast, and alpha-fetoprotein (AFP)-producing gastric cancers also express SALL4.<sup>34-37</sup> 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  $\mu\text{g}/\text{mL}$  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  $2 \times 10^3$  cells were seeded into 96-well plates and cultured in the presence or absence of 2  $\mu\text{g}/\text{mL}$  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- $\mu\text{m}$  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%  $\text{H}_2\text{O}_2$ . 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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Additional Supporting Information may be found in the online version of this article.

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  $1 \times 10^6$  cells of each of the cell lines stably expressing short hairpin RNA (shRNA) against *SALL4* or *luciferase* suspended in 200  $\mu$ L 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.<sup>38</sup> 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.<sup>39</sup> 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.<sup>40</sup> 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.



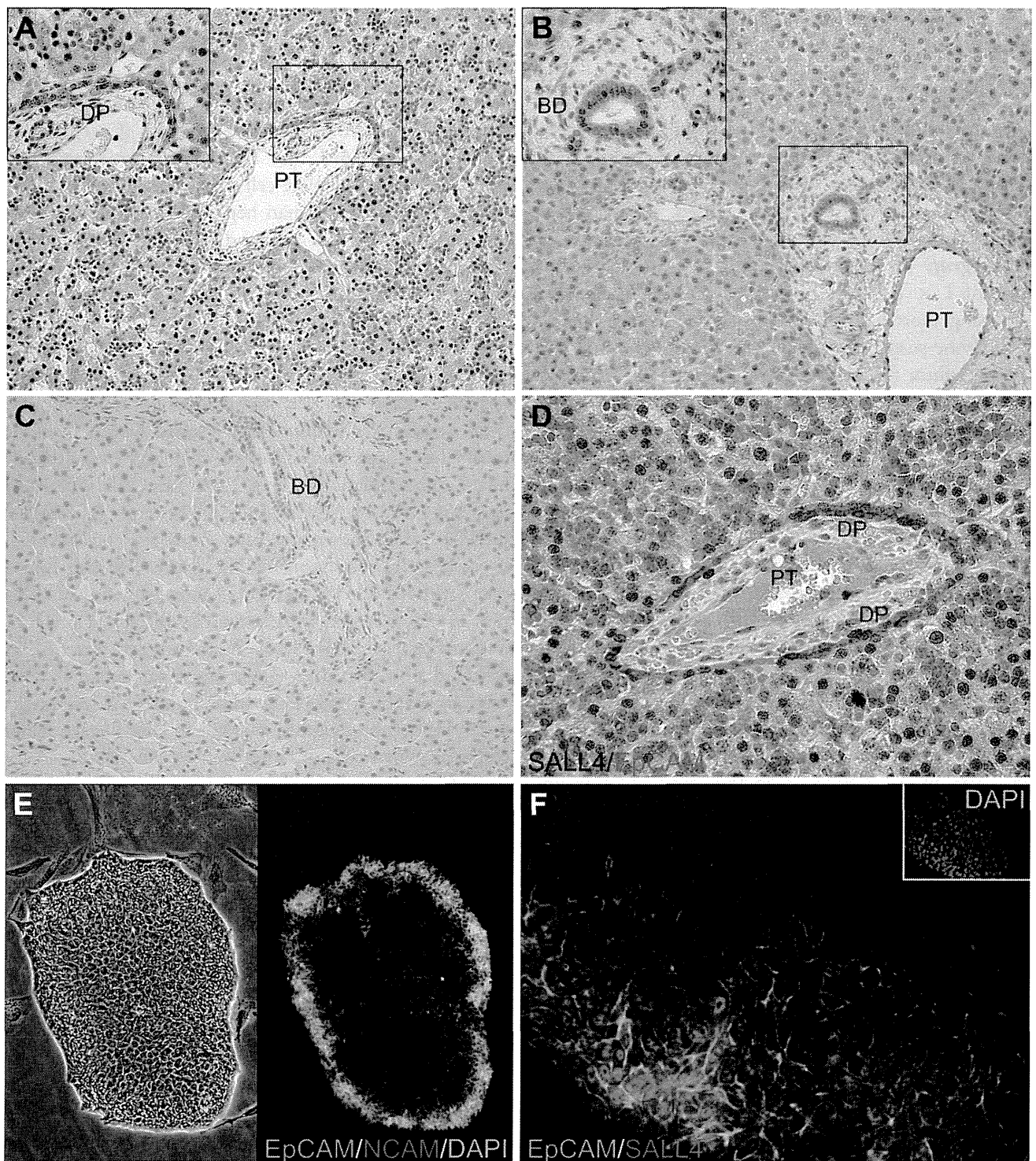


Fig. 1. Representative immunostaining of SALL4 and EpCAM expression in human normal livers and in a colony of hHpSCs. (A-D) Immunostaining of SALL4 expression during liver development. Fetal weeks gestation (A; 19 weeks, D; 16 weeks), neonatal (B; 4 months), and adult liver (C; 68 years) tissues. Sections 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  $\times 200$  (A-C),  $\times 400$  (D, F),  $\times 100$  (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 polymer-

ase chain reaction (qRT-PCR) analyses showed that both cell lines expressed SALL4A messenger RNA (mRNA). SALL4 protein was also detected using immunocytochemistry (Fig. 3A).

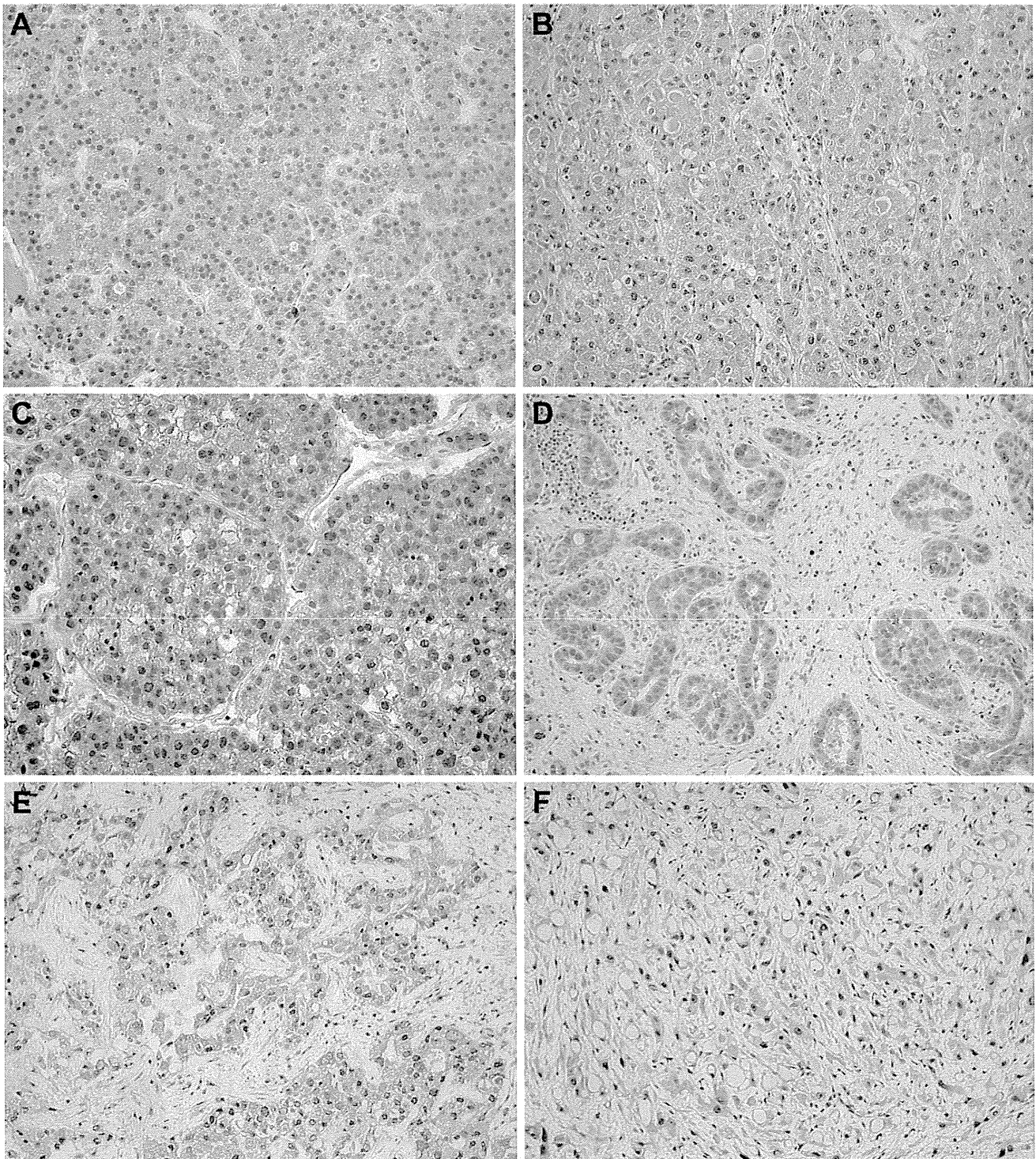


Fig. 2. Representative immunostaining of SALL4 in surgical specimens of liver cancers and transplantable tumor lines of FL-HCC. HCC (A; T41, well differentiated, B; T37, moderately differentiated, C; T49, poorly differentiated). CC (D; T5, poorly differentiated). HC-CC (E; T45, moderately differentiated).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

**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.<sup>28</sup> 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).

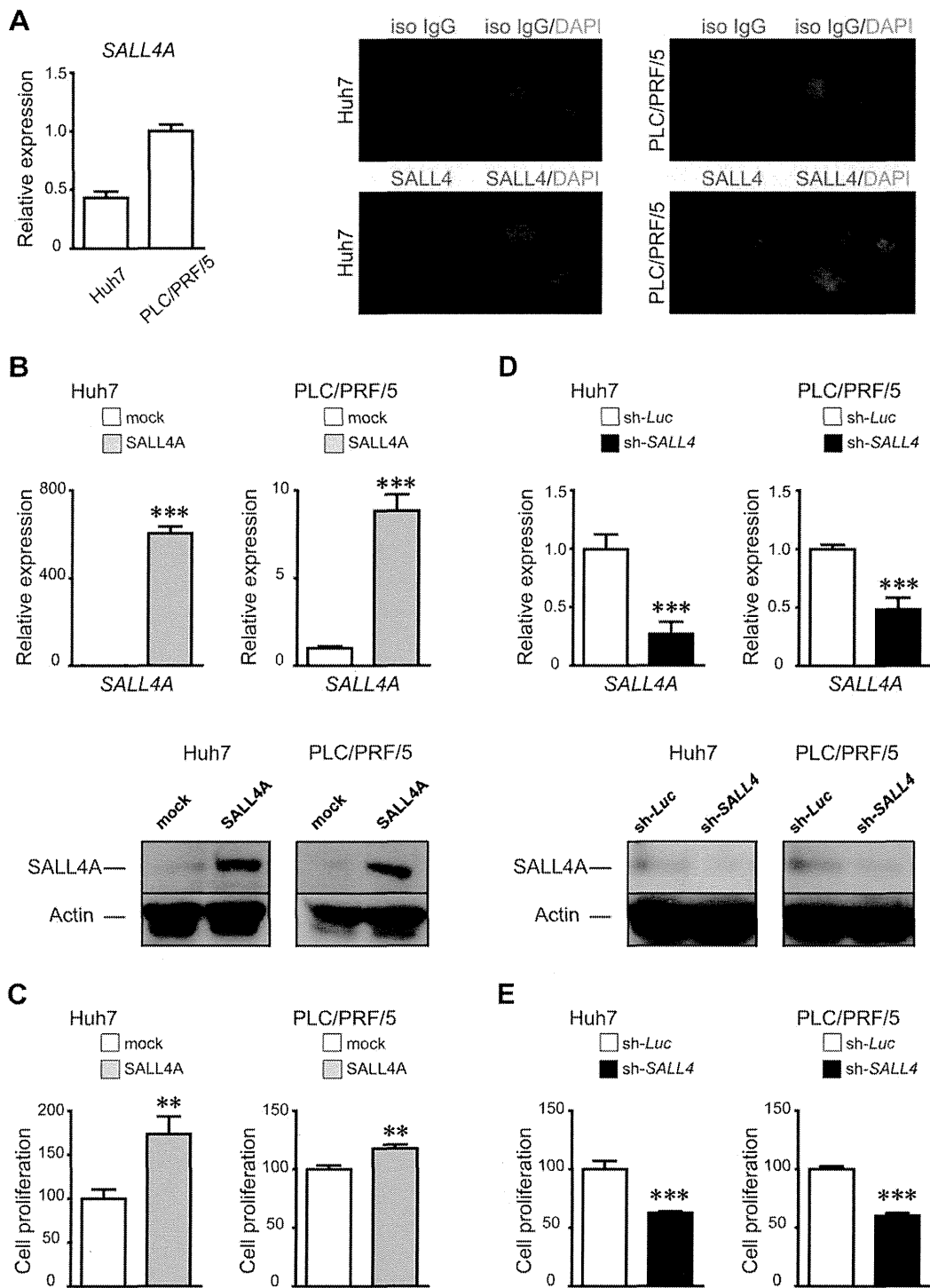


Fig. 3. SALL4 expression and the effects of SALL4 overexpression or knockdown on cell proliferation of liver cancer cells. (A) SALL4 mRNA and protein expression in liver cancer cells. (B,D) Expression of SALL4 mRNA and proteins in cultures derived from SALL4-overexpressing or SALL4-knockdown liver cancer 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  $\pm$  SD (triplicate samples, \*\*\* $P$  < 0.001, \*\* $P$  < 0.01).

Next, we conducted SALL4 expression knockdown studies using a lentiviral vector expressing-shRNA.<sup>32,39</sup> 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).

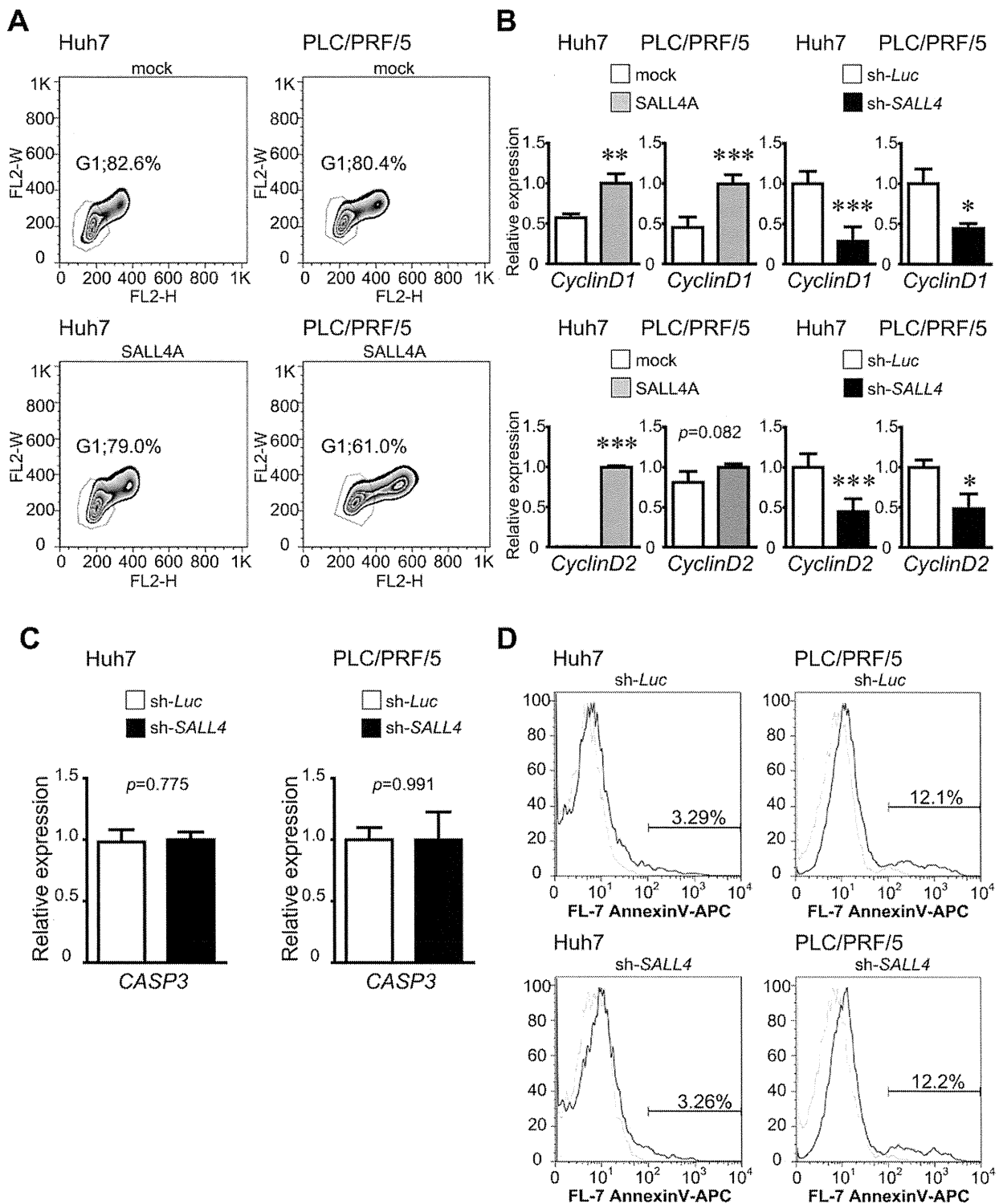


Fig. 4. Correlates of SALL4 overexpression or knockdown with respect to cell proliferation of liver cancer cells. (A) Cell-cycle analysis in SALL4-overexpressing liver cancer cells was estimated by flow cytometry. (B,C) Expression of Cyclin D1, Cyclin D2, and CASP3 in SALL4-overexpressing or SALL4-knockdown 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  $\pm$  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<sub>1</sub> 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,<sup>39</sup> 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- $\alpha$  (HNF4 $\alpha$ ), 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 HNF4 $\alpha$  (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.<sup>41</sup> OSM induced hepatocytic differentiation of EpCAM+ liver CSCs into EpCAM-negative cells and increased chemosensitivity to 5-FU.<sup>42</sup> 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

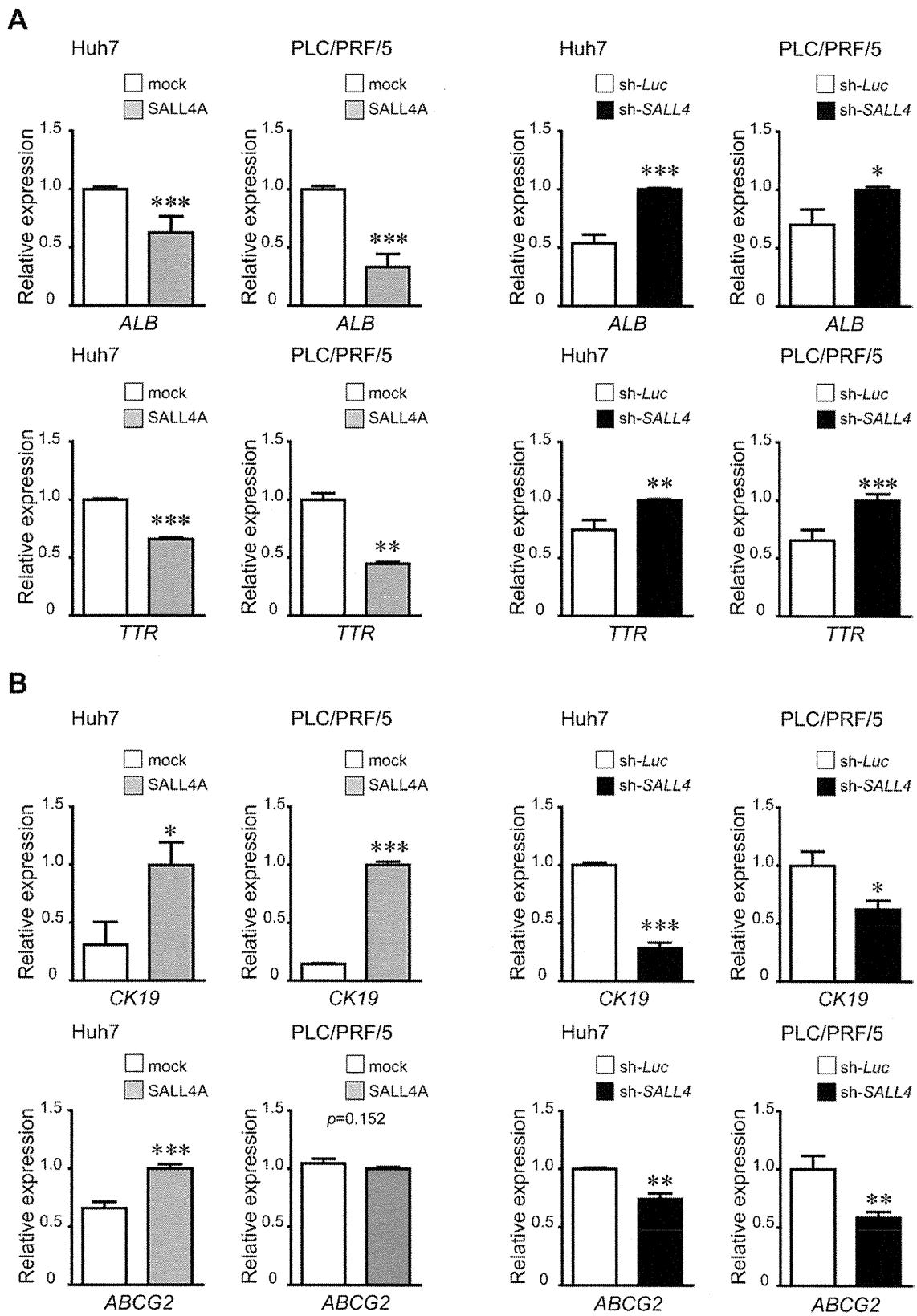


Fig. 5. Expression of hepatocytic differentiation (A) and stemness (B) genes in SALL4-overexpressing or SALL4-knockdown liver cancer cells. Cells transduced by a retroviral or lentiviral vector were cultured for 3 days. ALB, TTR, CK19, and ABCG2 mRNA expression was detected using qRT-PCR. Data are expressed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

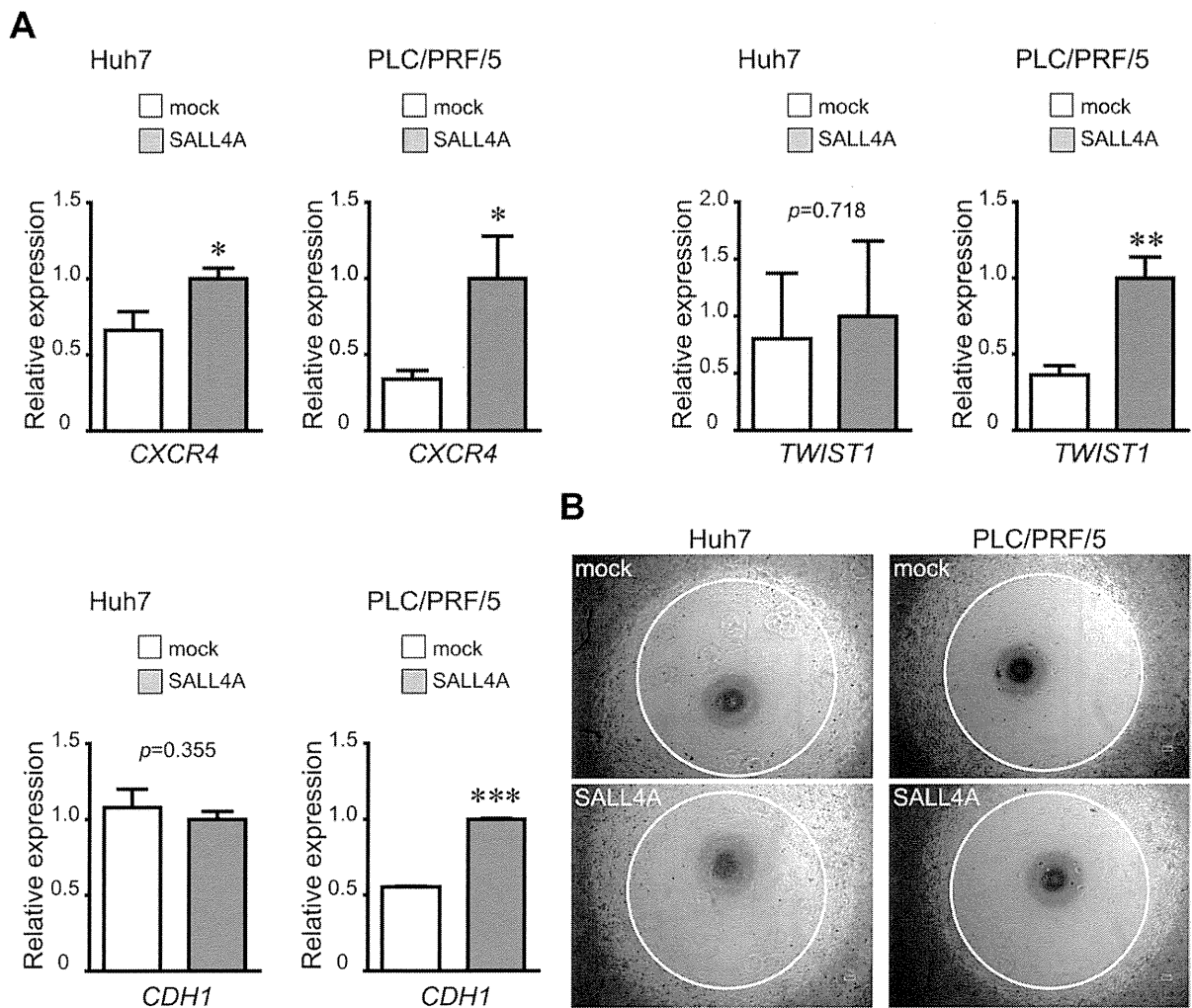


Fig. 6. Expression of EMT-related genes and migration assays in SALL4-overexpressing liver cancer cells. (A) Cells transduced by an overexpressing retroviral vector were cultured for 3 days. CXCR4, TWIST1, and CDH1 mRNA expression was detected using qRT-PCR. Data are expressed as mean  $\pm$  SD (triplicate 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.<sup>38</sup> 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.<sup>3</sup> Accordingly, fully understanding these common molecular mechanisms that regulate self-renewal and differentiation is a necessary step towards novel therapeutic

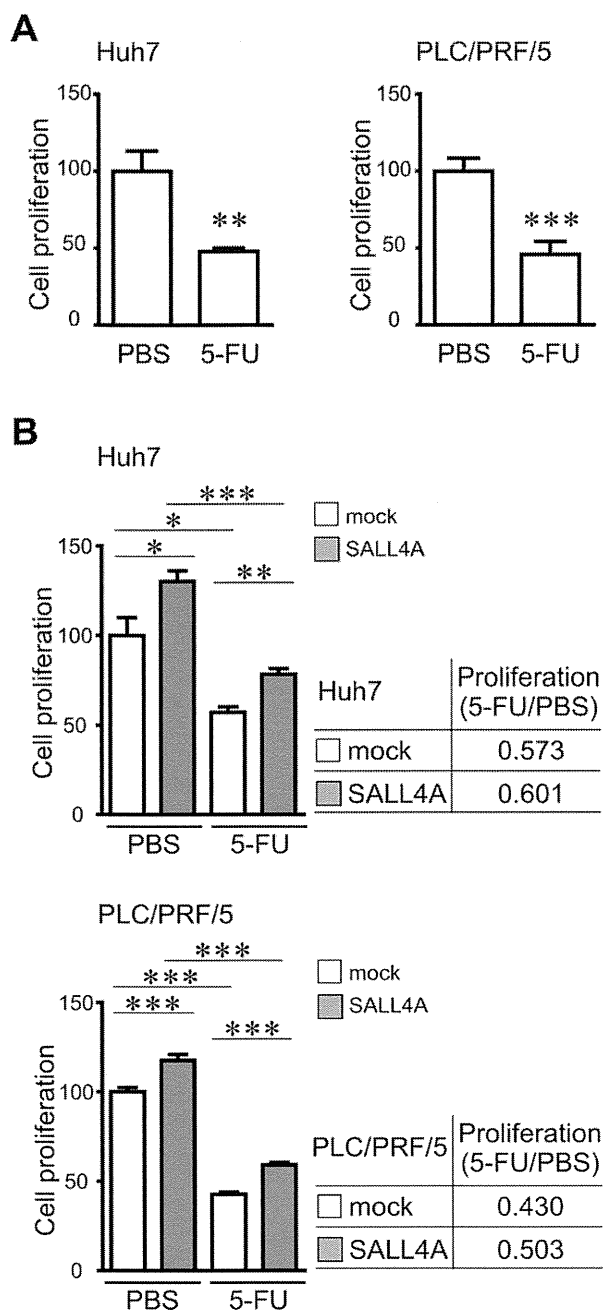


Fig. 7. Chemoresistance assays for SALL4-overexpressing liver cancer cells. Cells were transduced by a retroviral vector. Nontransduced (A) or transduced cells (B) were cultured in the presence or absence of 5-FU (2  $\mu$ g/mL) for 7 days. The relative cell proliferation between PBS- and 5-FU-treated liver cancer cells is shown. Data are expressed as mean  $\pm$  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 chemother-

apy, including sorafenib, are one of the options, but the effects are limited.<sup>14,17</sup> 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).<sup>21-26</sup> In addition, our prior investigations with mHBs revealed that inhibition of SALL4 contributes to cell differentiation.<sup>39</sup> 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.<sup>36,37</sup> 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.<sup>43</sup> 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<sub>1</sub>-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.<sup>44</sup> Moreover, the strong relationship of tumorigenesis and self-renewal by Ras-Cyclin D2 activation has been elucidated in spermatogonial stem cells.<sup>45</sup> With respect to SALL4's effects on growth, recent studies revealed that Cyclin D1 has been shown to bind to SALL4 and works



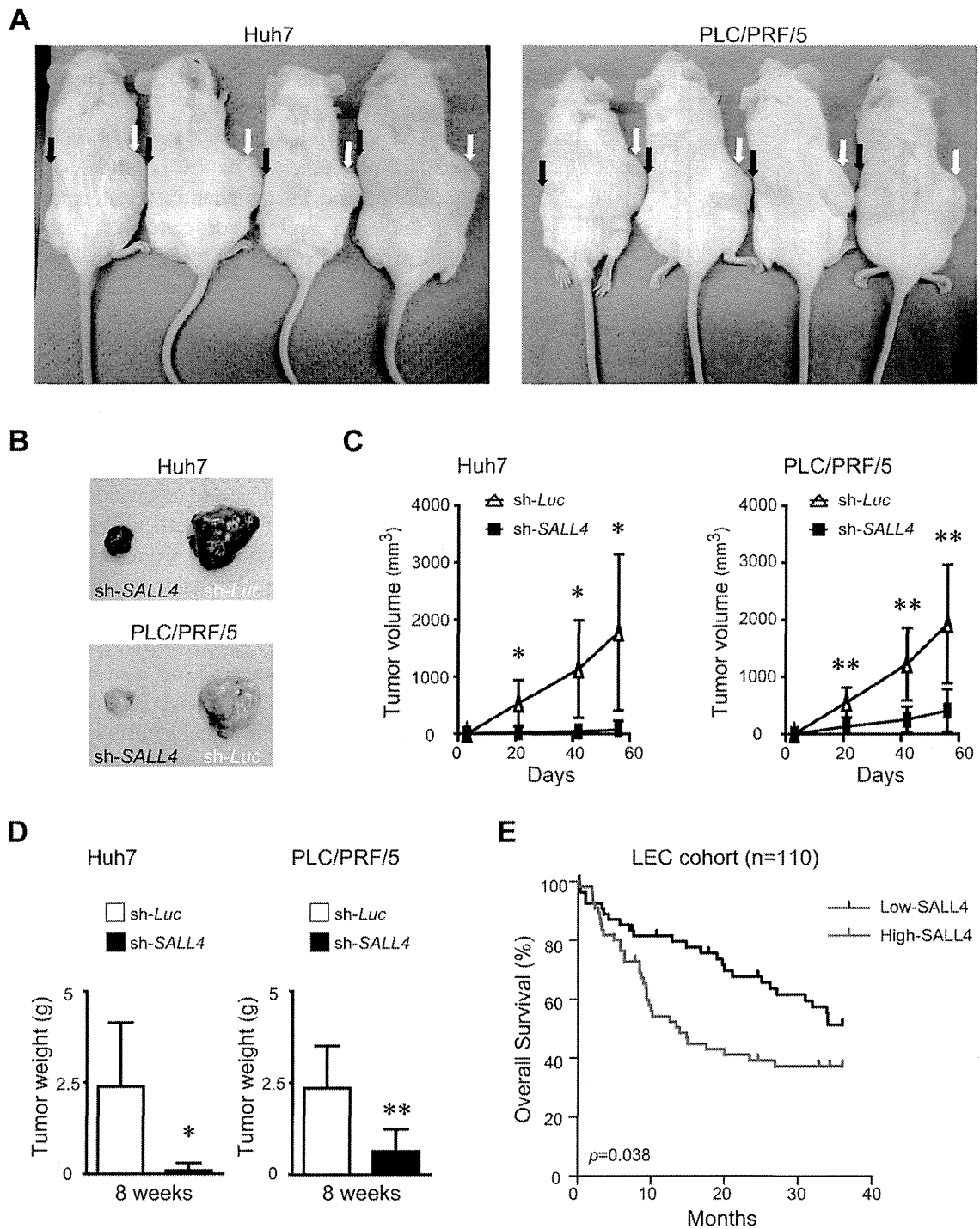


Fig. 8. Effect of SALL4 knockdown on xenograft tumor growth *in vivo*. (A) Control cells and SALL4-knockdown cells were implanted into recipient mice, respectively. White arrows show tumors derived from control cells and black arrows show tumors derived from SALL4-knockdown cells (Huh7 n = 5, PLC/PRF/5 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  $\pm$  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.<sup>25,31,46</sup> We found overexpressing SALL4

induced a shorter G<sub>1</sub> 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<sub>1</sub>-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,<sup>32</sup> 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 HNF4 $\alpha$  gene transfer into liver cancer cells resulted in more differentiated cells with reduced tumor-initiating ability and enhancement of sensitivity to 5-FU.<sup>42,47</sup> 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<sup>6,48</sup>; 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+).<sup>7,8,40</sup> 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.<sup>27,28</sup> This background makes interpretable published bioinformatics analyses<sup>49</sup> 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.<sup>9,38,50</sup>

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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