TWIST1, a direct transcriptional target of EMT inducers, was up-regulated by SALL4 over-expression. In contrast, another important EMT phenomenon, down-regulation of E-cadherin (encoded by the CDH1 gene) was not observed in SALL4-over-expressing liver cancer cells (**Fig.6A**) and 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 over-expression of SALL4 suppressed hepatocytic differentiation and induced stem cell-like phenotype in liver cancer cells. We thus analyzed whether over-expression 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-over-expression with or without 5-FU. Interestingly, over-expression of SALL4 increased cell proliferation (5-FU/PBS) in liver cancer cells (**Fig.7A-B**). These results suggest that SALL4 expression results in selection of cells that are chemo-resistant.

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 data set published in 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 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 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 which 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 cross-link 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 (**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 G1-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 synergistically in transcriptional repression; Cyclin D1 is a downstream target of SALL4 in malignant cells and in ESCs(25,31,46). We found over-expressing SALL4 induced a shorter G1 phase, and there was a positive correlation between expression of SALL4 and Cyclin D1 and D2 in liver cancer cell lines. This suggest that SALL4 regulates cell proliferation either by selection of early lineage stage cells or by controlling G1-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 observed 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 over-expression 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 since 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 via 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.

References

1.Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics,2002.CA Cancer J Clin2005;55:74-108.

2.Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells.Nature2001;414:105-111.

3.Lobo NA, Shimono Y, Qian D, Clarke MF. The biology of cancer stem cells.Annu Rev Cell Dev Biol2007;23:675-699.

4.Chiba T, Kita K, Zheng YW, Yokosuka O, Saisho H, Iwama A, Nakauchi H,et al. Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties.Hepatology2006;44:240-251.

5.Zen Y, Fujii T, Yoshikawa S, Takamura H, Tani T, Ohta T, Nakanuma Y. Histological and culture studies with respect to ABCG2 expression support the existence of a cancer

cell hierarchy in human hepatocellular carcinoma.Am J Pathol2007;170:1750-1762.

6.Schmelzer E, Zhang L, Bruce A, Wauthier E, Ludlow J, Yao HL, Moss N,et al. Human hepatic stem cells from fetal and postnatal donors.J Exp Med2007;204:1973-1987.

7.Cardinale V, Wang Y, Carpino G, Cui CB, Gatto M, Rossi M, Berloco PB,et al. Multipotent stem/progenitor cells in human biliary tree give rise to hepatocytes, cholangiocytes, and pancreatic islets.Hepatology2011;54:2159-2172.

8.Cardinale V, Wang Y, Carpino G, Mendel G, Alpini G, Gaudio E, Reid LM,et al. The biliary tree:a reservoir of multipotent stem cells.Nat Rev Gastroenterol Hepatol2012;9:231-240.

9.Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY, Jia H,et al. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features.Gastroenterology2009;136:1012-1024.

10.Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, Zheng BJ,et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology2007;132:2542-2556.

11.Yang ZF, Ho DW, Ng MN, Lau CK, Yu WC, Ngai P, Chu PW,et al. Significance of CD90+ cancer stem cells in human liver cancer.Cancer Cell2008;13:153-166.

12.Haraguchi N, Ishii H, Mimori K, Tanaka F, Ohkuma M, Kim HM, Akita H,et al. CD13 is a therapeutic target in human liver cancer stem cells.J Clin Invest2010;120:3326-3339.

13.Wang Y, Yao HL, Cui CB, Wauthier E, Barbier C, Costello MJ, Moss N,et al. Paracrine signals from mesenchymal cell populations govern the expansion and differentiation of human hepatic stem cells to adult liver fates. Hepatology2010;52:1443-1454.

14.Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular

carcinoma.Nat Genet2002;31:339-346.

15.Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment.Nat Rev Cancer2006;6:674-687.

16.Yamashita T, Budhu A, Forgues M, Wang XW. Activation of hepatic stem cell marker EpCAM by Wnt-beta-catenin signaling in hepatocellular carcinoma. Cancer Res2007;67:10831-10839.

17.Llovet JM, Bruix J. Molecular targeted therapies in hepatocellular carcinoma. Hepatology2008;48:1312-1327.

18.Mishra L, Banker T, Murray J, Byers S, Thenappan A, He AR, Shetty K,et al. Liver stem cells and hepatocellular carcinoma.Hepatology2009;49:318-329.

19.Marquardt JU, Factor VM, Thorgeirsson SS. Epigenetic regulation of cancer stem cells in liver cancer: current concepts and clinical implications. J Hepatol2010;53:568-577.

20.Nault JC, Zucman-Rossi J. Genetics of hepatobiliary carcinogenesis.Semin Liver Dis2011;31:173-187.

21.Sakaki-Yumoto M, Kobayashi C, Sato A, Fujimura S, Matsumoto Y, Takasato M, Kodama T,et al. The murine homolog of SALL4, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with Sall1 in anorectal, heart, brain and kidney development.Development2006;133:3005-3013.

22.Zhang J, Tam WL, Tong GQ, Wu Q, Chan HY, Soh BS, Lou Y,et al. Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1.Nat Cell Biol2006;8:1114-1123.

23.Elling U, Klasen C, Eisenberger T, Anlag K, Treier M. Murine inner cell mass-derived lineages depend on Sall4 function.Proc Natl Acad Sci USA2006;103:16319-16324.

24.Lim CY, Tam WL, Zhang J, Ang HS, Jia H, Lipovich L, Ng HH,et al. Sall4 regulates distinct transcription circuitries in different blastocyst-derived stem cell lineages.Cell Stem Cell2008;3:543-554.

25.Yuri S, Fujimura S, Nimura K, Takeda N, Toyooka Y, Fujimura Y, Aburatani H,et al. Sall4 is essential for stabilization, but not for pluripotency, of embryonic stem cells by repressing aberrant trophectoderm gene expression.Stem Cells2009;27:796-805.

26.Yang J, Gao C, Chai L, Ma Y. A novel SALL4/OCT4 transcriptional feedback network for pluripotency of embryonic stem cells.PLoS One2010;5:e10766.

27.Wong CC, Gaspar-Maia A, Ramalho-Santos M, Reijo Pera RA. High-efficiency stem cell fusion-mediated assay reveals Sall4 as an enhancer of reprogramming.PLoS One2008;3:e1955.

28.Tsubooka N, Ichisaka T, Okita K, Takahashi K, Nakagawa M, Yamanaka S. Roles of Sall4 in the generation of pluripotent stem cells from blastocysts and fibroblasts.Genes Cells2009;14:683-694.

29.Kohlhase J, Heinrich M, Schubert L, Liebers M, Kispert A, Laccone F, Turnpenny P, et al. Okihiro syndrome is caused by SALL4 mutations. Hum Mol Genet2002;11:2979-2987.

30.Aguila JR, Liao W, Yang J, Avila C, Hagag N, Senzel L, Ma Y. SALL4 is a robust stimulator for the expansion of hematopoietic stem cells.Blood2011;118:576-585.

31.Ma Y, Cui W, Yang J, Qu J, Di C, Amin HM, Lai R, et al. SALL4, a novel oncogene, is constitutively expressed in human acute myeloid leukemia (AML) and induces AML in transgenic mice.Blood2006;108:2726-2735.

32.Yang J, Chai L, Gao C, Fowles TC, Alipio Z, Dang H, Xu D,et al. SALL4 is a key regulator of survival and apoptosis in human leukemic cells.Blood2008;112:805-813.

33.Jeong HW, Cui W, Yang Y, Lu J, He J, Li A, Song D,et al. SALL4, a stem cell factor, affects the side population by regulation of the ATP-binding cassette drug transport genes.PLoS One2011;6:e18372.

34.Cao D, Humphrey PA, Allan RW. SALL4 is a novel sensitive and specific marker for metastatic germ cell tumors, with particular utility in detection of metastatic yolk sac tumors.Cancer2009;115:2640-2651.

35.Bard JD, Gelebart P, Amin HM, Young LC, Ma Y, Lai R. Signal transducer and activator of transcription3 is a transcriptional factor regulating the gene expression of SALL4.Faseb J2009;23:1405-1414.

36.Ushiku T, Shinozaki A, Shibahara J, Iwasaki Y, Tateishi Y, Funata N, Fukayama M. SALL4 represents fetal gut differentiation of gastric cancer, and is diagnostically useful in distinguishing hepatoid gastric carcinoma from hepatocellular carcinoma.Am J Surg Pathol2010;34:533-540.

37.Ikeda H, Sato Y, Yoneda N, Harada K, Sasaki M, Kitamura S, Sudo Y,et al. alpha-Fetoprotein-producing gastric carcinoma and combined hepatocellular and cholangiocarcinoma show similar morphology but different histogenesis with respect to SALL4 expression.Hum Pathol2012;in press.

38.Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A,et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat Med2006;12:410-416.

39.Oikawa T, Kamiya A, Kakinuma S, Zeniya M, Nishinakamura R, Tajiri H, Nakauchi H. Sall4 regulates cell fate decision in fetal hepatic stem/progenitor cells. Gastroenterology2009;136:1000-1011.

40.Wang Y LG, Carpino G, Cui C, Dominguez-Bendala J, Wauthier E, Cardinale V,et al.

Biliary Tree Stem Cells, Precursors to Pancreatic Committed Progenitors:Evidence for Possible Life-long Pancreatic Organogenesis.Stem Cell2012;in press.

41.Kamiya A, Kinoshita T, Ito Y, Matsui T, Morikawa Y, Senba E, Nakashima K,et al. Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer.Embo J1999;18:2127-2136.

42.Yamashita T, Honda M, Nio K, Nakamoto Y, Yamashita T, Takamura H, Tani T,et al. Oncostatin m renders epithelial cell adhesion molecule-positive liver cancer stem cells sensitive to 5-Fluorouracil by inducing hepatocytic differentiation.Cancer Res2010;70:4687-4697.

43.Shi SR, Imam SA, Young L, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies.J Histochem Cytochem1995;43:193-201.

44.Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer.Nat Rev Cancer2011;11:558-572.

45.Lee J, Kanatsu-Shinohara M, Morimoto H, Kazuki Y, Takashima S, Oshimura M, Toyokuni S,et al. Genetic reconstruction of mouse spermatogonial stem cell self-renewal in vitro by Ras-cyclin D2 activation.Cell Stem Cell2009;5:76-86.

46.Bohm J, Kaiser FJ, Borozdin W, Depping R, Kohlhase J. Synergistic cooperation of Sall4 and Cyclin D1 in transcriptional repression.Biochem Biophys Res Commun2007;356:773-779.

47.Yin C, Lin Y, Zhang X, Chen YX, Zeng X, Yue HY, Hou JL,et al. Differentiation therapy of hepatocellular carcinoma in mice with recombinant adenovirus carrying hepatocyte nuclear factor-4alpha gene.Hepatology2008;48:1528-1539.

48.Zhang L, Theise N, Chua M, Reid LM. The stem cell niche of human livers: symmetry

between development and regeneration.Hepatology2008;48:1598-1607.

49.Yamashita T, Forgues M, Wang W, Kim JW, Ye Q, Jia H, Budhu A,et al. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma.Cancer Res2008;68:1451-1461.

50.Yang XR, Xu Y, Yu B, Zhou J, Qiu SJ, Shi GM, Zhang BH,et al. High expression levels of putative hepatic stem/progenitor cell biomarkers related to tumour angiogenesis and poor prognosis of hepatocellular carcinoma.Gut2010;59:953-962.

Figure Legends

Figure 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.

Figure 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.

Figure 3. SALL4 expression and the effects of SALL4 over-expression 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-over-expressing 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-over-expressing retroviral vector or a SALL4-knockdown lentiviral vector were cultured for 7 days. Data are expressed as mean ± SD (triplicate samples,$^{***}p<0.001$,$^{**}p<0.01$).

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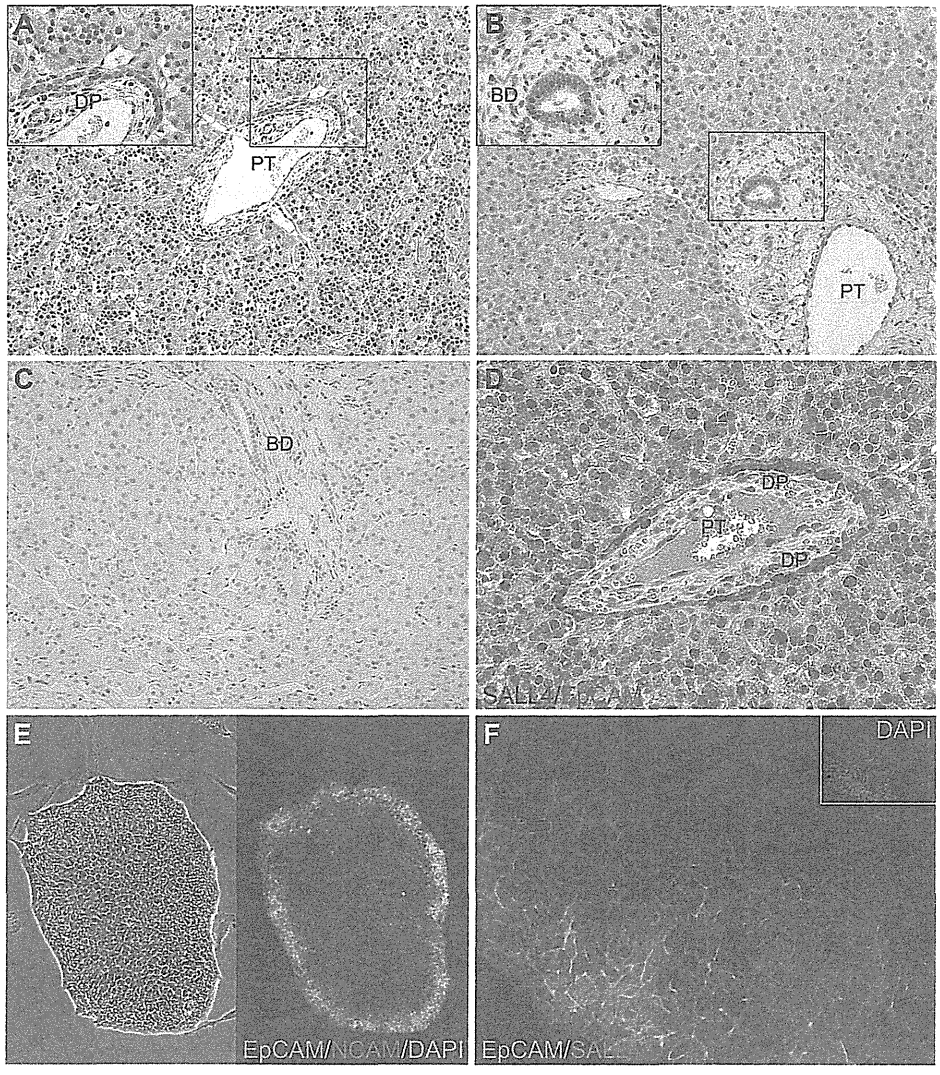
Figure 5. Expression of hepatocytic differentiation (A) and stemness (B) genes in SALL4-over-expressing or SALL4-knockdown liver cancer cells. Cells transduced by a retroviral or lentiviral vector were cultured for 3 days. ALB, TTR, CK19 and ABCG2 mRNA expression was detected using qRT-PCR. Data are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

Figure 6. Expression of EMT-related genes and migration assays in SALL4-over-expressing liver cancer cells. (A) Cells transduced by an over-expressing 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-over-expressing liver cancer cells.

Figure 7. Chemo-resistance assays for SALL4-over-expressing liver cancer cells. Cells were transduced by a retroviral vector. Non-transduced (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).

Figure 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.



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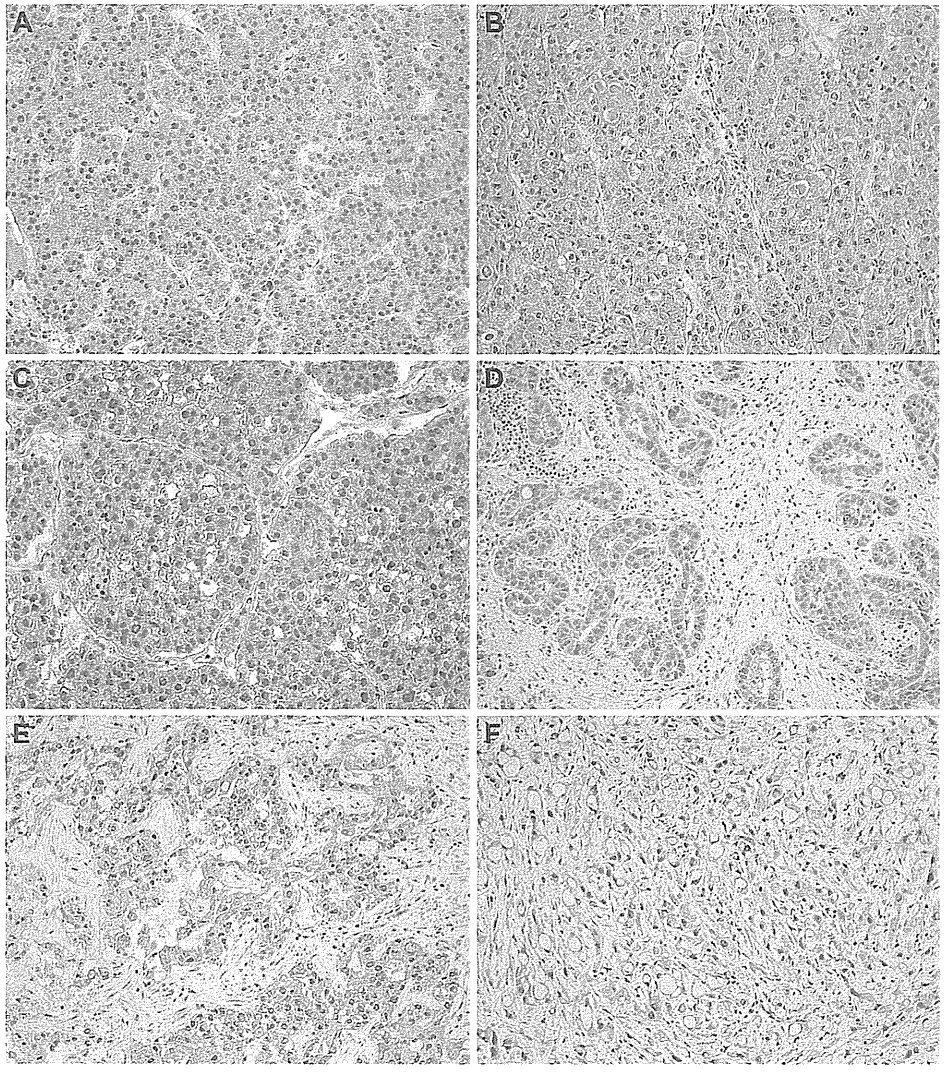

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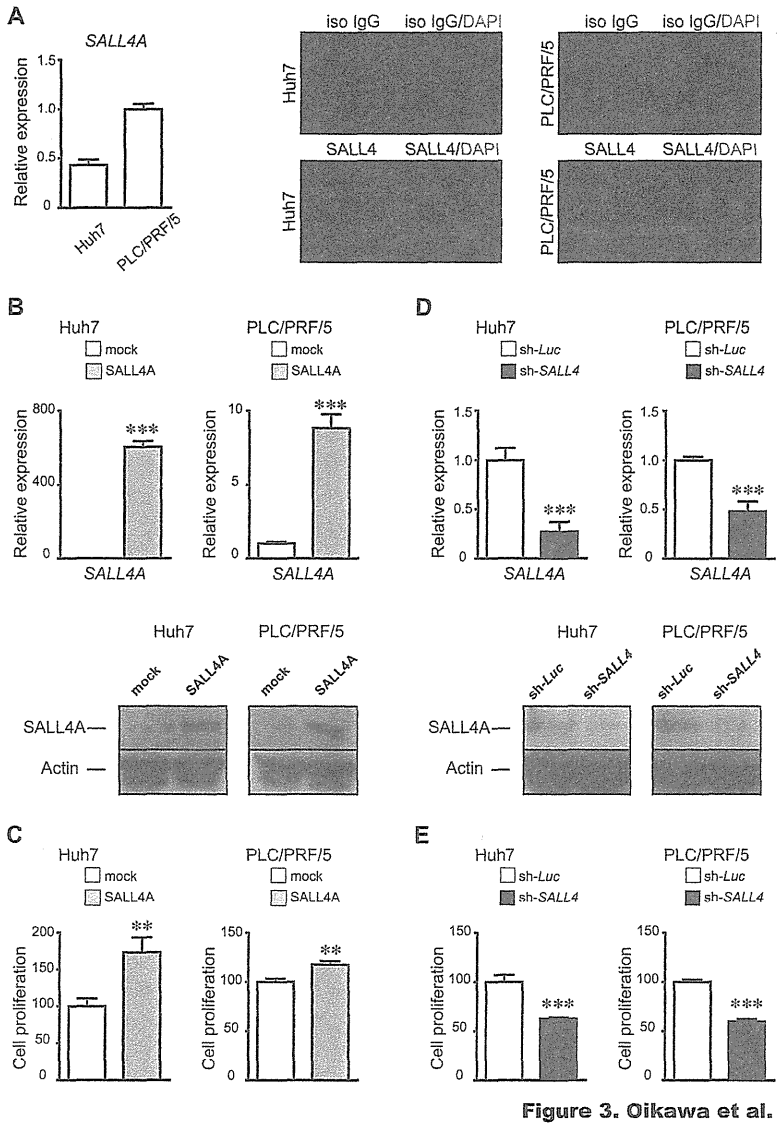

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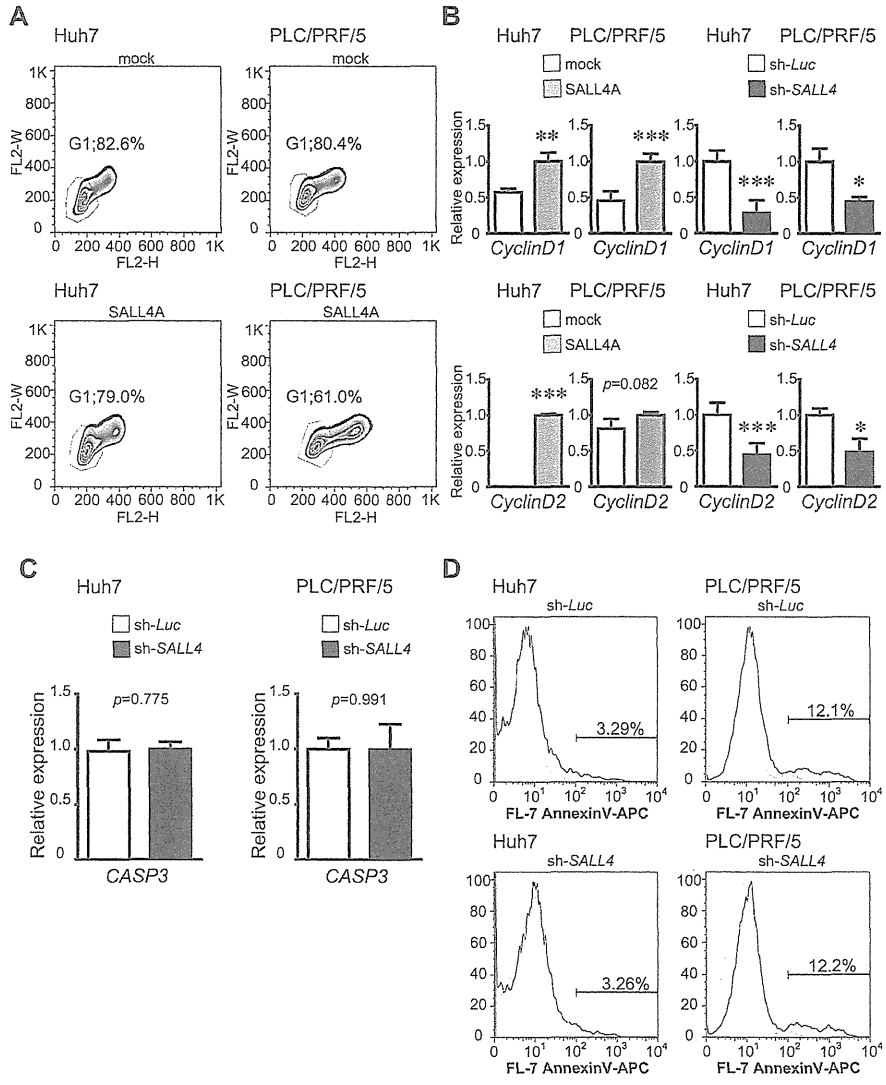

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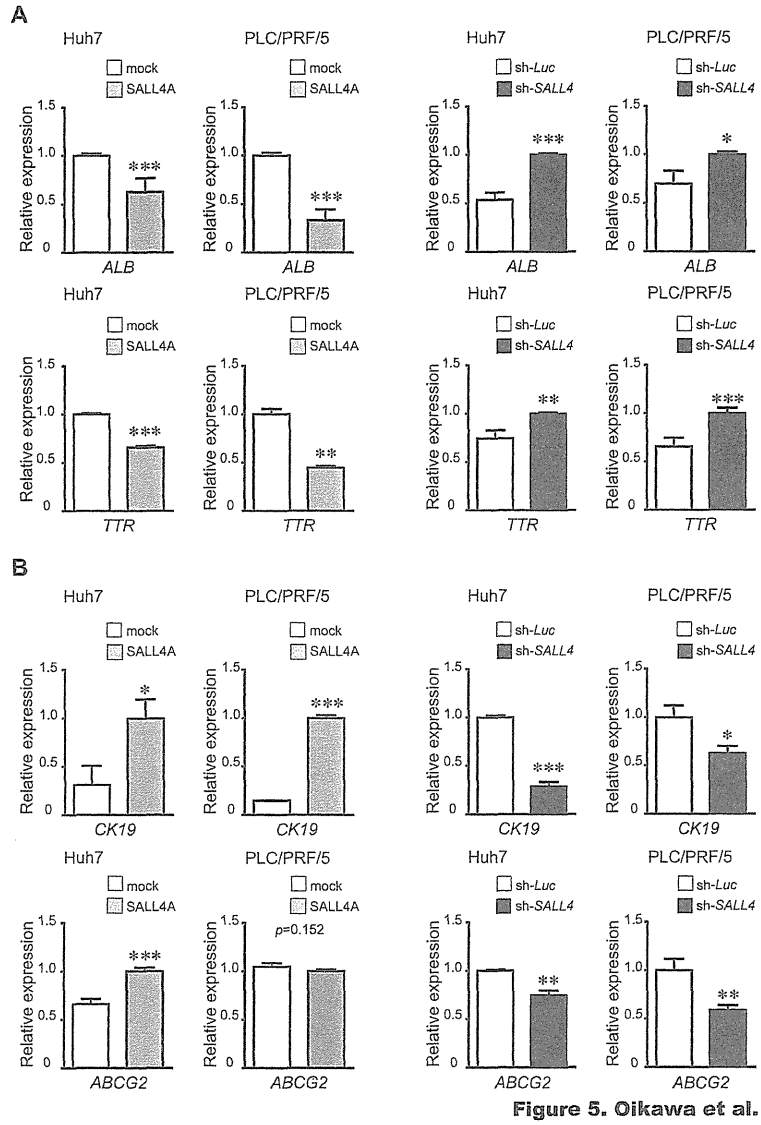

Figure 5. Oikawa et al.

Figure 5. Expression of hepatocytic differentiation (A) and stemness (B) genes in SALL4-over-expressing or SALL4-knockdown liver cancer cells. Cells transduced by a retroviral or lentiviral 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).
157x236mm (300 x 300 DPI)