Oncogene (2015) **34,** 3188–3198

www.nature.com/onc

ORIGINAL ARTICLE

Lysine-specific demethylase 1 promotes the stemness and chemoresistance of Lgr5$^+$ liver cancer initiating cells by suppressing negative regulators of β-catenin signaling

This article has been corrected since Advance Online Publication and a corrigendum is also printed in this issue

Z-J Lei[1,7], J Wang[1,7], H-L Xiao[2], Y Guo[1], T Wang[1], Q Li[1], L Liu[1], X Luo[1], L-L Fan[1], L Lin[2], C-Y Mao[2], S-N Wang[3], Y-L Wei[1], C-H Lan[1], J Jiang[4], X-J Yang[5], P-D Liu[6], D-F Chen[1] and B Wang[1]

Cancer initiating cells (CICs) are responsible for the unrestrained cell growth and chemoresistance of malignant tumors. Histone demethylation has been shown to be crucial for self-renewal/differentiation of stem cells, but it remains elusive whether lysine-specific demethylase 1 (LSD1) regulates the stemness properties of CICs. Here we report that the abundant expression of leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) is associated with the progression of hepatocellular carcinoma (HCC). Lgr5$^+$ HCC cells behave similarly to CICs and are highly tumorigenic and resistant to chemotherapeutic agents. Importantly, Lgr5$^+$ cells express higher levels of LSD1, which in turn regulates Lgr5 expression and promotes the self-renewal and drug resistance of Lgr5$^+$ CICs. Mechanistically, LSD1 promotes β-catenin activation by inhibiting the expression of several suppressors of β-catenin signaling, especially Prickle1 and APC in Lgr5$^+$ CICs, by directly regulating the levels of mono- and di-methylation of histone H3 lysine-4 at the promoters of these genes. Furthermore, LSD1-associated activation of the β-catenin signaling is essential for maintaining the activity of Lgr5$^+$ CICs. Together, our findings unravel the LSD1/Prickle1/APC/β-catenin signaling axis as a novel molecular circuit regulating the stemness and chemoresistance of hepatic Lgr5$^+$ CICs and provide potential targets to improve chemotherapeutic efficacies against HCC.

Oncogene (2015) **34,** 3188–3198; doi:10.1038/onc.2015.129; published online 20 April 2015

INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most common cancers worldwide, is the third leading cause of cancer-associated death in China. Despite multimodal therapy, such as surgical resection combined with chemotherapy with Sorafenib or transarterial chemo-embolization available, the prognosis of patients with HCC is generally dismal.[1] The low effectiveness of current therapies for HCC may stem from the phenotypic and functional diversities of tumor cells. Notably, cancer initiating cells (CICs), or cancer stem cells, with a potent capacity to promote self-renewal and differentiation, may have crucial roles in tumor initiation, progression and refractoriness to therapies.[1–4] Hence, CICs have been thought to be critical targets for cancer therapy.

The Wnt/β-catenin signaling is a potent regulator for the self-renewal of adult liver stem cells[5–6] and CICs.[1,7] Leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5), a target of the Wnt/β-catenin signaling, is expressed by putative stem cells of the intestinal epithelia[8–10] and is associated with murine intestinal adenomas and colorectal cancers.[11–13] Recent studies indicated that Lgr5 regulates the epithelial phenotype and proliferation of HCC.[14] However, it is unclear whether Lgr5 is expressed in hepatic CICs, and how its expression is regulated and is associated with the development of HCC in humans.

Histone methylation is regulated by histone methylases and demethylases, and has crucial roles in gene transcription. Its dysregulation has been implicated in cancer development.[15] Lysine-specific demethylase 1 (LSD1; or KDM1a, AOF2) demethylates mono- and di-methylated residues of lysine-4 on histone H3 (H3K4me1 or H3K4me2) and is typically associated with transcriptional repression.[16–17] On the other hand, when interacting with androgen receptor or estrogen receptor, LSD1 can also demethylate lysine-9 on histone H3 to promote transcription activation.[18] As a result, LSD1 is important for maintaining the pluripotency of embryonic stem cells[19] and embryonic teratocarcinoma cells,[20] and the tumorigenicity of MLL-AF9 leukemia stem cells.[21] Moreover, LSD1 overexpression is associated with malignancy of several types of cancers, including HCC.[22–24] However, it remains

[1]Department of Gastroenterology, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing, China; [2]Department of Pathology, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing, China; [3]Department of Radiology, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing, China; [4]Department of Urology, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing, China; [5]Center for Neuroscience, Shantou University Medical College, Shantou, China and [6]Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA. Correspondence: D-f Chen or Dr B Wang, Department of Gastroenterology, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Yuzhong District, Chongqing 400042, China.
E-mail: chendongfeng1981@126.com or wb_tmmu@126.com
[7]These authors contributed equally to this work.
Received 12 June 2014; revised 19 January 2015; accepted 20 March 2015; published online 20 April 2015

unknown whether LSD1 regulates Lgr5 expression and thus the self-renewal property of hepatic CICs.

In this study, we chose HCC as a model to examine the association of Lgr5 expression with stem-like properties of cancer cells and tumor progression. Moreover, we analyzed the potential role of LSD1 in regulating the stemness of $Lgr5^+$ hepatic CICs and potential underlying molecular mechanisms. Our findings reveal a novel mechanism by which depletion of LSD1 reactivates multiple suppressors of the Wnt/β-catenin signaling, including Prickle1, APC and Sfrp5, to inhibit the Wnt/β-catenin activity and the stemness and chemoresistance of $Lgr5^+$ CICs.

RESULTS

The abundant expression of Lgr5 is associated with the progression and poor outcome of HCC

We examined the Lgr5 expression levels in 12 HCC specimens and found that the relative levels of Lgr5 messenger RNA transcripts were significantly higher in HCC tissues compared with adjacent non-tumor tissues (Figure 1a). Using a phycoerythrin-conjugated anti-Lgr5 antibody, which readily recognizes exogenously expressed Lgr5 in L02 cells that contain barely detectable levels of Lgr5 proteins (Supplementary Figure S1a), a subset of $Lgr5^+$ cells was identified in several hepatocytes by flow cytometry analysis (Supplementary Figure S1b). Moreover, we validated that the anti-Lgr5 antibody used specifically recognized endogenous Lgr5 in PLC $Lgr5^+$ cells (Supplementary Figure S1c) and revealed the existence of $Lgr5^+$ tumor cells in primary HCC tissues (Supplementary Figure S1d). Further quantitative analysis indicated that the percentages of $Lgr5^+$ cells in HCC lines were significantly higher than that in non-tumor hepatic L-O2 cells (Figure 1b), suggesting that Lgr5 expression may be associated with tumorigenicity in HCC.

Using this Lgr5 antibody for immunohistochemistry analysis (validated in Supplementary Figure S1e), we observed that varying levels of Lgr5 were expressed predominantly on the membrane or in the cytoplasm in 188 HCC specimens (black arrow, Figure 1c). Semiquantitative analysis indicated that 77 (41%) cases of HCC were positive for the anti-Lgr5 staining ($Lgr5^+$ cells $>1\%$) and they had a higher frequency of $Lgr5^+$ cells in the tumor regions than adjacent areas (Figure 1d). These data identify an expanded pool of $Lgr5^+$ tumor cells in HCC.

Further stratification analysis revealed that the Lgr5 expression in HCC was associated significantly with multiple lesions, lymph node invasion, higher histological grades (III–IV) and metastasis, but not other clinical measures (Supplementary Table S1–11). Interestingly, Lgr5 expression was associated significantly with shorter periods of survival in patients with HCC (the median 19.1 ± 1.3 vs 34.1 ± 1.8 months of $Lgr5^-$ patients, Figure 1e). Furthermore, univariate analysis showed that high levels of Lgr5, poor differentiation, multiple lesions, microvascular invasion, lymph node invasion and higher histological grades were associated with the shorter survival of HCC patients (Supplementary Table S2). Notably, multivariate analysis indicated that high levels of Lgr5, along with microvascular invasion, lymph node invasion and higher histological grades, were independent risk predictors for the poor outcome of HCC (Supplementary Table S2). Together, these findings indicate that Lgr5 expression is associated with the HCC progression in this population, suggesting that the existence of $Lgr5^+$ HCC cells may be crucial for the poor outcome of HCC patients.

$Lgr5^+$ HCC cells exhibit CIC properties and are chemoresistant

To examine the properties of $Lgr5^+$ HCC cells, we isolated $Lgr5^+$ and $Lgr5^-$ cells from PLC cells by fluorescence-activated cell sorting (Figure 2a). Higher levels of Lgr5, Sox9, Nanog, CD90 and CD24, but lower levels of hepatocyte nuclear factor-4 (Hnf-4) and albumin (Alb) were observed in $Lgr5^+$, but not $Lgr5^-$ HCC cells (Figure 2b, Supplementary Figure S1c and S2a). Under the experimental conditions that induce CIC differentiation, PLC $Lgr5^+$ cells significantly lost the $Lgr5^+$ phenotype on day 10 post induction (Figure 2c). Conversely, the differentiated cells down-regulated Lgr5, Sox9 and Nanog expression, but upregulated the Alb and Hnf-4 expressions, which is indicative of mature hepatocytic differentiation *in vitro* (Figure 2d).

Analysis of tumorsphere formation, a hallmark of CICs,[25–30] indicated that $Lgr5^+$ HCC cells demonstrated a significantly stronger capacity to form tumorspheres than $Lgr5^-$ HCC cells *in vitro* (Figure 2e). As *in vitro* and *in vivo* serial passage/transplantation extreme limiting dilation assays are the golden standard for assessing self-renewal of a bona fide CIC,[25,31] to this end, we observed that $Lgr5^+$, but not $Lgr5^-$ HCC cells maintained the capacity to form tumorspheres (Figure 2f, Supplementary Figure S2b, Supplementary Table S3 and 4) and displayed potent tumorigenicity upon serial passages *in vitro* and *in vivo* (Figure 2g). Evidently, inoculation with 10^3 $Lgr5^+$ cells effectively induced solid tumors in majority of recipients and $Lgr5^+$ cells had a 42-fold higher frequency of CICs (Supplementary Table S5). Moreover, $Lgr5^+$ cells were more resistant to treatments with cisplatin or sorafenib (Figure 2h and Supplementary Figure S2c), although $Lgr5^+$ cells were more proliferative (Supplementary Figure S2d) and both $Lgr5^+$ and $Lgr5^-$ cells displaced comparable levels of spontaneous apoptosis (Supplementary Figure S2e). Collectively, these data clearly indicate that $Lgr5^+$ HCC cells are likely to be CICs with potent drug resistance.

LSD1 expression is positively associated with Lgr5 expression and poor survival in HCC patients

To examine the physiological role of LSD1 in the biology of HCC CICs, we first observed variable levels of LSD1 expression in different HCC cell lines (Supplementary Figure S3a). Immunohistochemistry analysis indicated that the levels of LSD1 expression in HCC tissues were positively associated with the HCC histological grades, lymph node invasion and Lgr5 expression, but negatively associated with the survival of HCC patients (Supplementary Figure S3b and c, Supplementary Table S1). Both univariate and multivariate analyses indicated that LSD1 or Lgr5 expression, lymph node invasion, microvascular invasion, a higher TNM stage were independent prognostic markers for the overall survival of HCC patients (Supplementary Table S2).

Induction of LSD1 overexpression expands the pool of $Lgr5^+$ cells, and enhances CIC properties and drug resistance in HCC cells

Given the positive association between LSD1 and Lgr5 in HCC tissues (Supplementary Table S1) and a panel of HCC cell lines (Figure 3a, Supplementary Figure S3d and e), we next examined the impact of LSD1 overexpression on the activity of HCC CICs. To this end, induced LSD1 expression significantly enhanced the messenger RNA levels of Sox9, Nanog, Lgr5, CD90 and CD24 in Hep3B cells (Lv-LSD1, Figure 3b and c). Fluorescence-activated cell sorting analysis indicated that Lv-LSD1 Hep3B cells contained a significantly larger population of $Lgr5^+$ cells (Figure 3d), possibly stemmed from the expansion of previously existing $Lgr5^+$ HCC CICs.

Consistent with a positive role of LSD1 in regulating stemess, Lv-LSD1 cells demonstrated a stronger capacity to form bigger tumorspheres (Figure 3e). Furthermore, expression of LSD1 in either Hep3B or PLC $Lgr5^-$ cells led to a higher frequency of sphere-initiating cells even after re-plating (Figure 3f, Supplementary Figure S3f, Supplementary Table S6 and 7). More importantly, compared with control cells, Lv-LSD1 cells contain a 11-fold higher abundance of CICs (Figure 3g, Supplementary Table S8) and exhibited a stronger potency to induce the formation of solid tumors in mice (Figure 3h, Supplementary

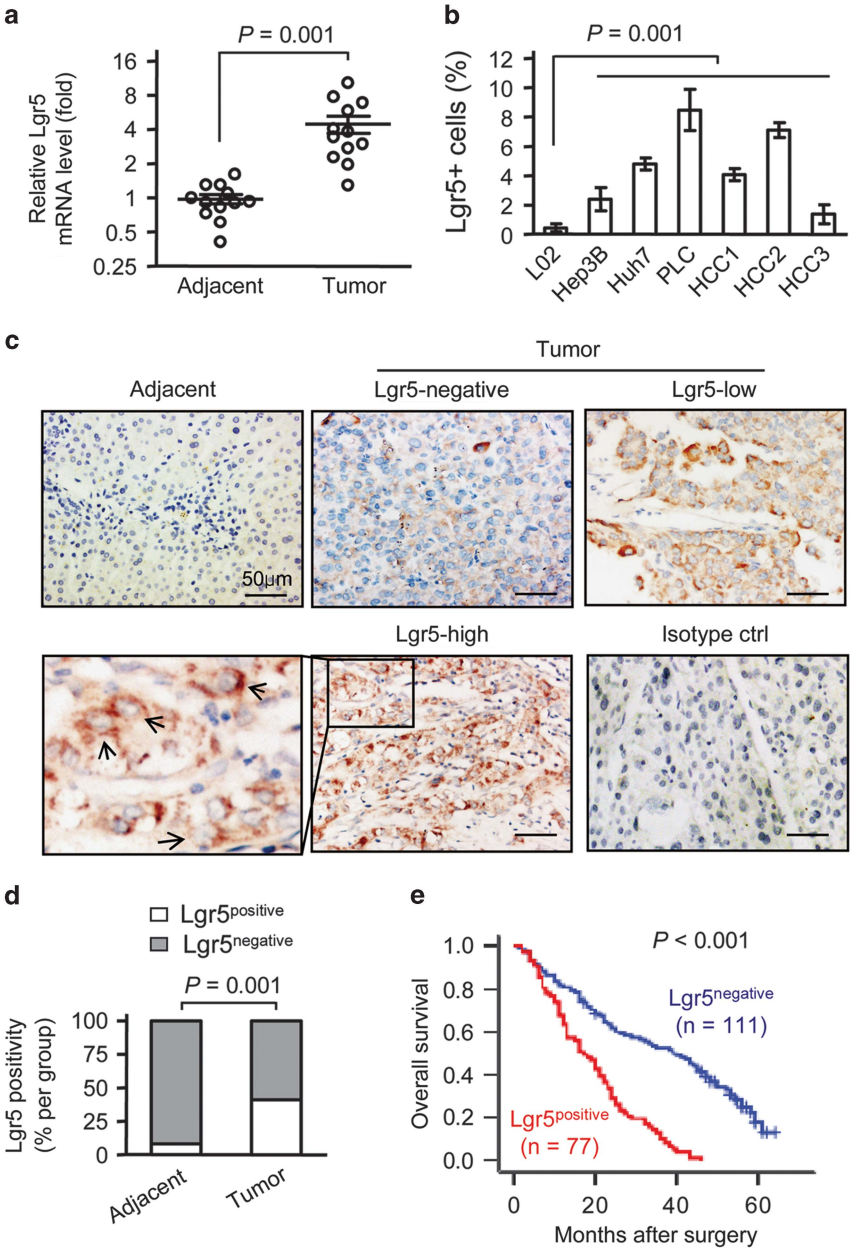

Figure 1. High Lgr5 expression levels are positively associated with poor prognosis of HCC. (**a**) The relative mRNA levels of Lgr5 in 12 HCC and their adjacent non-tumor tissues were determined by RT–PCR. (**b**) Flow cytometric analysis of the frequency of $Lgr5^+$ cells in non-tumor L-O2 cells, HCC cells and freshly isolated HCC cells (HCC1, HCC2 and HCC3). (**c**) Representative images of Lgr5 immunohistochemical staining in 188 HCC tissues as well as adjacent normal liver tissues. Black arrows indicate strong membranous and cytoplasmic Lgr5 staining in tumor cells. The specificity of the Lgr5 antibody was verified by using an isotype-specific antibody for IHC staining on HCC tissues with high levels of Lgr5 expression (Isotope trl) and was further validated in Supplementary figure 1. (**d**) Quantitative data show an expanded pool of $Lgr5^+$ cells in HCC compared with adjacent tissues. (**e**) The overall survival of 188 patients with $Lgr5^+$ or $Lgr5^-$ HCC was analyzed by the log-rank test. Data were presented as the individual means or mean ± s.d. of each group from at least three independent experiments.

Figure S3g). Histological analysis of extracted solid tumors demonstrated that higher levels of LSD1 and Lgr5 were co-expressed in Lv-LSD1-induced tumor tissues (Supplementary Figure S3h). Consistently, Lv-LSD1 Hep3B cells and Lv-LSD1 $Lgr5^-$ cells were more resistant to treatments with cisplatin or sorafenib (Figure 3i and Supplementary Figure S3i), although Lv-LSD1 cells were more proliferative (Supplementary Figure S3j) and Lv-LSD1 cells and controls displayed similar levels of spontaneous apoptosis (Supplementary Figure S3k). These results demonstrate that induction of LSD1 overexpression enhanced the activity of HCC CICs.

Depletion of LSD1 attenuates the self-renewal of $Lgr5^+$ CICs and their drug resistance

To further examine the impact of modulating LSD1 expression on the activity of CICs, $Lgr5^+$ PLC cells were sorted and infected with LSD1-specific or control scramble short hairpin RNA-expressing lenti-viruses to generate LSD1-Kd or Kd-ctrl cells, respectively (Figure 4a). Compared with Kd-ctrl cells, significantly decreased messenger RNA levels of Sox9, Nanog, Lgr5, CD90 and CD24 were observed in LSD1-Kd cells (Figure 4b), whereas the levels of Alb and Hnf-4 were upregulated (Supplementary Figure S4a),

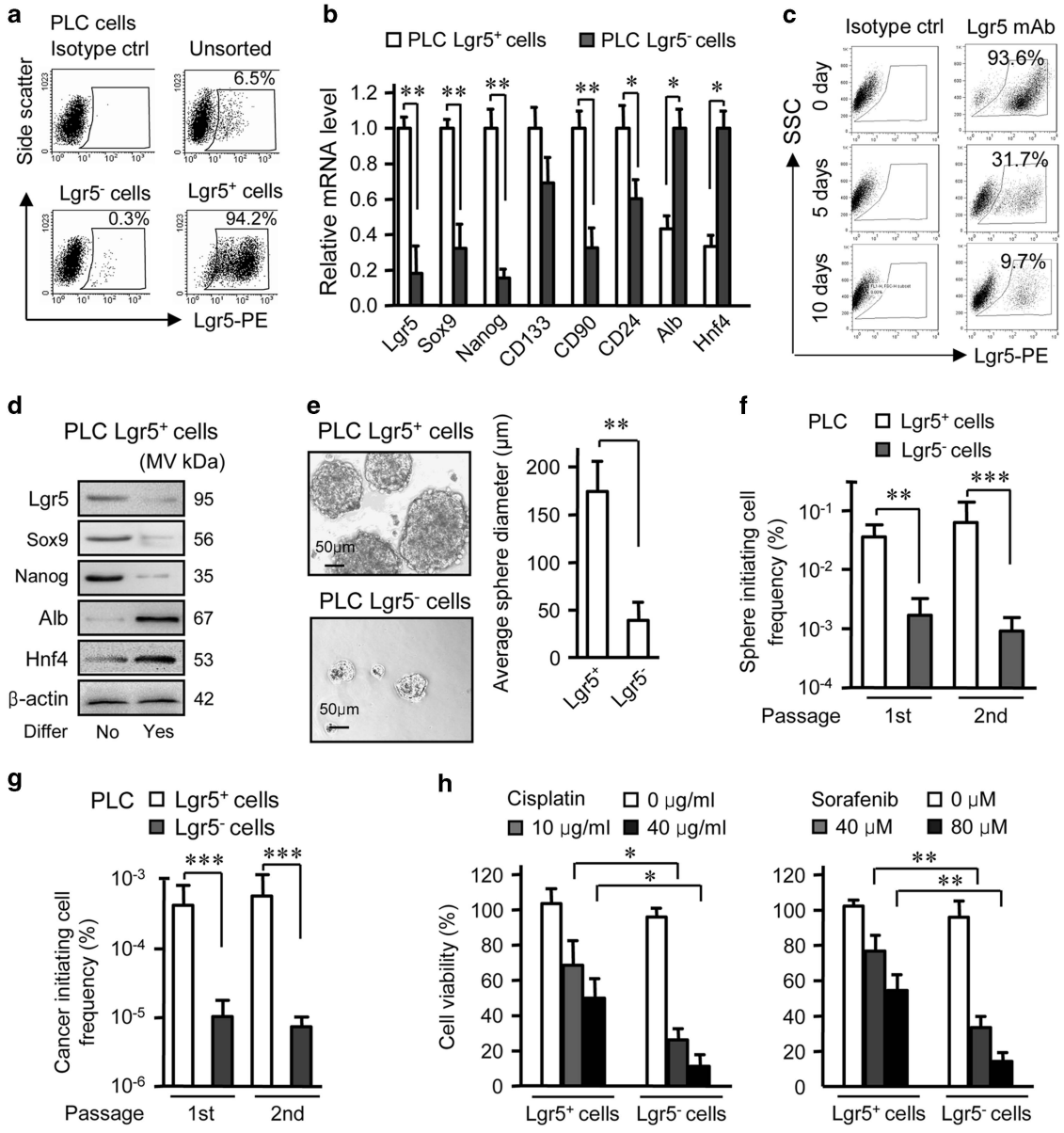

Figure 2. Lgr5$^+$, but not Lgr5$^-$ HCC cells have CIC properties and are resistant to chemotherapeutic agents. (**a**) Representative graphs showing efficient purification of Lgr5$^+$ or Lgr5$^-$ cells from PLC cells by FACS sorting. (**b**) The relative levels of mRNAs for stem cell or differentiation markers in PLC Lgr5$^+$ and Lgr5$^-$ cells were determined by qRT–PCR. (**c**, **d**) PLC Lgr5$^+$ cells were induced for differentiation for 5 or 10 days *in vitro*. A decreased pool of Lgr5$^+$ cells was examined by flow cytometry (**c**). The expression of stem cell and differentiation markers in differentiated and undifferentiated cells were determined by western blotting (**d**). (**e**) Average diameters of spheres generated by PLC Lgr5$^-$ or Lgr5$^+$ cells in stem cell medium. (**f**) Sphere-initiating cell frequencies of PLC Lgr5$^-$ or Lgr5$^+$ cells as analyzed by ELDAs upon serial passages. (**g**) Cancer initiating cell frequencies of PLC Lgr5$^+$ or Lgr5$^-$ cells as calculated by ELDAs in nude mice ($n = 6$ per group). (**h**) Viability of PLC Lgr5$^+$ or Lgr5$^-$ cells incubated with cisplatin or sorafenib for 24 h. Data from triplicates are presented as the mean ± s.d. (**b**, **e**, **h**), or mean and 95% confidence interval (CI, **f**, **g**) of triple replicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

suggesting that LSD1 may be crucial for the maintenance of the stemness properties of Lgr5$^+$ CICs.

Notably, knockdown of LSD1 demolished Lgr5 expression in Lgr5$^+$ CICs (Figure 4c and Supplementary Figure S4b) and reduced the capacity of Lgr5$^+$ CICs to form tumorspheres with much smaller sizes (Figure 4d). Moreover, loss of LSD1 in Lgr5$^+$ CICs in both PLC and Huh7 cell lines significantly impaired their self-renewal capacity *in vitro* (Figure 4e, Supplementary Figure S4c, Supplementary Table S9 and 10). In addition, knockdown of LSD1 significantly reduced the CIC frequency on serial transplantation *in vivo* (Figure 4f and Supplementary Table S11), thus repressed the ability of Lgr5$^+$ CICs to induce solid tumors (Figure 4g) with little Lgr5$^+$ cells *in vivo* (Supplementary Figure S4d). Finally, both PLC and Huh7 LSD1-Kd cells were more sensitive to treatments with cisplatin and sorafenib *in vitro* (Figure 4h and Supplementary Figure S4e), although depletion of LSD1 in Lgr5$^+$ CICs led to reduced percentage of cells that resided in proliferative phases (Supplementary Figure S4f) and did not significantly induce apoptosis (Supplementary Figure S4g). These data clearly demonstrate that depletion of LSD1 attenuated the activity of Lgr5$^+$ CICs and their drug resistance.

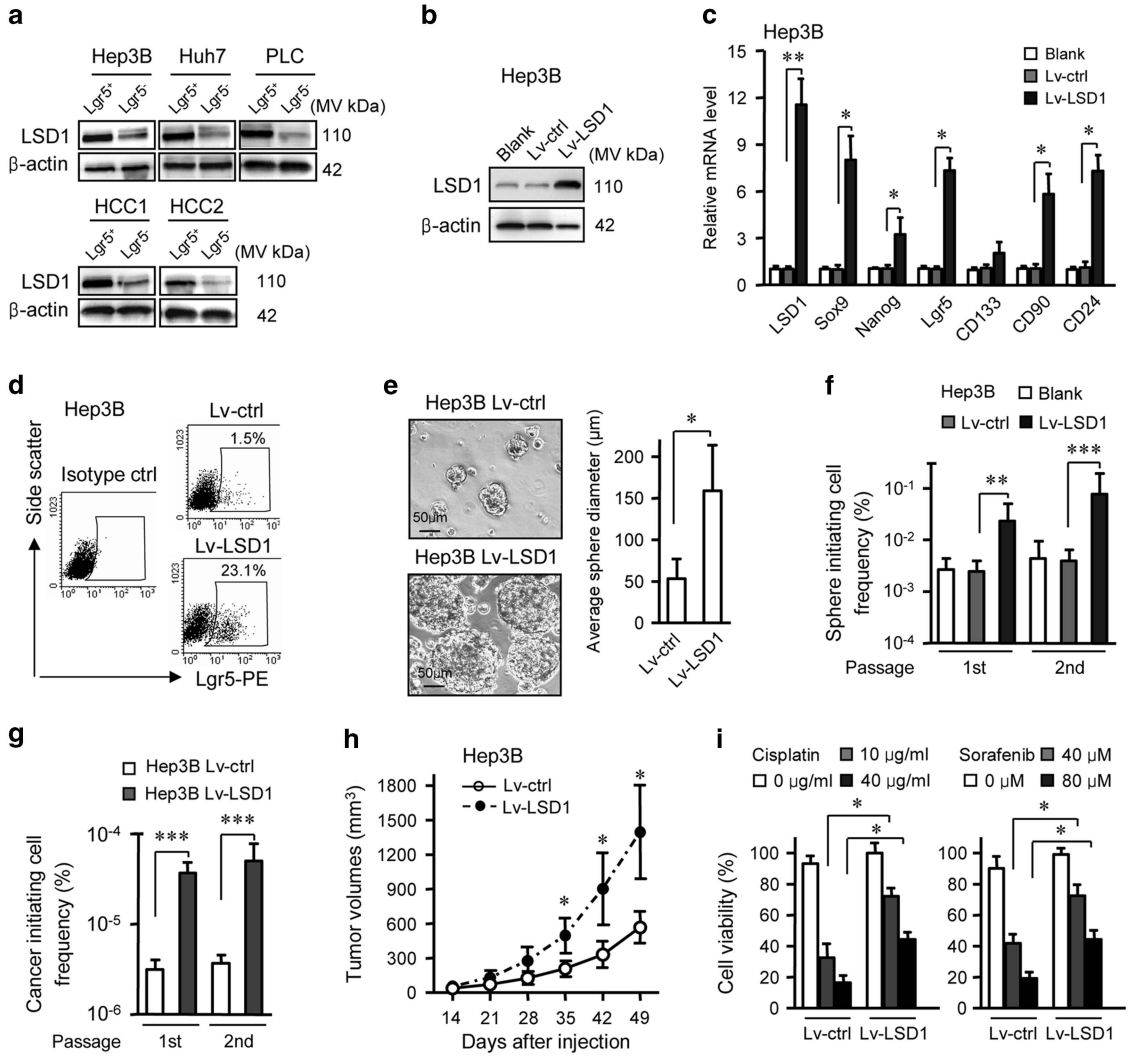

Figure 3. Induction of LSD1 expression enhances Lgr5 expression, cellular stemness and resistance to chemotherapies. (**a**) The levels of LSD1 in Lgr5$^+$ and Lgr5$^-$ HCC cells were determined by western blotting. (**b**) Hep3B cells were infected with indicated lenti-viruses to generate LSD1-overexpressing (Lv-LSD1) or control (Lv-ctrl) cells. (**c**, **d**) The relative levels of mRNA transcripts of LSD1 and indicated stem cell markers (**c**) and Lgr5$^+$ expression (**d**) in Hep3B Lv-LSD1 and Lv-ctrl cells were determined by qRT–PCR and FACS analyses. (**e**) Average diameters of spheres generated using Hep3B cells in stem cell medium. (**f**) Sphere-initiating cell frequencies of serially seeded Hep3B cells was analyzed by ELDAs. (**g**) Cancer initiating cell frequencies of Hep3B cells as calculated by ELDAs *in vivo* ($n = 6$ per group). (**h**) Growth curves of subcutaneous tumors formed by the Hep3B cells in nude mice (5×10^6 cells per injection, $n = 5$). (**i**) The sensitivity of Hep3B cells to cisplatin or sorafenib treatment for 24 h. Representative images (**a**, **b**, **e**) or graphs (**d**) are shown. Data are presented as the mean ± s.d. (**c**, **e**, **h**, **i**) or mean and 95% CI (**f**, **g**) of each group from triple replicate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

LSD1 reduces the H3K4me1/2 methylation at the promoters of several repressors of β-catenin signaling to enhance β-catenin activity in Lgr5$^+$ CICs

To identify potential downstream targets underlying the functions of LSD1, we analyzed the ENCODE Epigenomic Data Base, which contained chromatin immunoprecipitation-sequence data sets of LSD1 and H3K4me1/2 from K562 cells. Genome-wide analysis demonstrated that the promoters of 2644 genes were enriched with LSD1, but not H3K4me1/2 (Supplementary Table S12). The kyoto encyclopedia of genes and genomes pathway analysis revealed that 89 out of 2644 genes were associated with several developmental signaling pathways (Supplementary Table S13), among which the MAPK, Jak/Stat, and Wnt/β-catenin, TGF-β and PI3k/AKT pathways were ranked as the top five pathways where most genes were regulated by LSD1 (Supplementary Figure S5a). Interestingly, we found significantly increased levels of the β-catenin activation, but not the p38, ERK, JNK, Smad, STAT3 and AKT activation, accompanied by increased levels of TOP/FOP activity in Lgr5$^+$ HCC cells, as compared with Lgr5$^-$ cells (Figure 5a and Supplementary Figure S5b and c), suggesting that LSD1 and Lgr5 expressions may be associated with the activation of β-catenin signaling. Indeed, knockdown of LSD1 downregulated, whereas induction of LSD1 upregulated the relative levels of the levels of dephosphorylated, active β-catenin (Figure 5b) and T-cell factor transcriptional activity (Supplementary Figure S5d) in PLC and Hep3B HCC cell lines, respectively. Hence, LSD1 appeared to promote the β-catenin activation in Lgr5$^+$ HCC CICs in part through demetylation of H3K4Me1/2 at the promoters of regulators of β-catenin pathway.

To examine whether LSD1 also governs the transcription of known β-catenin regulators, we further analyzed the expression patterns of genes upstream of β-catenin that were predicted to be potential LSD1 targets (Supplementary Table S13). The relative messenger RNA levels of Prickle1,[32] APC and Sfrp5, three putative antagonists of the β-catenin signaling, were significantly higher in

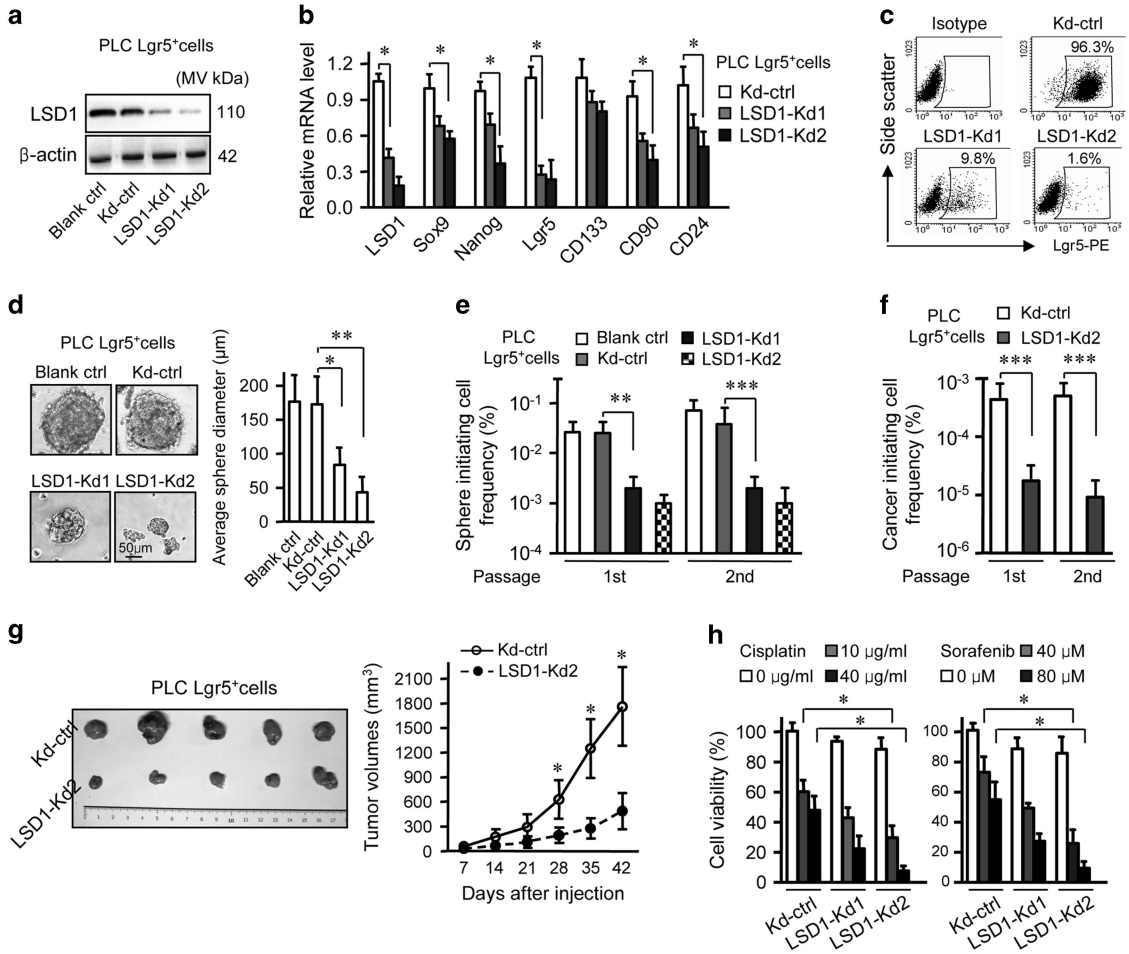

Figure 4. Depletion of LSD1 decreases Lgr5 expression, cellular stemness and resistance to chemotherapies in Lgr5$^+$ CICs. (**a**) The Lgr5$^+$ cells were purified by FACS from PLC cells and transfected with vectors expressing LSD1 shRNAs or scramble shRNA to generate LSD1-kd1, LSD1-kd2, Kd-ctrl PLC cells. LSD1 expression in the manipulated cells and parental cells (Blank ctrl) were verified by western blotting. (**b**) qRT–PCR analysis of the relative mRNA levels of stem cell markers in these PLC Lgr5$^+$ cells. (**c**–**e**) Decreases in the frequency of Lgr5$^+$ population (**c**), the capacity to sustain growth of tumorspheres (**d**), and the ability of sphere regeneration upon serial passages (**e**) were examined in PLC Lgr5$^+$ cells with or without LSD1 knockdown. (**f**) Frequencies of cancer initiating cells in PLC Lgr5$^+$ cells with or without LSD1 knockdown was calculated by ELDAs *in vivo* ($n=6$ per group). (**g**) The growth kinetics of subcutaneous tumors induced by PLC Lgr5$^+$ cells with Kd-ctrl or LSD1-kd2 in nude mice (1×10^6 cells per injection, $n=5$). Pictures shown are representative dissected xenografts (left panel). (**h**) The sensitivity of these PLC Lgr5$^+$ cells to chemotherapeutic treatment for 24 h. Representative images (**a**, **c**, **d**, **g**) or data were presented as the mean ± s.d. (**b**, **d**, **g**, **h**) or mean and 95% CI (**e**, **f**) of each group from triple replicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

LSD1-Kd Lgr5$^+$ cells compared with Kd-ctrl cells (Figure 5c and Supplementary Figure S5e). Moreover, knockdown of LSD1 remarkably increased, whereas induction of LSD1 decreased the levels of H3K4me1, H3K4me2, Prickle1, APC and Sfrp5 (Figure 5d). Indeed, the potential enrichment of LSD1-binding signals was found on the promoters of Prickle1, APC and Sfrp5 genes (Supplementary Table S12). Using Prickle1 as an example, we detected significantly increased levels of H3K4me1 and H3K4me2, but not H3K4me3, in the promoter of Prickle1 gene in LSD1-Kd cells by chromatin immunoprecipitation assays (Figure 5e).

Moreover, induced expression of Prickle1 and/or APC in Lv-LSD1 Hep3B cells inhibited upregulation of Lgr5 expression and β-catenin signaling (Figure 5f), whereas knockdown of Prickle1 and/or APC in LSD1-kd PLC Lgr5$^+$ cells rescued Lgr5 expression and β-catenin activation (Figure 5g). TOP/FOP luciferase reporter assays further confirmed that manipulation of Prickle1 and/or APC expression affected LSD1-assicated β-catenin signaling activity (Supplementary Figure S5f and g). Thus, endogenous LSD1 may enhance the β-catenin activation by inhibiting H3K4me1/2 methylation and the expression of Prickle1, APC and Sfrp5 in hepatic Lgr5$^+$ CICs.

The β-catenin signaling activity is crucial for LSD1 to regulate the tumorsphere formation and chemoresistance in Lgr5$^+$ CICs

We next tested the functional significance of the LSD1-related β-catenin activation in HCC cells. We found that treatment with XAV-939, a tatankyrase inhibitor to stabilize Axin2,[33] not only inhibited tumorsphere growth (Figure 6a), abrogated the self-renewal potential (Figure 6b, Supplementary Figure S6a, Supplementary Table S14 and 15), but also repressed the cellular resistance to cisplatin and sorafenib (Figure 6c and Supplementary Figure S6b) in both Lv-LSD1 Hep3B cells and Lgr5$^-$ PLC cells. In contrast, treatment with CT99021, a selective GSK-3 inhibitor,[34] rescued the cellular capacity to form tumorspheres (Figure 6d), self-renewal (Figure 6e, Supplementary Figure S6c, Supplementary Table S16 and 17) and cellular resistance to cisplatin and sorafenib in both LSD1-Kd Lgr5$^+$ PLC cells and Lgr5$^+$ Huh7 cells (Figure 6f and Supplementary Figure S6d). Collectively these data indicate that the β-catenin signaling is critical for LSD1 to regulate the tumorsphere formation and resistance to the drugs in hepatic Lgr5$^+$ CICs.

Moreover, expression of Prickle1 and/or APC in both Hep3B and Lgr5$^-$ PLC cells efficiently attenuated the cellular self-renewal (Figure 6b, Supplementary Figure S6a, Supplementary Table S14

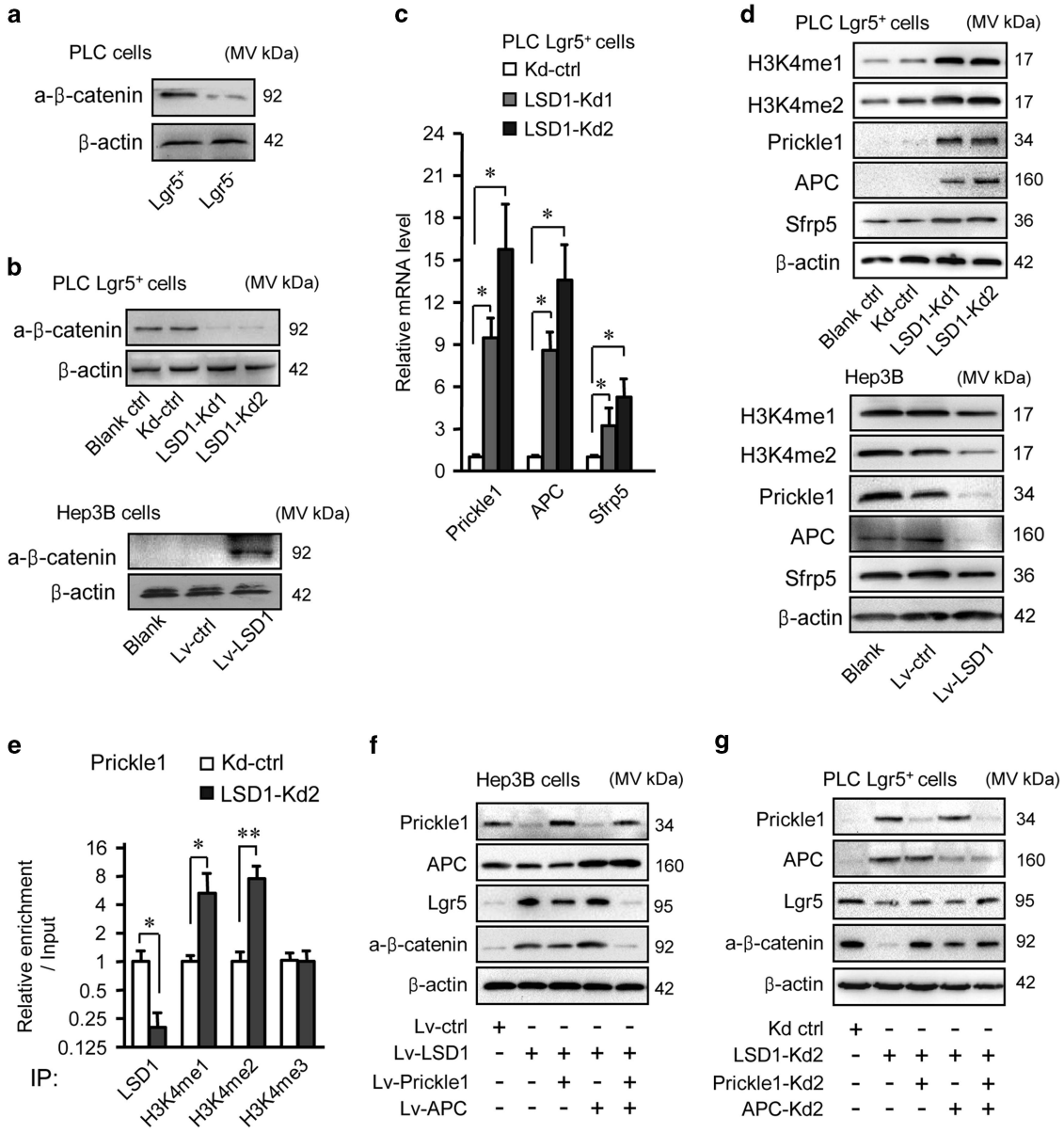

Figure 5. LSD1 enhances the β-catenin activity by demethylating H3K4me1/2 in the promoters of multiple β-catenin antagonists in Lgr5$^+$ CICs. (**a**, **b**) The activation status of the β-catenin signaling pathway in PLC Lgr5$^+$ or Lgr5$^-$ cells (**a**), or LSD1-silencing PLC Lgr5$^+$ cells and LSD1-overexpressing Hep3B cells (**b**) were characterized by western blotting using an antibody recognizing the active dephosphorylated forms of β-catenin (a-β-catenin). (**c**) qRT–PCR analysis demonstrates upregulation of Prickle1, APC and Sfrp5 mRNAs in PLC Lgr5$^+$ cells with LSD1 knockdown. (**d**) Western blot analysis of the levels of Prickle1, APC and Sfrp5 as well as activating H3K4me1 and H3K4me2 in PLC Lgr5$^+$ cells and Hep3B cells following LSD1 manipulation. (**e**) The abundance of LSD1 and HeK4me1/2 on the promoter of Prickle1 in LSD1-Kd2 Lgr5$^+$ PLC cells was characterized by Chip assays. (**f**) Hep3B cells stably expressing LSD1 were transfected with vectors carrying full-length Prickle1 and/or APC. Lgr5 expression and the β-catenin activation status were examined. (**g**) PLC Lgr5$^+$ cells with LSD1 knockdown were further infected with lenti-viruses expressing shRNAs specific for Prickle1 and/or APC. The levels of Lgr5 expression and activation of β-catenin signaling were determined by western blotting. Representative images (**a**, **b**, **d**, **f**, **g**) and data were presented as the mean ± s.d. (**c**, **e**) of each group of cells from triplicates. $^*P < 0.05$, $^{**}P < 0.01$.

and 15) and chemoresistance (Figure 6c, Supplementary Figure S6b) that were conferred by LSD1 upregulation in these cells, whereas depletion of Prickle1 and/or APC in LSD1-Kd Lgr5$^+$ CICs obviously phenocopied the effects of CT99021 in promoting the self-renewal and drug resistance *in vitro* (Figure 6e and f, Supplementary Figure S6c and d, Supplementary Table S16 and 17). Therefore, repression of Prickle1 and to a lesser extent, APC, contribute to LSD1-assciated activation of the β-catenin signaling and subsequent aberrant self-renewal and chemoresistance of Lgr5$^+$ CICs.

DISCUSSION

The precise cell type(s) responsible for the development of HCC and the mechanisms underlying resistance of HCC to conventional therapies remain unclear.[1–2] Lgr5$^+$ is a unique biomarker expressed in stem/progenitor cells in epithelia of the intestine and colon,[9–10] mammary gland,[35] ovarian,[36] kidney and endometrium.[37] However, it remains unclear whether the Lgr5$^+$ neoplastic counterparts contribute to the progression of HCC. In this study, we found that 41% of HCC tissues were positive for anti-Lgr5 staining and the frequency of Lgr5$^+$ cells in the HCC

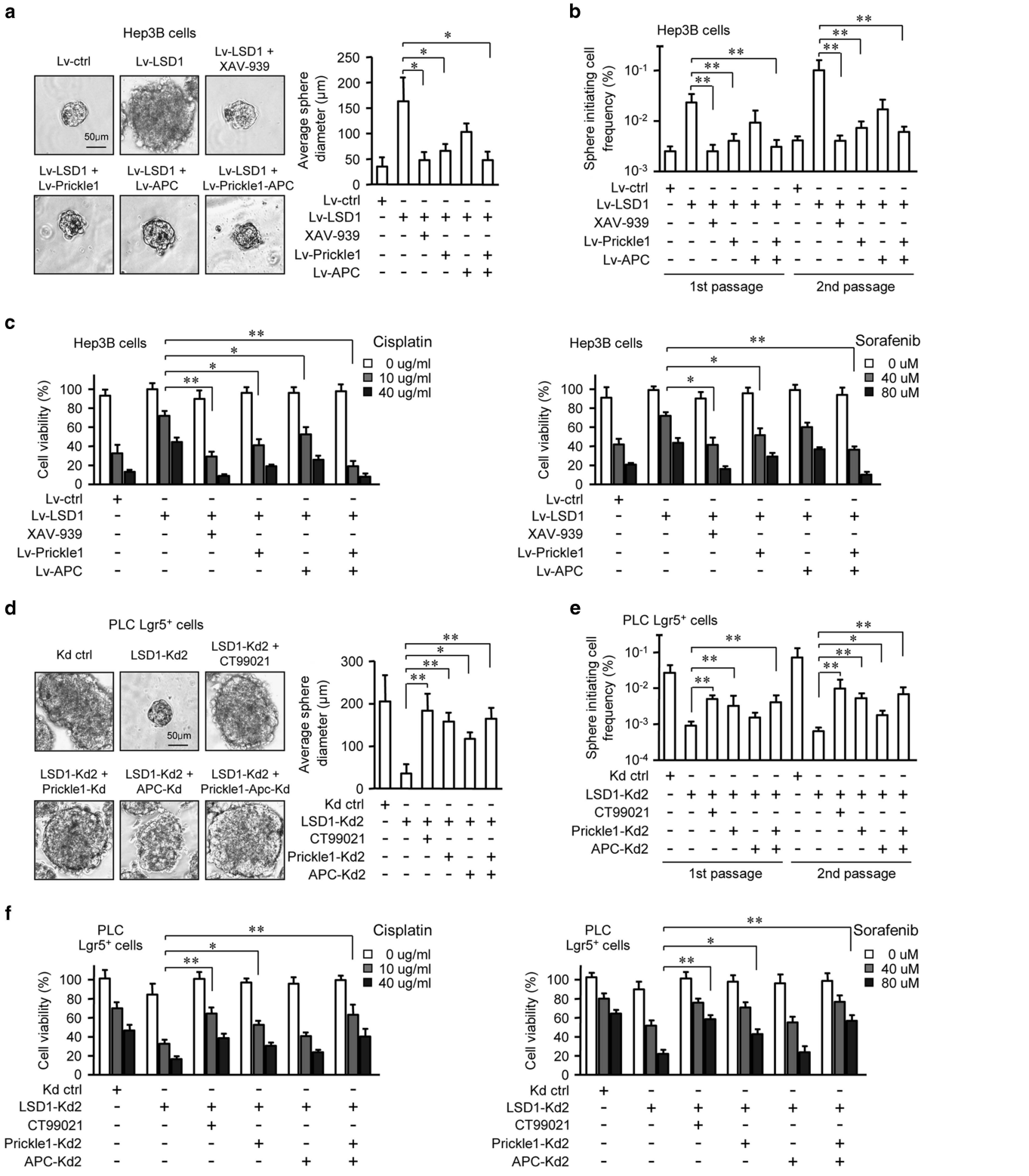

Figure 6. Prickle1 and APC alter the tumorsphere formation and chemoresistance of Lv-LSD1 Hep3B cells and LSD1-Kd2 PLC Lgr5$^+$ cells. (**a**–**c**) Hep3B cells stably expressing LSD1 were incubated with XAV-939 (5 μM), or infected with lenti-viruses encoding Prickle1 and/or APC. The growth of tumorspheres (**a**), the frequency of sphere-initiating cells in serial seeding (**b**) and the sensitivity to cisplain or sorafenib (**c**) were determined. (**d**–**f**) PLC Lgr5$^+$cells with LSD1 knockdown were treated with CT99021 (3 μM), or infected with lenti-viruses to induce expression of scramble shRNA or shRNAs targeting Prickle1 and/or APC. These PLC Lgr5$^+$cells were tested for their capacity for growth as tumorspheres in the stem cell medium (**d**), the numbers of sphere-initiating cells (**e**) and the sensitivity to chemotherapies (**f**). Representative images were shown (**a**, **d**) and data were represented as the mean ± s.d. (**a**, **c**, **d**, **f**) or 95% CI (**b**, **e**) of each group of cells from three separate experiments. $^*P < 0.05$, $^{**}P < 0.01$.

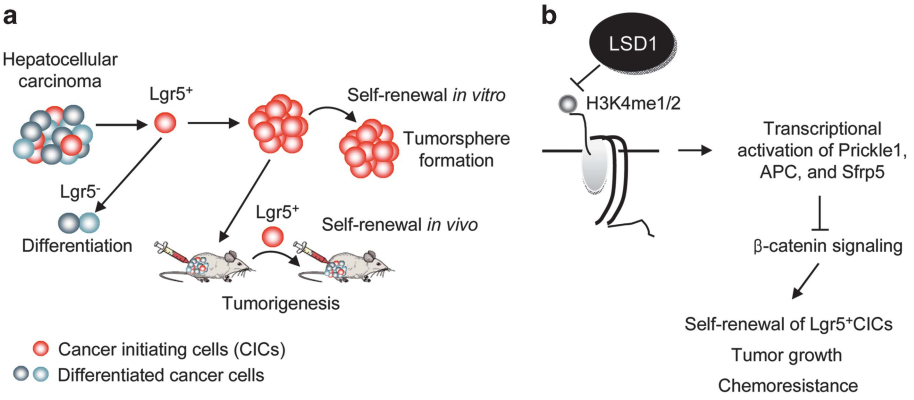

Figure 7. Cartoon illustration of the characterization of $Lgr5^+$ CICs in HCC and the underlying mechanisms by which LSD1 regulates the β-catenin signaling pathway and stemness of $Lgr5^+$ CICs. (**a**) A subset of $Lgr5^+$ CICs in HCC tissues were endowed with differentiation potential and robust stem-like characteristics. These cells are capable of self-renewal as tumorspheres on serial passages *in vitro* and maintenance of tumorigenicity on serial transplantation *in vivo*. (**b**) Aberrant expression of LSD1 in $Lgr5^+$ CICs reduces mono- and di- methylation on lysine-4 of histone H3 (H3K4me1 or H3K4me2), thereby inhibiting transcriptional activity of several suppressors for the β-catenin activity, including Prickle1, APC and Sfrp5. The low expression of negative regulators of β-catenin signaling allows activation of the β-catenin pathway, thus contributing to the maintenance of stemness and chemoresistance of $Lgr5^+$ CICs.

tissues and cell lines was significantly higher than that in the non-tumor regions. Furthermore, the Lgr5 expression in tumor cells was positively associated significantly with progression of HCC, and was an independent prognostic factor for the shorter patient-survival period. Therefore, Lgr5 expression may be valuable for the prognosis of HCC and other types of human cancers, including gastric cancer.[38]

The present study reveals that $Lgr5^+$ HCC cells were a subset of previously unrecognized tumor cells that displayed the properties of CICs (Figure 7a). $Lgr5^+$ HCC cells expressed higher levels of Sox9 and Nanog, transcription factors commonly expressed in hepatic progenitor cells,[39] but lower levels of Alb and Hnf-4, two markers for differentiated and mature hepatocytes. This profile of gene expression is consistent with that of the recently identified $Lgr5^+$ adult liver stem cells.[5] More importantly, functional analyses indicated that $Lgr5^+$ HCC cells had potent self-renewing capacity *in vitro* (serial tumorsphere formation) and *in vivo* (serial transplantation and tumor initiation), and were more resistant to therapeutic agents. This functional phenotype is similar to that of CD133,[30] CD90,[40] EpCAM,[29] CD13,[28] CD24[27] and Nanog[26] expressing HCC cells. Given that $Lgr5^+$ tumor cells are crucial for the development and progression of colorectal cancer[11–13] and gastric adenoma,[8,38] $Lgr5^+$ CICs may be important for the pathogenesis and drug resistance of HCC. Although different types of CICs have distinct biological properties,[41] our findings may have profound implications in understanding tumor biology and designing effective anti-CIC therapies for HCC.

In addition, we also report a novel for the histone demethylase LSD1 in regulating the stemness and chemoresistance of $Lgr5^+$ CICs in HCC (Figure 7b). Previous studies have shown that LSD1 regulates the expression of E-cadherin and the growth and metastasis of colorectal cancers.[42] Moreover, LSD1 and hypoxia inducible factor-1α synergistically enhance glycolysis to promote the progression of pancreatic cancer.[43] Thus, chemical inhibitors targeting LSD1 activity display potent anticancer activity.[20–21] In this study, we found that upregulation of LSD1 in HCC was associated with disease progression and shorter survival of the patients, which was consistent with previous observations.[22,24] More importantly, we demonstrated that LSD1 was also upregulated with Lgr5 in HCC and had a important role in regulating self-renewal of $Lgr5^+$ CICs. Specifically, knockdown of LSD1 remarkably reduced the frequency and chemoresistance of $Lgr5^+$ CICs, whereas induction of LSD1 enhanced Lgr5 expression in HCC cells. Thus, our data support the notion that LSD1 has an oncogenic function to maintain the pool size and the activity of CICs.[21,44–45] It is notable that knockdown of LSD1 in $Lgr5^+$ CICs increased the levels of Alb and Hnf-4 expression, a hallmark of differentiated HCC. Given that LSD1 has been shown to be crucial for maintaining the pluripotency of different types of stem cells,[15,19,21] it is possible that LSD1 may also be a critical regulator of the differentiation of HCC CICs. We are interested in further investigating how LSD1 regulates Lgr5 expression and CIC differentiation.

Furthermore, our findings reveal that LSD1 epigenetically inhibited the expression of several repressors of the β-catenin activity, including Prickle1, APC and Sfrp5 in $Lgr5^+$ CICs, which was possibly mediated by demethylating H3K4me1/2 in the promoters of these antagonists (Figure 7b). Consistently, inhibition of LSD1 increases H3K4me2 and upregulates the expression of only myeloid differentiation-associated genes in acute promyelocytic leukemia cells.[46] Similarly, LSD1 expression is selectively upregulated in $Sox2^+$ lung squamous cell carcinomas and acts synergistically with Sox2 to regulate lung squamous cell carcinoma cell growth.[47] In this study, we detected significantly higher levels of β-catenin, but not MAPK, Jak/Stat, TGF-β, and PI3k/AKT signaling activity in $Lgr5^+$ CICs. Knockdown of LSD1 not only demolished Lgr5 expression, but also inactivated the β-catenin signaling, accompanied by increased levels of Prickle1, APC and Sfrp5, as well as increased H3K4me1/2 in the promoter of Prickle1 in HCC cells. Thus, our findings are in agreement with previous observations that LSD1 positively regulates the Wnt/β-catenin signaling in colorectal cancers.[48] It is possible that LSD1 may regulate the β-catenin activation and stemness properties of CICs through different targets in different types of cancers. Indeed, our findings demonstrated that modulation of the β-catenin activation significantly altered the tumorigenicity in LSD1-overexpressing $Lgr5^+$ CICs. Hence, activation of the β-catenin signaling through loss of its negative regulators is crucial for the stemness of $Lgr5^+$ CICs and understanding this specific regulatory mechanism by LSD1 may aid the design of reseanable targeted therapies for a specific type of cancer.

Finally, our data suggest the possibility of targeted inhibition of LSD1/Prickle1/APC/β-catenin signaling axis to achieve chemo-sensitization in HCC therapies. Unresponsiveness to traditional chemotherapy is a major obstacle for HCC treatment. Although sorafenib has been used for patients with inoperable HCC, the therapeutic efficacy of the available therapeutic strategies is limited,[49] which may be due to drug resistance of CICs. In this regard, we found that $Lgr5^+$ CICs were relatively insensitive to cisplatin and sorafenib, associated with their high levels of LSD1

activity and aberrantly elevated β-catenin activity. Moreover, induction of LSD1 and activation of β-catenin enhance chemoresistance, whereas loss of LSD1 and inhibition of β-catenin activity renders $Lgr5^+$ CICs sensitive to cisplatin and sorafenib. As multiple LSD1 inhibitors demonstrated promising effects for treating cancer in preclinical settings,[20–21,44] further studies are warranted to examine whether targeted inhibition of the LSD1/Prickle1/APC/β-catenin signaling axis could act synergistically with current chemotherapeutic agents to cure HCC.

In summary, our data indicate that $Lgr5^+$ HCC cells represent a subset of HCC CICs, which are associated tumor progression and shorter survival in cancer patients. LSD1 is crucial for the stemness, tumorigenicity and drug resistance of $Lgr5^+$ HCC CICs by demethylating H3K4me1/2 and inhibiting the expression of negative regulators of β-catenin pathway, especially Prickle1, leading to aberrant activation of the β-catenin signaling (Figure 7b). Therefore, our findings may provide new insights in the molecular regulation on the progression of HCC and provide a rationale for the design of new therapeutic strategies, such as combinated usage of LSD1 inhibitors and chemotherapeutic agents, for intervention of HCC.

MATERIALS AND METHODS

Patients and HCC tissues

Written informed consent was obtained from individual patients and the experimental protocols were approved by the Institutional Review Board of Daping Hospital of Third Military Medical University (Chongqing, China). A total of 188 patients with primary HCC underwent curative surgery at the Department of Hepatology of our hospital between 2005 and 2008. Patients with HCC were diagnosed, according to the guidelines on the diagnosis and treatment of primary liver cancer in China (2011 edition) and the pathogenic degrees of individual tumor tissues were evaluated by histology. Individual patients with secondary liver tumor, radiotherapy or chemotherapy prior to the surgery were excluded. The dissected tumor specimens were fixed and paraffin-embedded for pathological examination and some fresh liver tumor tissues (Supplementary Table S18) were used for isolation of tumor cells by primary culture. The demographic and clinical characteristics of the patients are summarized in Supplementary Table S1.

Primary cell culture and flow cytometry

Preparation and primary culture of HCC cells were performed, as described previously.[3–4,27] In brief, fresh tumor samples were mechanically minced, and digested with type IV collagenase (100 units/ml; Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's Modified Eagle's medium at 37 °C for 1 h. After lysis of red blood cells, the remaining cells were cultured in10% fetal bovine serum Dulbecco's Modified Eagle's medium. Some cells were stained with 7-amino-actinomycin D (7-AAD0, ebioscience, San Diego, CA, USA), anti-CD45 and anti-Lgr5 or isotype controls. The percentages of $Lgr5^+$ cells were analyzed or sorted using a BDAria II Sorter (BD Biosciences, San Jose, CA, USA) by gating on $7\text{-AAD}^-\text{CD45}^-$ live HCC cells.

Tumorosphere formation and limiting dilution assay

For tumorosphere culture, the isolated cells at 200 cells/well were cultured in 10 replicate in ultra-low attached 24-well plates (Corning, Union City, CA, USA) in stem cell medium.[26–27] The stem cell medium was Dulbecco's Modified Eagle's medium/F12 medium (GIBCO, Beijing, China) containing 20 ng/ml of epidermal growth factor, fibroblast growth factor, 10 ng/ml of HGF (PeproTech, Rocky Hill, NJ, USA), B27 supplement (Invitrogen, Grand Island, NY, USA), and 4 μg/ml of insulin (Sigma-Aldrich, Natick, MA, USA)[26–27] as well as 1% methyl cellulose (Sigma-Aldrich) for 2 weeks. The cells were exposed to fresh medium every 4 days. The diameters of at least 20 tumorospheres were examined under a microscope to calculate the average diameters of tumorospheres.

In limiting dilution assay of tumorosphere formation, the cells at different densities were cultured 20 wells per cell density in stem cell medium in 96-well plates for 2 weeks. The numbers of wells with at least one tumorsphere (diameter >75 μm) were counted in a blinded manner. The frequency of sphere-initiating cells was calculated by extreme limiting dilation assays.[25,31] For serial passage, some tumorospheres were dissociated enzymatically and single cells were re-seeded in the same manner.

Chromatin immunoprecipitation

The enrichment of LSD1 and histone marks on gene promoters was determined by chromatin immunoprecipitation-quantitative PCR assay.[3] In brief, 2×10^6cells were fixed in 1% formaldehyde for protein cross-linking. The DNA fragment was precipitated with antibodies against LSD1, H3K4me2, H3K4me1 and H3K4me3 (Supplementary Table S19), using genomic DNA as positive input. The changes in histone marks at the promoter were quantified by quantitative PCR using primers specific for the LSD1_peak_5254 of Prickle1locus (forward: 5′-TGGGCTTGCTTTGAGGAT-3′, reverse: 5′-CAGGTCACGCGATGTACTAAC-3′). The results were expressed as fold changes relative to input DNA.

Tumorigenicity *in vivo*

Animal protocols were approved by the Animal Studies Ethics Committee of Third Military Medical University.[50] Male nude mice at 4-week of age were housed in a specific pathogen-free facility in the animal center. Tumor cell suspension was mixed with Matrigel (1:1) and injected subcutaneously into the flanks of the mice. The tumor volumes were measured weekly using a caliper and calculated as $(\text{length}\times\text{width}^2)/2$. The tumors were harvested or fixed with 10% buffered formalin for further analysis.

For *in vivo* limiting dilution assays, cells were diluted and injected at defined cell doses subcutaneously. To assess self-renewal capacity of CICs, cells were isolated from xenograft tumors and diluted serially to the desired cell doses. Secondary limiting dilution assays were carried out by injecting the cells subcutaneously into the flanks of nude mice. For all experiments, six injection sites were tested. All mice were killed humanely 3 months later and the number of injection sites containing tumors was counted. The CIC frequency was calculated using extreme limiting dilation assays software.[25,31]

Statistical analysis

Data are expressed as the mean ± s.d. or percentage, and analyzed by Student's *t*-test, χ^2-test or analysis of variance using a SPSS software (version 17.0). Survival data were estimated by Kaplan–Meier method analyzed by log-rank test. The association between factors for the survival was tested by univariate and multivariate analyses using Cox regression analysis. A *P*-value of <0.05 was considered statistically significant. See Supplementary Materials and Methods and Supplementary Figure legends for other experimental procedures.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are indebted to Prof Xiang Xu (Research Institute of Surgery, Daping Hospital, Third Military Medical University, Chongqing, China) for his technical assistance. We are grateful to Miss Chunhua Quan, Ya Li and Qing Li (Department of Gastroenterology, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing, China) for their help in collecting clinical data. This work was supported by the grants from the National Natural Science Foundation of China (NSFC Nos. 81472294, 81201949, and 81372558), the Natural Science Foundation Project of CQ CSTC (No. CSTC2012JJA10124), and the Science Foundation of Third Military Medical University (No. 2012XJQ22).

REFERENCES

1 Yamashita T, Wang XW. Cancer stem cells in the development of liver cancer. *J Clin Invest* 2013; **123**: 1911–1918.
2 Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. *Nature* 2013; **501**: 328–337.
3 Wang Z, Wang B, Shi Y, Xu C, Xiao HL, Ma LN *et al.* Oncogenic miR-20a and miR-106a enhance the invasiveness of human glioma stem cells by directly targeting TIMP-2. *Oncogene* 2015; **34**: 1407–1419.
4 Wang B, Wang Q, Wang Z, Jiang J, Yu SC, Ping YF *et al.* Metastatic consequences of immune escape from NK cell cytotoxicity by human breast cancer stem cells. *Cancer Res* 2014; **74**: 5746–5757.

5 Huch M, Dorrell C, Boj SF, van Es JH, Li VS, van de Wetering M *et al.* In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature* 2013; **494**: 247–250.
6 Hu M, Kurobe M, Jeong YJ, Fuerer C, Ghole S, Nusse R *et al.* Wnt/beta-catenin signaling in murine hepatic transit amplifying progenitor cells. *Gastroenterology* 2007; **133**: 1579–1591.
7 Yang W, Yan HX, Chen L, Liu Q, He YQ, Yu LX *et al.* Wnt/beta-catenin signaling contributes to activation of normal and tumorigenic liver progenitor cells. *Cancer Res* 2008; **68**: 4287–4295.
8 Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH *et al.* $Lgr5^{+ve}$ stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* 2010; **6**: 25–36.
9 Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M *et al.* Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 2007; **449**: 1003–1007.
10 Qiu W, Wang X, Buchanan M, He K, Sharma R, Zhang L *et al.* ADAR1 is essential for intestinal homeostasis and stem cell maintenance. *Cell Death Dis* 2013; **4**: e599.
11 Hirsch D, Barker N, McNeil N, Hu Y, Camps J, McKinnon K *et al.* LGR5 positivity defines stem-like cells in colorectal cancer. *Carcinogenesis* 2014; **35**: 849–858.
12 Schepers AG, Snippert HJ, Stange DE, van den Born M, van Es JH, van de Wetering M *et al.* Lineage tracing reveals $Lgr5^{+}$ stem cell activity in mouse intestinal adenomas. *Science* 2012; **337**: 730–735.
13 Kemper K, Prasetyanti PR, De Lau W, Rodermond H, Clevers H, Medema JP. Monoclonal antibodies against Lgr5 identify human colorectal cancer stem cells. *Stem Cells* 2012; **30**: 2378–2386.
14 Fukuma M, Tanese K, Effendi K, Yamazaki K, Masugi Y, Suda M *et al.* Leucine-rich repeat-containing G protein-coupled receptor 5 regulates epithelial cell phenotype and survival of hepatocellular carcinoma cells. *Exp Cell Res* 2013; **319**: 113–121.
15 Greer EL, Shi Y. Histone methylation: a dynamic mark in health, disease and inheritance. *Nat Rev Genet* 2012; **13**: 343–357.
16 Wang Y, Zhang H, Chen Y, Sun Y, Yang F, Yu W *et al.* LSD1 is a subunit of the NuRD complex and targets the metastasis programs in breast cancer. *Cell* 2009; **138**: 660–672.
17 Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA *et al.* Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 2004; **119**: 941–953.
18 Metzger E, Wissmann M, Yin N, Muller JM, Schneider R, Peters AH *et al.* LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature* 2005; **437**: 436–439.
19 Adamo A, Sese B, Boue S, Castano J, Paramonov I, Barrero MJ *et al.* LSD1 regulates the balance between self-renewal and differentiation in human embryonic stem cells. *Nat Cell Biol* 2011; **13**: 652–659.
20 Wang J, Lu F, Ren Q, Sun H, Xu Z, Lan R *et al.* Novel histone demethylase LSD1 inhibitors selectively target cancer cells with pluripotent stem cell properties. *Cancer Res* 2011; **71**: 7238–7249.
21 Harris WJ, Huang X, Lynch JT, Spencer GJ, Hitchin JR, Li Y *et al.* The histone demethylase KDM1A sustains the oncogenic potential of MLL-AF9 leukemia stem cells. *Cancer Cell* 2012; **21**: 473–487.
22 Zhao ZK, Dong P, Gu J, Chen L, Zhuang M, Lu WJ *et al.* Overexpression of LSD1 in hepatocellular carcinoma: a latent target for the diagnosis and therapy of hepatoma. *Tumour Biol* 2013; **34**: 173–180.
23 Yu Y, Wang B, Zhang K, Lei Z, Guo Y, Xiao H *et al.* High expression of lysine-specific demethylase 1 correlates with poor prognosis of patients with esophageal squamous cell carcinoma. *Biochem Biophys Res Commun* 2013; **437**: 192–198.
24 Hayami S, Kelly JD, Cho HS, Yoshimatsu M, Unoki M, Tsunoda T *et al.* Overexpression of LSD1 contributes to human carcinogenesis through chromatin regulation in various cancers. *Int J Cancer* 2011; **128**: 574–586.
25 Kreso A, van Galen P, Pedley NM, Lima-Fernandes E, Frelin C, Davis T *et al.* Self-renewal as a therapeutic target in human colorectal cancer. *Nat Med* 2014; **20**: 29–36.
26 Shan J, Shen J, Liu L, Xia F, Xu C, Duan G *et al.* Nanog regulates self-renewal of cancer stem cells through the insulin-like growth factor pathway in human hepatocellular carcinoma. *Hepatology* 2012; **56**: 1004–1014.
27 Lee TK, Castilho A, Cheung VC, Tang KH, Ma S, Ng IO. $CD24^{+}$ liver tumor-initiating cells drive self-renewal and tumor initiation through STAT3-mediated NANOG regulation. *Cell Stem Cell* 2011; **9**: 50–63.
28 Haraguchi N, Ishii H, Mimori K, Tanaka F, Ohkuma M, Kim HM *et al.* CD13 is a therapeutic target in human liver cancer stem cells. *J Clin Invest* 2010; **120**: 3326–3339.
29 Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY *et al.* EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology* 2009; **136**: 1012–1024.
30 Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO *et al.* Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology* 2007; **132**: 2542–2556.
31 O'Brien CA, Kreso A, Ryan P, Hermans KG, Gibson L, Wang Y *et al.* ID1 and ID3 regulate the self-renewal capacity of human colon cancer-initiating cells through p21. *Cancer Cell* 2012; **21**: 777–792.
32 Chan DW, Chan CY, Yam JW, Ching YP, Ng IO. Prickle-1 negatively regulates Wnt/beta-catenin pathway by promoting Dishevelled ubiquitination/degradation in liver cancer. *Gastroenterology* 2006; **131**: 1218–1227.
33 Blaschke K, Ebata KT, Karimi MM, Zepeda-Martinez JA, Goyal P, Mahapatra S *et al.* Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. *Nature* 2013; **500**: 222–226.
34 Wang B, Liu J, Ma LN, Xiao HL, Wang YZ, Li Y *et al.* Chimeric 5/35 adenovirus-mediated Dickkopf-1 overexpression suppressed tumorigenicity of $CD44^{+}$ gastric cancer cells *via* attenuating Wnt signaling. *J Gastroenterol* 2013; **48**: 798–808.
35 Plaks V, Brenot A, Lawson DA, Linnemann JR, Van Kappel EC, Wong KC *et al.* Lgr5-expressing cells are sufficient and necessary for postnatal mammary gland organogenesis. *Cell Rep* 2013; **3**: 70–78.
36 Flesken-Nikitin A, Hwang CI, Cheng CY, Michurina TV, Enikolopov G, Nikitin AY. Ovarian surface epithelium at the junction area contains a cancer-prone stem cell niche. *Nature* 2013; **495**: 241–245.
37 Gil-Sanchis C, Cervello I, Mas A, Faus A, Pellicer A, Simon C. Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) as a putative human endometrial stem cell marker. *Mol Hum Reprod* 2013; **19**: 407–414.
38 Xi HQ, Cai AZ, Wu XS, Cui JX, Shen WS, Bian SB *et al.* Leucine-rich repeat-containing G-protein-coupled receptor 5 is associated with invasion, metastasis, and could be a potential therapeutic target in human gastric cancer. *Br J Cancer* 2014; **110**: 2011–2020.
39 Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T *et al.* Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. *Nat Genet* 2011; **43**: 34–41.
40 Yang ZF, Ho DW, Ng MN, Lau CK, Yu WC, Ngai P *et al.* Significance of $CD90^{+}$ cancer stem cells in human liver cancer. *Cancer Cell* 2008; **13**: 153–166.
41 Yamashita T, Honda M, Nakamoto Y, Baba M, Nio K, Hara Y *et al.* Discrete nature of $EpCAM^{+}$ and $CD90^{+}$ cancer stem cells in human hepatocellular carcinoma. *Hepatology* 2013; **57**: 1484–1497.
42 Ding J, Zhang ZM, Xia Y, Liao GQ, Pan Y, Liu S *et al.* LSD1-mediated epigenetic modification contributes to proliferation and metastasis of colon cancer. *Br J Cancer* 2013; **109**: 994–1003.
43 Qin Y, Zhu W, Xu W, Zhang B, Shi S, Ji S *et al.* LSD1 sustains pancreatic cancer growth *via* maintaining HIF1alpha-dependent glycolytic process. *Cancer Lett* 2014; **347**: 225–232.
44 Suva ML, Rheinbay E, Gillespie SM, Patel AP, Wakimoto H, Rabkin SD *et al.* Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. *Cell* 2014; **157**: 580–594.
45 Wu Y, Wang Y, Yang XH, Kang T, Zhao Y, Wang C *et al.* The deubiquitinase USP28 stabilizes LSD1 and confers stem-cell-like traits to breast cancer cells. *Cell Rep* 2013; **5**: 224–236.
46 Schenk T, Chen WC, Gollner S, Howell L, Jin L, Hebestreit K *et al.* Inhibition of the LSD1 (KDM1A) demethylase reactivates the all-trans-retinoic acid differentiation pathway in acute myeloid leukemia. *Nat Med* 2012; **18**: 605–611.
47 Zhang X, Lu F, Wang J, Yin F, Xu Z, Qi D *et al.* Pluripotent stem cell protein Sox2 confers sensitivity to LSD1 inhibition in cancer cells. *Cell Rep* 2013; **5**: 445–457.
48 Huang Z, Li S, Song W, Li X, Li Q, Zhang Z *et al.* Lysine-specific demethylase 1 (LSD1/KDM1A) contributes to colorectal tumorigenesis *via* activation of the Wnt/Beta-catenin pathway by down-regulating Dickkopf-1 (DKK1). *PLoS One* 2013; **8**: e70077.
49 Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF *et al.* Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378–390.
50 Wang B, Yu SC, Jiang JY, Porter GW, Zhao LT, Wang Z *et al.* An inhibitor of arachidonate 5-lipoxygenase, Nordy, induces differentiation and inhibits self-renewal of glioma stem-like cells. *Stem Cell Rev* 2011; **7**: 458–470.

Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)