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Lymphocyte-Specific Protein-1 Controls Sorafenib Sensitivity and Hepatocellular Proliferation through Extracellular Signal-Regulated Kinase 1/2 Activation

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The gene leukocyte-specific protein-1 (*LSP1*), encodes an F-actin binding protein that directly interacts with the mitogen-activated protein kinase pathway. *LSP1* has copy number variations in 52% of human hepatocellular carcinoma (HCC). LSP1 suppresses proliferation and migration in hepatocytes. LSP1 binds to the rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein/extracellular signal-regulated kinase (ERK)/ERK signaling cassette, the target for sorafenib, a crucial chemotherapeutic agent for HCC. This study addresses the role of LSP1 in liver regeneration and sensitivity to sorafenib in normal and neoplastic hepatocytes. Two mouse models, an Lsp1 global knockout (LSP1KO) and a hepatocyte-specific Lsp1 transgenic (LSP1TG) mouse, were used. After two-thirds hepatectomy (PHx), LSP1KO mice displayed increased proliferation and ERK activation, whereas LSP1TG mice displayed suppressed proliferation and decreased ERK activation. LSP1KO hepatocytes cultured without growth factors exhibited increased proliferation, whereas LSP1TG hepatocytes showed decreased proliferation. Rat and human hepatoma cells expressing Lsp1 shRNA displayed increased sensitivity to sorafenib, as evidenced by decreased cell numbers and phosphorylated ERK expression compared with control. LSP1 KO mice treated with sorafenib before PHx displayed decreased hepatocyte proliferation. Our data show that loss of LSP1 function, observed in HCC, leads to increased sensitivity to sorafenib treatment and enhanced hepatocellular proliferation after PHx in vivo and in cultured cells. *(Am J Pathol 2018, 188: 2074–2086; https://doi.org/10.1016/j.ajpath.2018.06.005)*

Hepatocellular carcinoma (HCC), the most commonly diagnosed form of liver cancer, is increasing in incidence throughout the world. Along with increasing incidence, mortality from HCC is also on the rise.[1,2] Treatment options for HCC remain limited, and advanced-stage HCC continues to have a poor prognosis.[3] Sorafenib remains the main chemotherapeutic choice for HCC, but approximately 50% of cases respond and the response is limited, typically to 6 to 8 months. Sorafenib, one of the only US Food and Drug Administration–approved molecular targeted therapies for advanced HCC, is a tyrosine kinase inhibitor that targets Ras–mitogen-activated protein kinase pathway with higher sensitivity compared with other targets.[1,3,4] A phase 3 clinical trial has demonstrated that sorafenib treatment increased overall survival of patients with advanced HCC from 7.9 to 10.7 months. This improved survival is modest, and not all HCCs respond to this therapy.[4,5] Therefore, a greater understanding of the molecular basis of HCC response to sorafenib will enable better prediction of chemotherapeutic responses; modulation of the signaling molecules controlling response to sorafenib may also facilitate development of novel therapeutics to combat this deadly disease.

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Previous work from our laboratory has demonstrated that the gene encoding lymphocyte-specific protein-1 (LSP1; alias leukocyte-specific protein-1) had the highest copy number variation in human HCC, with >50% of the samples studied containing copy number ratio of LSP1 associated with C-terminal deletions or C-terminal dominant negative amplifications.[6] LSP1 is an F-actin–binding protein that is a scaffold for the Ras–mitogen-activated protein kinase pathway and regulates migration of hematopoietic cells.[7,8] Previous work has shown that loss of LSP1 expression leads to enhanced skin wound healing, suggesting a role for LSP1 in cell proliferation.[9] LSP1 is also known to interact with the scaffold kinase suppressor of Ras (KSR) to regulate the activation of the extracellular signal-regulated kinase (ERK) pathway.[10] We have previously shown that LSP1 acts as a negative regulator of hepatocellular migration and proliferation both *in vitro*, using an *Lsp1* shRNA stable rat hepatoma cell line, and *in vivo*, during liver regeneration after partial hepatectomy (PHx).[11] Additional studies have shown that loss of LSP1 expression correlates with larger tumor size and advanced tumor-node-metastasis stage as well as decreased overall and disease-free survival.[12] Loss of LSP1 expression in rat hepatoma cells leads to enhanced ERK activation [increased phosphorylated ERK (pERK) expression], whereas increased LSP1 expression causes a decrease in ERK phosphorylation.[11,12] These findings suggest that HCC loss of LSP1 function leads to increased proliferation and migration because of increased activation of ERK. Given the high affinity of sorafenib for the rapidly accelerated fibrosarcoma (RAF) kinase, a component of the RAF/mitogen-activated protein/ERK (MEK)/ERK signaling cascade, it is reasonable to hypothesize that LSP1, by way of binding to KSR and suppressing the RAF/MEK/ERK signal, may be a controlling protein for response of HCC to sorafenib.

To better understand the interaction between LSP1, ERK, and sorafenib, studies were conducted both in normal liver during liver regeneration after partial hepatectomy and hepatocytes in primary culture and with hepatoma cell lines with modified expression of LSP1. A global *Lsp1* knockout (KO) mouse strain and a hepatocyte-specific *Lsp1* transgenic (TG) mouse model were used. The overexpression of LSP1 through hydrodynamic tail vein injection of LSP1 plasmid DNA leads to decreased hepatocyte proliferation on day 2 after PHx.[11] However, how the loss of LSP1 globally as well as overexpression of LSP1 specifically in hepatocytes affect liver regeneration after PHx remains unknown. In this study, we demonstrate that LSP1 KO mice display increased proliferation and ERK activation, whereas LSP1 TG mice demonstrate the opposite phenotype with decreased proliferation and pERK expression. Because loss of LSP1 expression leads to increased activation of the ERK pathway, the role of LSP1 loss was studied in the context of sensitivity to sorafenib in liver regeneration and hepatoma cell lines. Because loss of LSP1 expression leads to increased ERK activation, we hypothesize that sorafenib treatment will be more efficacious in LSP1-negative tumors than in the tumors in which LSP1 expression is intact. Using both *Lsp*1 shRNA–expressing hepatoma cells and LSP1 KO mice, we demonstrate that loss of LSP1 expression leads to increased sensitivity to sorafenib.

Materials and Methods

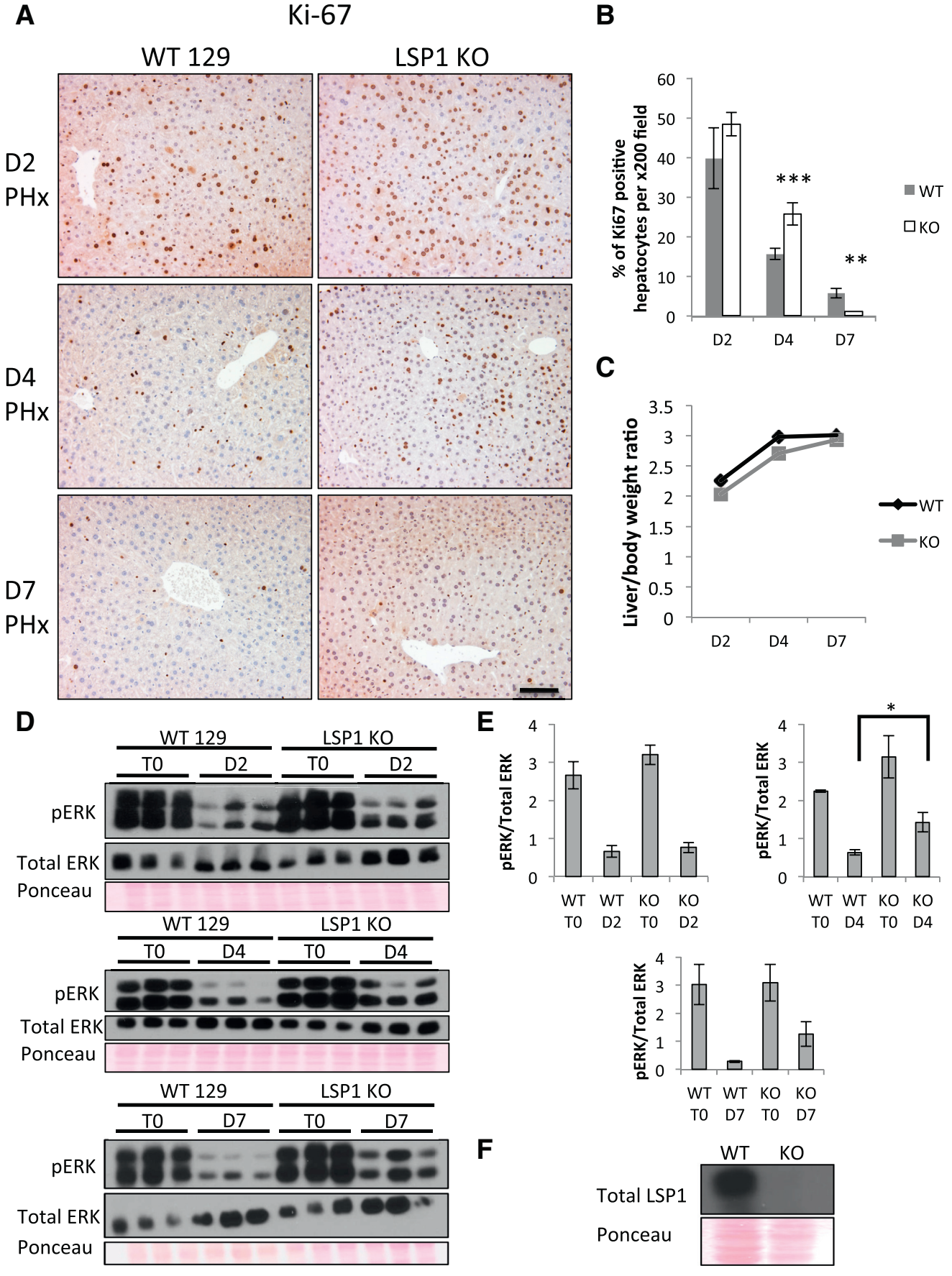
Materials and Reagents

Rabbit anti-LSP1 primary antibody was a generous gift from Dr. Jan Jongstra (University Health Network, Toronto, ON, Canada). Additional antibodies that were used for Western blotting, immunofluorescence, immunohistochemistry, and immunoprecipitation include cyclin D1 (Neomarkers, Fremont, CA), KSR (Santa Cruz Biotechnology, Dallas, TX), pERK1/2 (Tyr202/204) and total ERK1/2 (Cell Signaling Technology Inc., Danvers, MA), proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology), and Ki-67 (ThermoFisher, Pittsburgh, PA). Green fluorescent protein–tagged rat *Lsp1* shRNA plasmid and control scrambled shRNA green fluorescent protein plasmid were purchased from Origene (Rockville, MD; number TG702934). Sorafenib, p-toluenesulfonate salt (S-8502), was purchased from LC Laboratories (Woburn, MA) and dissolved in dimethyl sulfoxide.

Animals

Lsp1 knockout mice were generated on a 129/SvJ background in the laboratory of Dr. Jenny Jongstra-Bilen, as previously described,[13] and given to our laboratory by Dr. Lixin Liu (University of Saskatchewan, Saskatoon, SK, Canada). LSP1 KO mice and 129/SvJ wild-type (WT) mice were used for liver regeneration studies after PHx and liver perfusion to obtain hepatocytes for *in vitro* culture.

*Lsp*1 transgenic mice were generated on a C57/BL6 background in conjunction with Dr. Kyle Orwig (Magee-Womens Research Institute Transgenic and Molecular Research Core Facility, Pittsburgh, PA). Mouse LSP1 cDNA was cloned into a plasmid with an albumin promoter and an α-fetoprotein enhancer to ensure expression of LSP1 in hepatocytes. Using the pronuclear injection technique, *Lsp1*-albumin promoter plasmid DNA was injected into donor zygotes, which were implanted into pseudopregnant female mice. PCR was used to screen the offspring for the presence of the transgene. To determine the transgene copy number, mice positive for the transgene were mated to control mice.[14] Once a pure line was established, a homozygous transgenic mouse strain was used in the PHx studies as well as hepatocyte cultures. LSP1 protein expression was assessed by Western blot. For all animal experiments, 15- to 22-week–old male mice were used. Previous literature has demonstrated that there is no impairment in hepatocyte proliferation after partial hepatectomy in mice of this age


A
Ki-67
WT 129
LSP1 KO
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D7 PHx
B
% of Ki67 positive hepatocytes per x200 field
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50
40
30
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10
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D2
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WT
KO
C
Liver/body weight ratio
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3
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2
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Ponceau

group.[15] In addition to the lack of an effect on hepatocyte proliferation, mice <12 weeks of age experience changes in the polyploidization of the hepatocytes; therefore, mice >12 weeks of age were used to ensure that this phenomenon would not affect the findings.[16] All procedures performed on mice were approved under University of Pittsburgh (Pittsburgh, PA) Institutional Animal Care and Use Committee protocols and conducted in accordance with the NIH animal care and use guidelines.

Generation of a Stable *Lsp1* shRNA Cell Line and Sorafenib Treatment

Stable *Lsp1* shRNA JM1 rat hepatoma cells as well as scrambled shRNA control JM1 cells were generated using green fluorescent protein—*Lsp1* shRNA plasmid from Origene (number TG702934), as previously described.[11,17] Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO) and gentamicin (1:1000) and maintained in an incubator at 37°C with 5% CO_2. For sorafenib treatment, 400,000 cells were seeded into each well of a 6-well plate in complete media. Two days after seeding, cells were treated with 10, 20, and 40 μmol/L sorafenib in dimethyl sulfoxide for 48 hours in complete media. After 48 hours, protein lysates were collected using radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors, and DNA was fixed using cold 5% trichloroacetic acid. DNA was dissolved in NaOH, and concentration was measured at wavelength 260 nm on a plate reader.

Transient Transfection of Hep3B Cells with *LSP1* shRNA and Sorafenib Treatment

Hep3B cells [Hep 3B2.1-7 (Hep 3B, Hep3-B, and Hep3B); HB-8064; ATCC, Manassas, VA] were transiently transfected with either human *LSP1* shRNA or scrambled control shRNA (Origene; TG311654) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). Cells were transfected at approximately 70% to 80% confluency, following the manufacturer's protocol. Forty-eight hours after transfection, cells were treated with either 2 μmol/L sorafenib in dimethyl sulfoxide or dimethyl sulfoxide as control in complete medium [Eagle's minimal essential medium (ATCC) with 10% fetal bovine serum (Atlas) and gentamicin; 1:1000]. The cells were treated with sorafenib for 48 hours, after which protein lysates were collected in radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors and cells were fixed in formalin to allow counting of cells per high-power field.

Two-Thirds Partial Hepatectomy and Administration of Sorafenib

LSP1 KO, TG, and WT controls were subjected to a two-thirds partial hepatectomy, as previously described.[18] The livers were harvested on days 2, 4, and 6 after PHx, and the tissue was processed for paraffin embedding, frozen OCT embedding, and protein isolation. For *in vivo* sorafenib experiments, LSP1 KO and WT 129svJ mice were gavaged with 100 μL of 100 mg/kg of sorafenib in 12.5% Cremophor (Sigma-Aldrich, St. Louis, MO) and 8.75% ethanol aqueous solution.[19] Sorafenib was administered to the mice twice, one dose a day before PHx and one dose 2 hours before PHx. Livers were harvested on day 2 after PHx, and the tissue was formalin fixed, embedded in OCT, and stored for protein isolation.

Western Blotting

Protein whole-cell lysates of liver tissue and cells were prepared in radioimmunoprecipitation assay buffer with 1% SDS (10 mmol/L Na_2HPO_4, 10 mmol/L NaH_2PO_4, 150 mmol/L NaCl, 1% NP-40, and protease inhibitor cocktail; P8340; Sigma-Aldrich), phosphatase inhibitor cocktail I and II (P2850 and P5726; Sigma-Aldrich), 0.26 mg/mL amiloride, and 0.05 mg/mL 4-benzenesulfonyl fluoride hydrochloride and homogenized. Bicinchoninic acid assay (Pierce Chemical Co, Rockford, IL) was used to determine protein concentrations, and 30 μg of protein was loaded and separated onto 10% SDS polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). After transfer, Ponceau S was used to stain membranes to ensure equal loading and protein transfer. Blots were probed overnight with primary antibodies, followed by a 1-hour incubation with horseradish peroxidase—conjugated secondary antibodies separately in tris-buffered saline with Tween 20 containing 5% fish gelatin (Sigma-Aldrich). The membranes were processed with SuperSignal West Pico chemiluminescence substrate (Pierce Chemical Co) and exposed to X-ray film (Lab Product Sales, Rochester, NY).

Immunohistochemistry

Formalin-fixed, paraffin-embedded liver tissue were divided into sections (4 μm thick) and incubated with Ki-67 antibody (ThermoFisher) using the avidin-biotin-

Figure 1 LSP1 knockout (KO) livers display increased proliferation and phosphorylated extracellular signal-regulated kinase (pERK) expression after PHx. **A:** Representative images of Ki-67 immunohistochemistry of wild-type (WT) 129 and LSP1 KO livers at various time points after PHx. **B:** Quantification of the percentage of Ki-67—positive hepatocytes. **C:** Liver/body weight ratios of WT and KO mice after PHx. **D:** Western blots of pERK and total ERK expression in WT and KO livers at days (D) 2, 4, and 7 after PHx. Each lane represents a different animal. **E:** Quantification of the ratio of pERK/total ERK from the Western blots in **D**. **F:** Western blot of LSP1 expression in baseline WT and KO mouse livers. Ponceau S was used as a loading control in **D** and **F**. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus WT. Scale bar = 100 μm (**A**). Original magnification, ×200 (**A**). T0, the beginning of the experiment.

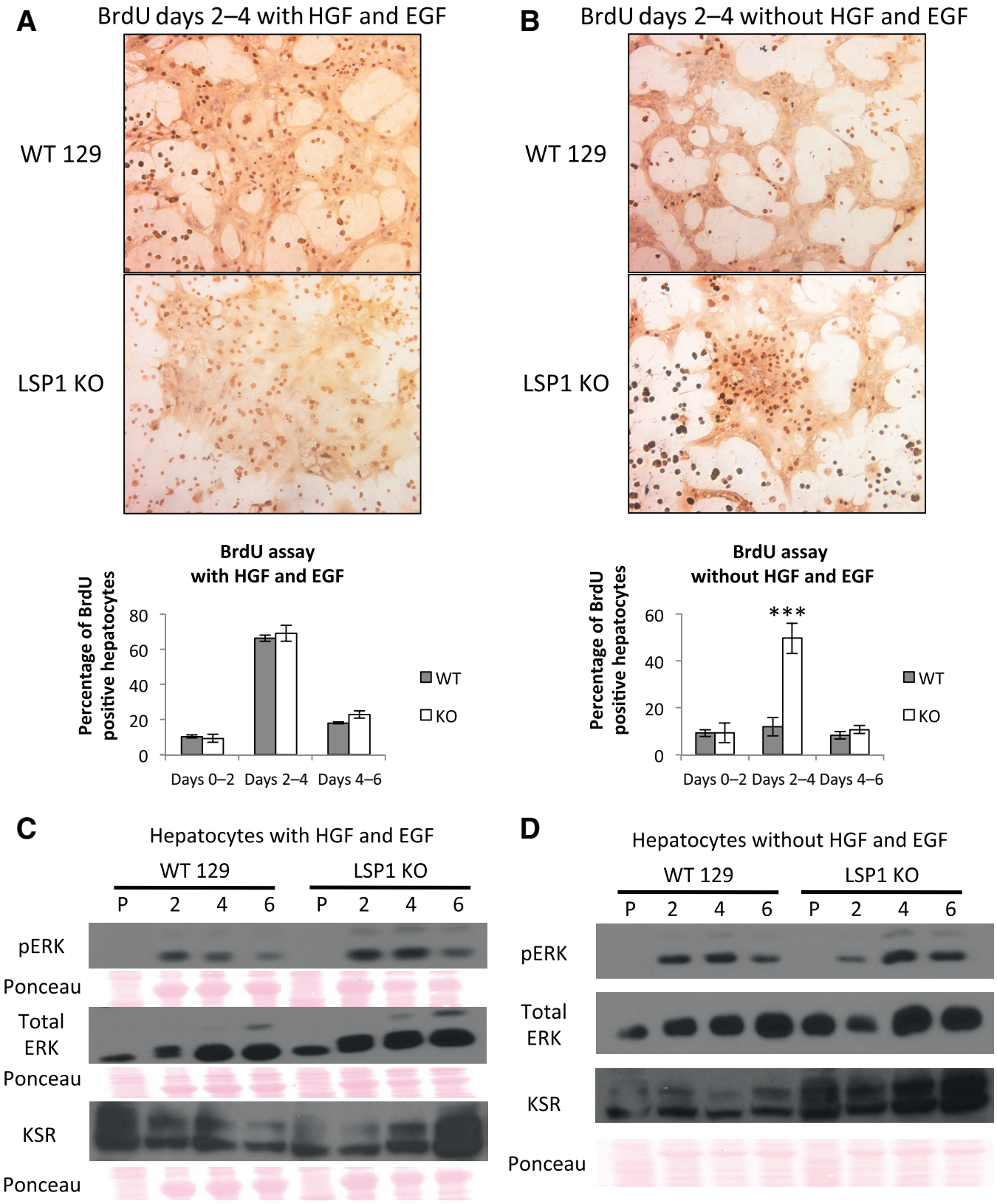


Figure 2 LSP1 knockout (KO) hepatocytes exhibit enhanced proliferation and extracellular signal-regulated kinase (ERK) phosphorylation in the absence of growth factors, hepatocyte growth factor (HGF), and epidermal growth factor (EGF). Representative images of bromodeoxyuridine (BrdU) immunohistochemistry on days 2 to 4 and quantification of the percentage of BrdU-positive wild-type (WT) and KO hepatocytes in culture with HGF and EGF (**A**) and without HGF and EGF (**B**). Western blot analysis of phosphorylated ERK (pERK), total ERK, and kinase suppressor of Ras (KSR) expression in WT and KO hepatocytes cultured in the presence (**C**) and absence (**D**) of growth factors. Ponceau S was used as a loading control. ***$P < 0.001$ versus WT. Original magnification, ×100 (**A** and **B**). P, hepatocyte pellet.

peroxidase complex technique (Vectastain ABC kit and DAB peroxidase substrate kit; Vector Laboratories, Burlingame, CA). Hepatocytes in culture were formalin fixed and stained with bromodeoxyuridine (BrdU) antibody (Accurate Chemical and Scientific Corp., Westbury, NY). The sections and cells were counterstained with hematoxylin. The stained tissue sections and cells were imaged using an Olympus inverted microscope at ×200 and ×100 magnification, respectively. The percentage of Ki-67− and BrdU-positive hepatocytes was quantified using ImageJ software version 1.52b (NIH, Bethesda, MD; *http://imagej.nih.gov/ij*) in at least six random fields per section.

Statistical Analysis

t-Tests and two-way analysis of variance test were performed to determine statistical significance. Data are expressed as means ± SEM. $P < 0.05$ was considered significant.

Results

Loss of LSP1 Expression Leads to Increased Proliferation and pERK Expression after Two-Thirds PHx

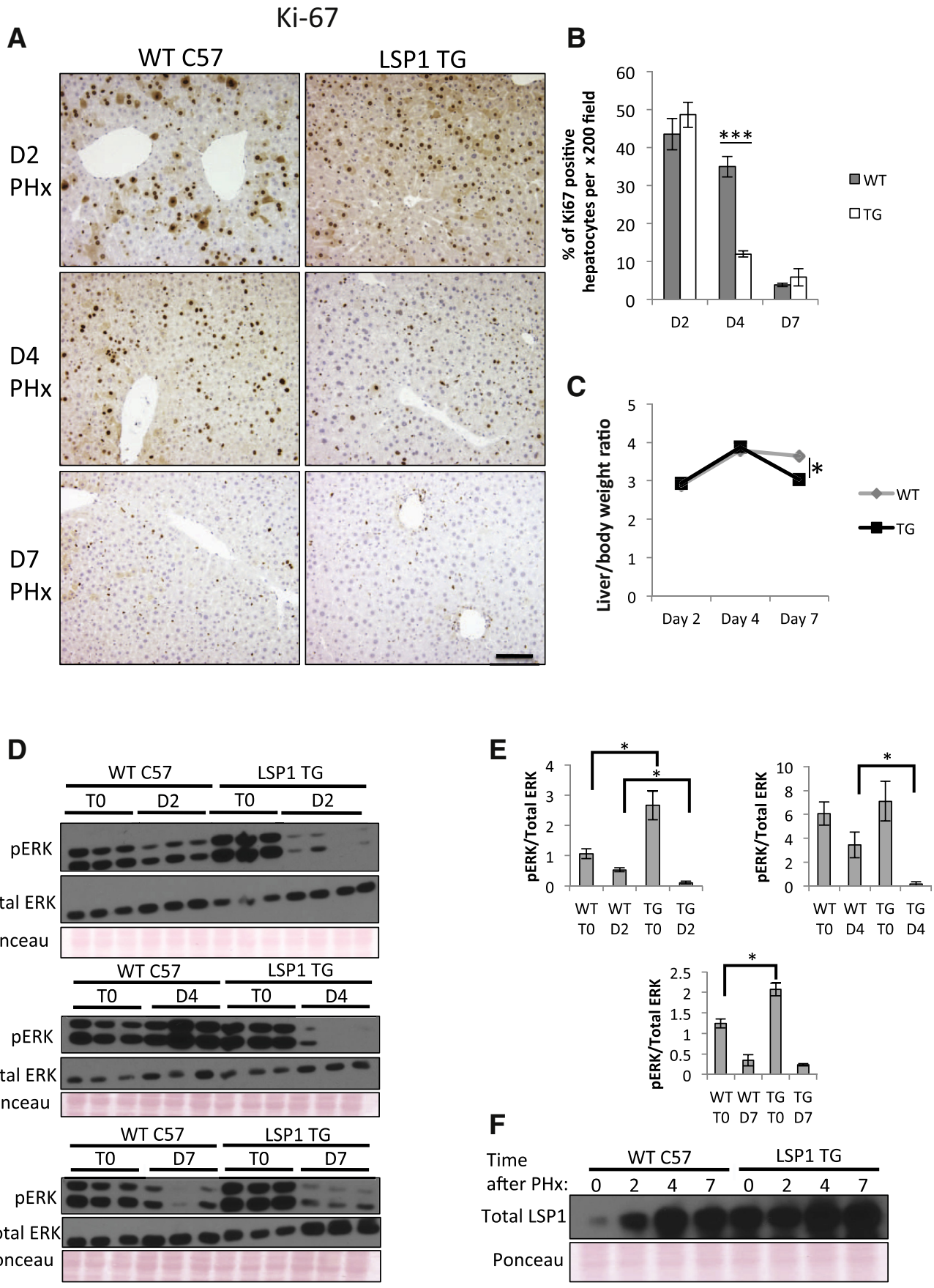
Our previous study demonstrated that LSP1 overexpression through hydrodynamic tail vein injection results in decreased proliferation after PHx[11]; therefore, in this study, it was determined how loss of LSP1 expression would affect liver regeneration after PHx. We hypothesized that the lack of LSP1 expression would cause increased proliferation because LSP1 negatively regulates hepatocellular growth. Therefore, PHx was performed on a global LSP1 KO mouse model (Figure 1F), and the percentage of proliferating hepatocytes was determined at various time points after surgery. No difference in the number of dividing hepatocytes was detected between the WT and KO mice at day 2 after PHx. However, on day 4, there was a significant increase in the percentage of Ki-67−positive hepatocytes in the KO mice (25.7%) compared with the WT mice (15.6%) (Figure 1, A and B). On day 7, the KO livers displayed significantly less proliferating hepatocytes than in the WT mice (Figure 1, A and B). No statistically significant differences were observed in the liver/body weight ratios between the KO and WT mice, despite the increase in dividing hepatocytes on day 4 (Figure 1, A−C). The KO mice display significantly increased ERK activation on day 4 after PHx in comparison to WT mice (Figure 1, D and E). These results demonstrate that loss of LSP1 expression leads to enhanced ERK activation with enhanced hepatocyte proliferation on day 4 after PHx. This is consistent with previous studies demonstrating multiple complex roles of the RAF/MEK/ERK signaling cascade besides cell proliferation, involving cell migration, size control, and cell differentiation.[20,21] We have previously shown that such processes continue beyond day 5 after PHx.[22]

LSP1 KO Hepatocytes in Culture Display Increased Proliferation in the Absence of Growth Factors

Next, hepatocytes were isolated from WT and KO mice to determine the role of LSP1 on proliferation in culture. BrdU incorporation assays were performed on hepatocytes cultured in the presence and absence of hepatocyte growth factor (HGF) and epidermal growth factor (EGF). In the presence of growth factors, there was no difference in the percentage of proliferating hepatocytes between the WT and KO mice at every time point assessed (Figure 2A). However, in the absence of HGF and EGF, the percentage of proliferating KO hepatocytes was increased significantly in comparison to the WT hepatocytes (50% BrdU-positive KO hepatocytes compared with 10% BrdU-positive WT hepatocytes) on days 2 to 4 in culture (Figure 2B). In the presence of growth factors, KO hepatocytes displayed increased ERK phosphorylation at all of the time points assessed in comparison to WT hepatocytes (Figure 2C). Expression of KSR increased in the KO hepatocytes on day 6 in culture compared with WT hepatocytes in both the presence and absence of growth factors. Without growth factors, hepatocytes from KO mice exhibited increased pERK expression on days 4 and 6 in culture as well as increased KSR expression on days 2, 4, and 6 compared with the WT controls (Figure 2D). Absence of hepatocellular LSP1 expression leads to increased proliferation and KSR expression in cultures without growth factors. ERK phosphorylation is enhanced in KO cultures with HGF and EGF and increased at later time points in cultures in the absence of growth factors.

Enhanced Transgenic Expression of LSP1 Specifically in Hepatocytes Results in Decreased Proliferation and pERK Expression during Liver Regeneration

LSP1 functions to inhibit proliferation after PHx by using hydrodynamic tail vein injection of plasmid DNA to exogenously express LSP1 in the liver.[11] However, the function of LSP1 on the kinetics of liver regeneration at later time points after PHx could not be studied because the expression of the plasmid DNA is transient. An additional caveat to this previous study is that, although hydrodynamic injection of plasmid DNA has been demonstrated to mostly target hepatocytes, it is possible that other cell types of the liver are transfected as well and contributed to the observed decrease in hepatocellular proliferation.[23] Therefore, an LSP1 TG mouse model, in which expression of LSP1 is linked with the albumin promoter and α-fetoprotein enhancer, was generated to ensure specific hepatocellular expression. Using this TG model, PHx was performed and the number of proliferating hepatocytes was measured using Ki-67 immunohistochemistry. On day 2 after PHx, no significant differences in the percentage of proliferating hepatocytes were observed between the WT and TG livers (Figure 3, A and B). However, on day 4, there was a


A
Ki-67
WT C57
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% of Ki67 positive hepatocytes per x200 field
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Day 7
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WT C57
LSP1 TG
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D4
D7
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Time after PHx:
0 2 4 7 0 2 4 7
Total LSP1
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significant decrease in the number of Ki-67–positive hepatocytes in the TG livers compared with the WT livers (35% Ki-67 positive in the WT to 12% positive in the TG) (Figure 3, A and B). By day 7, the number of dividing hepatocytes remains unchanged between the WT and TG mice (Figure 3, A and B). No differences were measured in the liver/body weight ratios between the WT and TG mice on days 2 and 4 after PHx; however, on day 7, a significant decrease in the liver/body weight ratio of the TG mice was observed ($P = 0.043$) (Figure 3C). In the TG livers, decreased ERK phosphorylation and a decrease in the ratio of pERK/total ERK, compared with WT, were measured on days 2 and 4 (Figure 3, D and E). LSP1 TG mice displayed increased total LSP1 expression at all time points after PHx (Figure 3F). Increased LSP1 expression in hepatocytes leads to decreased proliferation and ERK activation on day 4 after PHx.

Increased LSP1 Expression Leads to Decreased Proliferation in LSP1 TG Hepatocytes in Culture

Next, livers from both WT and TG mice were perfused to isolate hepatocytes and the cells were cultured with and without HGF and EGF to determine how the expression of LSP1 in hepatocytes affects proliferation *in vitro*. In a BrdU assay to measure proliferation, the TG hepatocytes exhibited decreased cell division in comparison to WT hepatocytes in both the presence and absence of HGF and EGF (Figure 4, A and B). With growth factors, a fourfold decrease was observed on days 2 to 4 and a 2.5-fold decrease was observed on days 4 to 6 in the percentage of dividing TG hepatocytes compared with WT (Figure 4A). In the absence of growth factors, an approximately 2.5-fold difference in the number of proliferating hepatocytes was observed between the TG and WT mice on days 2 to 4 in culture (Figure 4B). These findings demonstrate that LSP1 functions to negatively regulate hepatocellular proliferation in both the absence and presence of growth factors. In cultures with HGF and EGF, pERK expression is decreased in the TG hepatocytes on all of the time points assessed in comparison to WT (Figure 4C). In the absence of growth factors, a decrease was detected in ERK phosphorylation in TG hepatocytes on days 4 and 6 in culture (Figure 4D). TG hepatocytes expressed the greatest amount of KSR at time 0, with expression decreasing on days 2 and 4 and then increasing on day 6 in both the presence and absence of growth factors (Figure 4, C and D). KSR expression in the WT hepatocytes remains relatively unchanged in culture with growth factors and increases on day 4 in the absence of growth factors (Figure 4, C and D). Therefore, in the TG hepatocytes, enhanced LSP1 expression causes decreased pERK and KSR expression as well as inhibition of proliferation.

Loss of LSP1 Expression Leads to Increased Sensitivity to Sorafenib in Hepatoma Cell Lines

Sorafenib, one of the only two US Food and Drug Administration–approved targeted therapies for advanced HCC,[24] targets the Raf–mitogen-activated protein kinase pathway, which is activated in the absence of LSP1 expression. Therefore, it was studied whether the loss of LSP1 expression affects sorafenib sensitivity. LSP1 and scrambled shRNA expressing JM1 rat hepatoma cells were treated with increasing concentrations of sorafenib in culture. Decreased cell numbers were measured in the LSP1 shRNA JM1 cells treated with 20 and 40 μmol/L of sorafenib in comparison to scrambled shRNA control JM1 cells (Figure 5, A and B). An approximately 30% decrease in DNA was measured per plate at 20 μmol/L sorafenib, and approximately 60% less LSP1 shRNA cells were measured in comparison to scrambled control cells at 40 μmol/L sorafenib (Figure 5B). ERK phosphorylation and PCNA expression were decreased in the hepatoma cells lacking LSP1 expression at 20 and 40 μmol/L of sorafenib compared with the sorafenib-treated scrambled control JM1 cells (Figure 5C). Sensitivity to sorafenib was concentration dependent, with the 40 μmol/L concentration displaying the greatest decrease in cell numbers and ERK activation (Figure 5C). LSP1-shRNA–transfected human Hep3B cells also exhibited a significant decrease in cell numbers and pERK1 and PCNA expression when treated with 2 μmol/L sorafenib in comparison to scrambled control (Figure 5, D and E). The increased concentration of sorafenib used in the rat hepatoma studies is because of the relative resistance of rodent cell lines to sorafenib in comparison to cell lines of human origin.[19] Loss of LSP1 expression leads to increased sensitivity to sorafenib treatment with decreased cell numbers and pERK and PCNA expression.

LSP1 KO Mice Treated with Sorafenib Display Significantly Decreased Proliferation after PHx

Next, it was determined if the absence of LSP1 leads to increased sorafenib sensitivity *in vivo* during liver

Figure 3 LSP1 overexpression in transgenic (TG) mice livers results in decreased proliferation and extracellular signal-regulated kinase (ERK) phosphorylation after PHx. **A:** Representative images of Ki-67 immunohistochemistry of wild-type (WT) C57 and LSP1 transgenic (TG) livers at various time points after PHx. **B:** Quantification of the percentage of Ki-67–positive hepatocytes. **C:** Liver/body weight ratios of WT and TG mice after PHx. **D:** Western blots of phosphorylated ERK (pERK) and total ERK expression in WT and TG livers at days (D) 2, 4, and 7 after PHx. Each lane represents a different animal. **E:** Quantification of the ratio of pERK/total ERK from the Western blots in **D**. **F:** Western blot of LSP1 expression in WT and TG mouse livers at various time points after PHx. Ponceau S was used as a loading control in **D** and **F**. $*P < 0.05$, $***P < 0.001$. Scale bar = 100 μm (**A**). Original magnification, ×200 (**A**). T0, the beginning of the experiment.

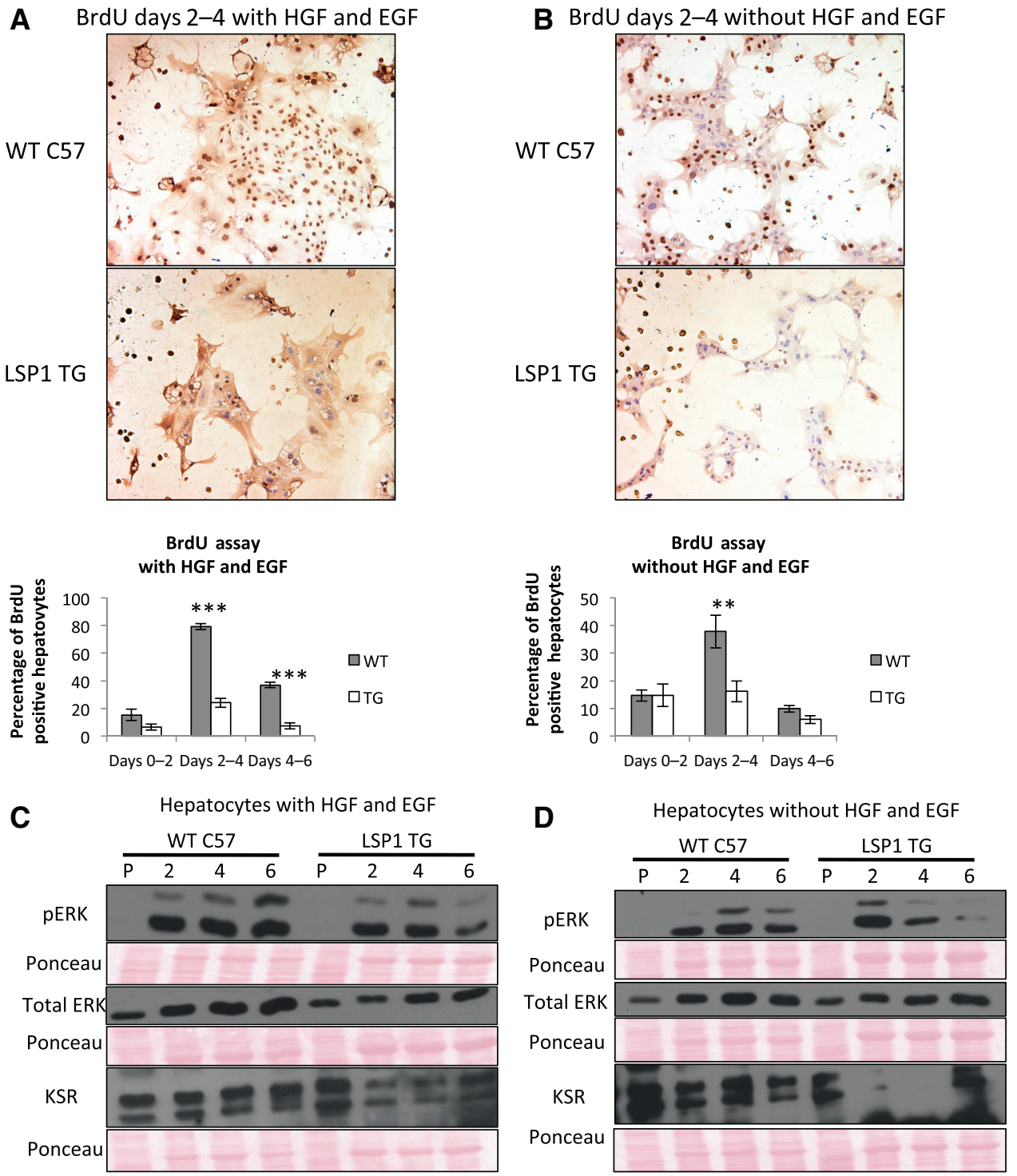

Figure 4 Hepatocytes from LSP1 transgenic (TG) mice display decreased cellular division and extracellular signal-regulated kinase (ERK) activation in both the presence and absence of growth factors, hepatocyte growth factor (HGF), and epidermal growth factor (EGF). Representative images of bromodeoxyuridine (BrdU) immunohistochemistry on days 2 to 4 and quantification of the percentage of BrdU-positive wild-type (WT) and TG hepatocytes in culture with (**A**) and without (**B**) HGF and EGF. Western blot analysis of phosphorylated ERK (pERK), total ERK, and kinase suppressor of Ras (KSR) expression in WT and TG hepatocytes cultured in the presence (**C**) and absence (**D**) of growth factors. Ponceau S was used as a loading control. $^{**}P < 0.01$, $^{***}P < 0.001$ versus WT. Original magnification, ×100. P, hepatocyte pellet.

regeneration after PHx. LSP1 KO and WT 129/svJ mice were treated with 100 mg/kg sorafenib the day before and 2 hours before PHx, and the livers were harvested on day 2 after surgery. Previous literature has demonstrated that the average half-life of sorafenib is 20 to 48 hours; therefore, sorafenib should be bioavailable in the mice until day 2 after surgery.[25] A significant decrease in the percentage of Ki-67–positive hepatocytes was observed in

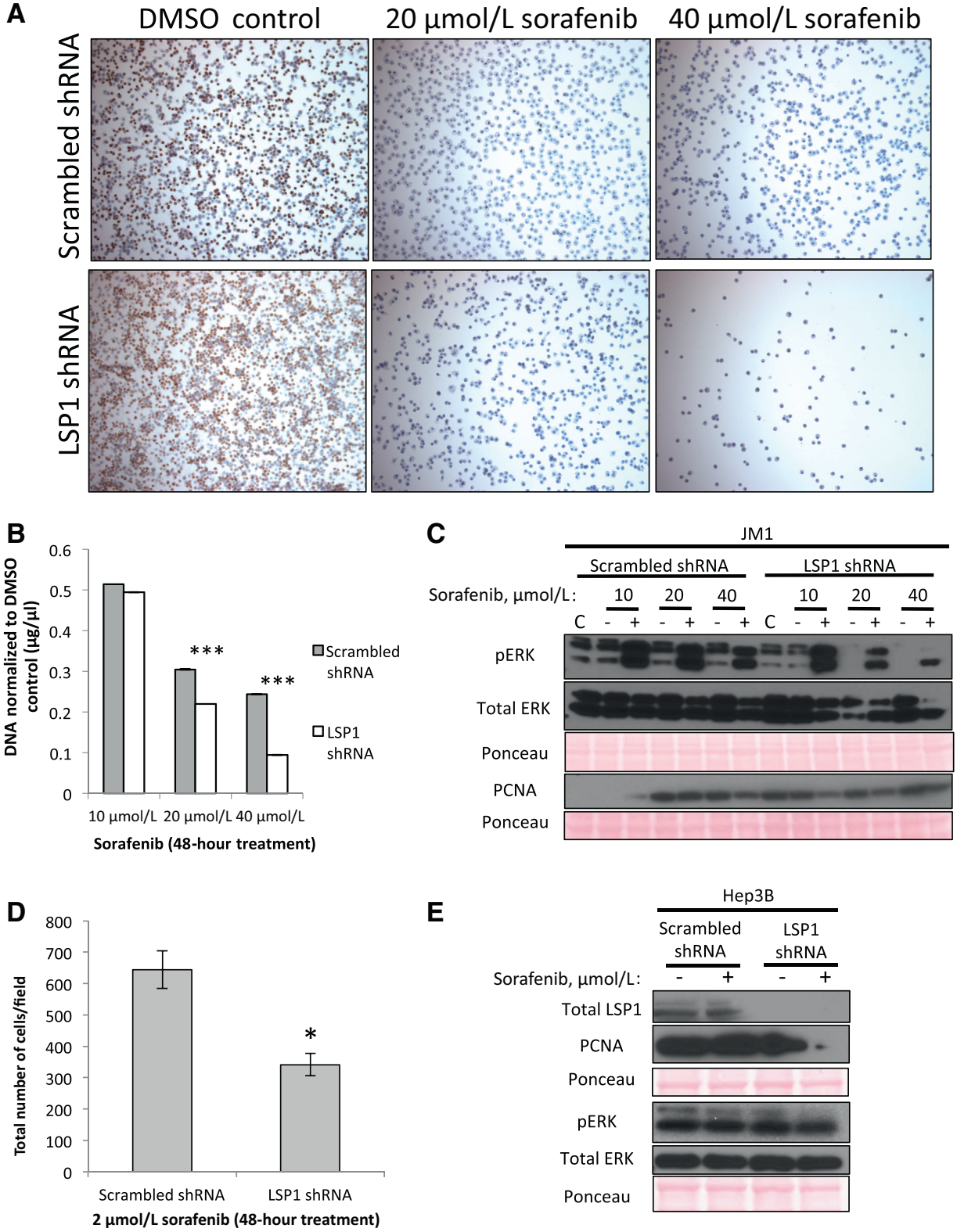

Figure 5 Loss of LSP1 expression in the JM1 rat hepatoma cell line leads to increased sorafenib sensitivity with decreased cell numbers and phosphorylated extracellular signal-regulated kinase (pERK) expression. **A:** Representative bright-field images of scrambled and LSP1 shRNA JM1 hepatoma cells treated with dimethyl sulfoxide (DMSO), 20 µmol/L sorafenib, and 40 µmol/L sorafenib. **B:** Quantification of the concentration of DNA per well normalized to the DMSO control. **C:** Western blot analysis of pERK, total ERK, and proliferating cell nuclear antigen (PCNA) expression in scrambled and LSP1 shRNA JM1 cells. Ponceau S was used as a loading control. **D:** Quantification of the total number of cells per field of the LSP1 and scrambled control shRNA transfected Hep3B treated with 2 µmol/L sorafenib for 48 hours. **E:** Western blot analysis of total LSP1, PCNA, pERK, and total ERK in the LSP1 and scrambled shRNA transfected Hep3B treated with 2 µmol/L sorafenib or DMSO as control. $^{*}P < 0.05$, $^{***}P < 0.001$ versus scrambled. Original magnification, ×200 (**A**). −, DMSO control; +, sorafenib treated; C, untreated control (10, 20, and 40 µmol/L).

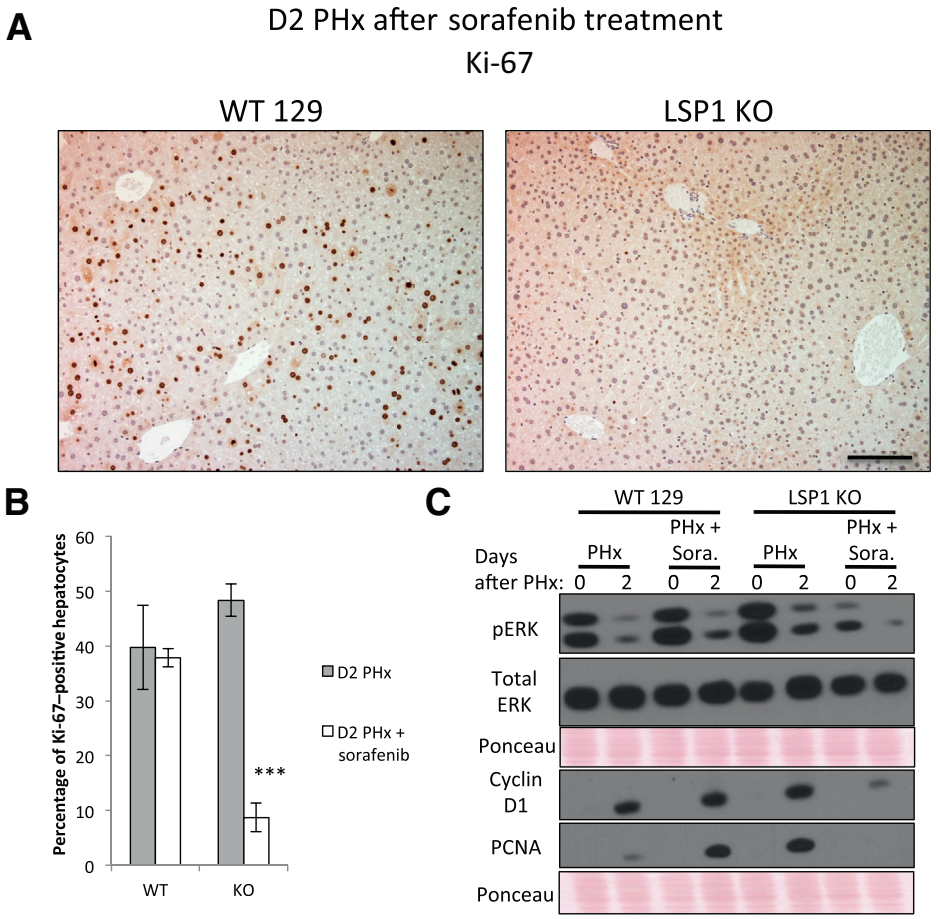

Figure 6 LSP1 knockout (KO) mice treated with sorafenib display decreased proliferation and phosphorylated extracellular signal-regulated kinase (pERK), cyclin D1, and proliferating cell nuclear antigen (PCNA) expression on day (D) 2 after PHx. **A:** Representative Ki-67 immunohistochemistry images from wild-type (WT) 129 and LSP1 KO livers on day 2 after PHx. **B:** Quantification of Ki-67–positive hepatocytes in WT and LSP1 KO livers after PHx only and PHx with sorafenib pretreatment. **C:** Western blot analysis of pERK, total ERK, cyclin D1, and PCNA in WT and KO livers after PHx only and PHx with sorafenib. Ponceau S was used as a loading control. ***$P < 0.001$ versus without sorafenib. Scale bar = 100 μm (**A**). Original magnification, ×200 (**A**). Sora., sorafenib.

the sorafenib-treated LSP1 KO mice compared with WT controls (Figure 6, A and B). In the WT controls, there was no effect of sorafenib on hepatocellular proliferation (Figure 6B). However, the LSP1 KO mice pretreated with sorafenib exhibited a fivefold decrease in the percentage of dividing hepatocytes compared with day 2 after PHx only (Figure 6B). Sorafenib-treated LSP1 KO mouse livers display decreased pERK, cyclin D1, and PCNA expression on day 2 after PHx, whereas WT mice treated with sorafenib display slightly increased ERK phosphorylation as well as cyclin D1 and PCNA expression 2 days after PHx (Figure 6C). These results indicate that the absence of LSP1 expression causes decreased proliferation and pERK expression in response to sorafenib after PHx.

Discussion

In human hepatocellular carcinoma, LSP1 has the highest number of small deletions, as assessed by copy number ratio analysis.[6] In addition to the small deletions of the carboxy terminal portion of LSP1 (47% of HCC), another 5% of cases have amplification of only the carboxy terminal portion of LSP1.[11] Amplification of the carboxy terminal portion of LSP1 acts as a dominant negative, blocking sites that are bound normally by the intact LSP1 protein.[10,26] LSP1 affects HCC cell line proliferation and migration, and its binding to F-actin as well as LSP1 phosphorylation occur as a regulated phenomenon during liver regeneration.[11]

HCC patients with low LSP1 expression display decreased overall survival and decreased disease-free survival in comparison to patients with high LSP1 expression. LSP1 expression reduces tumor growth *in vivo* and leads to decreased ERK activation in hepatoma cell lines *in vitro*.[12]

All of these findings are of interest to LSP1 as a biomarker for HCC behavior per se; they are also interrelated to the current therapeutic method for human HCC. LSP1 acts primarily by regulating the activation of the RAF/MEK/ERK signaling pathway. The latter is bound to the scaffold protein KSR. The exact site of binding of LSP1 (KSR or RAF) is not clearly determined. The RAF/MEK/ERK pathway, however, is the main target of sorafenib, currently the most effective chemotherapeutic agent for human HCC. Data from clinical studies have shown that only approximately 50% of patients with HCC respond to sorafenib. We hypothesized that the ERK activation regulator LSP1 (also inactivated in approximately 50% of human HCC cases)[6] is the key protein regulating the effectiveness of sorafenib. The current study aimed to

determine the relationship between LSP1 expression, ERK activation, and sensitivity to sorafenib to assess the validity of this hypothesis.

It is well known that ERK activation, in addition to cell proliferation, also affects multiple other processes.[20,21] Thus, a simple correlation between ERK activation and hepatocyte proliferation was not expected in all instances. The data demonstrate that, although there is a certain relationship between ERK activation and hepatocyte proliferation, the relationship is complex. This is shown in the findings with liver regeneration (Figures 1 and 3) and cell cultures (Figures 2 and 4). The overall data show, however, that there is a different pattern in regulation of ERK activation during liver regeneration between LSP1 WT, KO, and TG mice, especially at the later stages of liver regeneration in the KO mice and at all stages in the TG mice. There is overall enhanced ERK activation in KO mice and decreased ERK activation in TG mice. Notably, the latter had a decreased liver/body weight ratio at day 7 after PHx, which is rarely observed in liver regeneration studies.

The data in Figures 2 and 4 with primary cultures also demonstrate that, although the correlation between ERK activation and hepatocyte proliferation is not always linearly simple, there is enhanced proliferation of the KO hepatocytes, suppressed proliferation of TG hepatocytes, and different patterns of ERK activation between WT, KO, and TG cultures.

The role of LSP1 as a regulator of the sensitivity of neoplastic hepatocytes to sorafenib was directly assessed (Figures 5 and 6). Absence of LSP1 enhances the sensitivity of JM1 and Hep3B HCC cells to sorafenib (Figure 5), and in the absence of LSP1, the peak (day 2) of hepatocyte proliferation during liver regeneration is much more affected by sorafenib treatment in the LSP1 KO mice than that of the WT mice.

Our data are the first to demonstrate that there is validity to the hypothesis that sorafenib, mainly targeting RAF kinase,[4] should be more effective in HCC in which LSP1 function has been eliminated. Further studies are required to demonstrate whether this would be more valid when LSP1 function is eliminated through carboxy terminal deletion (47% of cases) or dominant negative acting carboxy terminal amplification (5% of HCC).[6] Either way, there is reasonable basis from our studies to examine the role of LSP1 as a critical biomarker in determining whether to administer sorafenib in HCC patients, given the serious adverse effects of the treatment.

Recently, it was announced that another chemotherapeutic agent, regorafenib, has been approved for chemotherapy of HCC. Notably, this agent acts through the RAF/MEK/ERK pathway as well.[27,28] Thus, it is likely that LSP1 will be a useful biomarker to use for HCC treatment decisions, not only for sorafenib but also for any other new chemotherapeutic agents acting through the RAF/MEK/ERK pathway. It is also worth exploring whether this may be true not only for HCC but for other neoplasms in which chemotherapy via regulation of the ERK pathway becomes a chemotherapeutic approach.

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