AWARD NUMBER: W81XWH-17-1-0342

TITLE: LSP1 Involved in Liver Regeneration Termination, Deleted in 50% of Human

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CONTRACTING ORGANIZATION: University of Pittsburgh

REPORT DATE: October 2020

TYPE OF REPORT: Final Technical Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2020	Final Technical Report	01Jul2017-30Jun2020

4. TITLE AND SUBTITLE

LSP1 Involved in Liver Regeneration Termination, Deleted in 50% of Human Liver Cancer and Major Determinant of Response to Sorafenib

5a. CONTRACT NUMBER
W81XWH-17-1-0342

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our studies on LSP1 and its role in hepatocyte signaling pathways aimed at control of growth and hepatocyte differentiation, started in 2011, with a paper unexpectedly demonstrating that LSP1 protein carboxyterminal was deleted in approximately 46% of hepatocellular carcinomas (HCC) and amplified in 5% of HCC, with the latter amplification acting as a dominant negative to eliminate the effects of the normal protein. While there was abundant literature on LSP1 in leucocytes and lymphocytes, there was no literature whatsoever to link it to hepatocyte growth and function. In a subsequent paper published in 2014, we demonstrated that upregulation of LSP1in an HCC cell line dramatically suppressed cell proliferation while downregulation in an HCC cell line expressing LSP1 increased cell proliferation and cell migration. Forced expression of LSP1in mouse liver also decreased liver regeneration after partial hepatectomy. The data obtained supported the hypothesis that LSP1overall is a growth suppressor for normal hepatocytes and HCC. In a subsequent paper published in 2018, we published some of the data generated by the current DoD award. (Please note: We gave credit to the DoD award for support of the part of the research published in that paper by acknowledging DoD and stating the CDMRP Log Number: CA160119). We used mice with transgenic overexpression of LSP1 (LSP1 TG) and genomic deletion "knockout" of LSP1 (LSP1 KO), and showed that the TG mice had suppressed liver regeneration whereas the KO mice had enhanced regeneration. In the latter paper, we showed that LSP1controls a very important signaling pathway, RAF/MEK/ERK, which is held together by the scaffolding protein KSR. That pathway receives signaling from many upstream proteins and regulates phosphorylation of ERK1 and ERK2, important components of optimization and regulation of many cellular pathways in all cell types, including cell growth and differentiation. The RAF protein is one of the targets of Sorafenib, the main chemotherapeutic agents shown to prolong survival and disease-free interval in human patients with HCC. Our data showed that suppression of expression of LSP1 in human HCC cell lines enhanced their sensitivity to Sorafenib and that KO mice treated with Sorafenib had severely suppressed liver regeneration compared to control "wild type" (WT). An overall summary of our main findings will be presented in this abstract. Details of our findings in relation to each of the specific aims and the various tasks under each of the specific aims will be given in the section of Accomplishments, below. Based on the data obtained from the studies funded this award, we have found the following:

1. In normal liver, following liver regeneration after partial hepatectomy, LSP1 acts as a growth suppressor. The same was seen with hepatocytes in primary culture. Liver regeneration in LSP1 KO mice was more robust, but, in support of the main hypothesis, it was substantially inhibited by Sorafenib compared to WT (control) mice.
2. In several HCC cell lines, LSP1 also acted as growth suppressor. Suppression of LSP1 enhanced HCC cell line growth but made the cells more responsive to Sorafenib.
3. Contrary to our hypothesis, LSP1 elimination did not result in disassembly of the KSR[RAF/MEK/ERK1/2] complex. The complex continued to be assembled, but the phosphorylation of ERK was more sensitive to Sorafenib.
4. The effects of inhibitors of PKCβ1 and MAPkAPK2 (a.k.a. MK2), the main enzymes involved in LSP1 phosphorylation, were variable, depending on different HCC cell lines. All inhibitors tried were relatively weak. Stronger inhibitors, as they may become available in the future, could have an effect against HCC. We extended the studies using silencing RNA against each of the two enzymes. Elimination of MK2 was more effective in decreasing LSP1 phosphorylation. We found, however, that, contrary to our hypothesis, decreased LSP1 phosphorylation actually led to decreased ERK activation and decreased cell proliferation. This does open the possibility of designing therapeutic applications against HCC using silencing RNA against MK2 and/or PKCβ1, based on lipid nanoparticles to be used for their in vivo administration.
5. Carcinogenesis experiment . We used diethylnitrosamine (DEN) as the standard, most often used, chemical carcinogen to induce liver tumors in rodents. We administered DEN at 14 days after birth, to LSP1 TG, LSP1 KO and heir control WT counterparts specific for the strains. We administered Sorafenib to the mice in these four groups after the tumors had appeared in the livers, at about 5 months after DEN administration. We chose this approach because administration of Sorafenib to already formed tumors is similar to the human reality, since Sorafenib is never administered to prevent liver tumors; it is only used a chemotherapeutic agent. The mice were injected with DEN 14 days after birth and the second protocol was applied, keeping track of the different dates for each mouse. The results of the DEN carcinogenesis were not conclusive. Data details are provided in the Accomplishments section, below. The reasons for the results may be related to the fact that the carcinogen DEN, used as standard in mouse liver carcinogenesis studies, does induce mutations in Ras gene and it fundamentally alters the relationship between RAS protein and Raf protein, the site of action of sorafenib and the site of attachment of LSP1. Furthermore, as cancer cells develop, they do undergo major genomic changes. We found that many of the liver tumors seen in LSP1 TG mice had actually lost the LSP1 transgene and thus their behavior was not constrained by the assumptions made based on the genomic make-up of the liver from which they arose

15. SUBJECT TERMS

None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	36	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION:

In previous studies, we had discovered that the protein LSP1 (Leukocyte Specific Protein 1) is deleted in 46% of human liver cancers. We subsequently documented that LSP1 suppresses liver regeneration and migration of hepatocellular carcinoma (HCC) cell lines. Absence of LSP1 rendered several HCC cell lines more susceptible to Sorafenib. The purpose of this grant award was to determine whether LSP1 could be used for diagnostic and therapeutic purposes for HCC and whether its presence or absence determined the response to Sorafenib, the major chemotherapeutic agent against human HCC.

2. KEYWORDS:

Hepatocellular carcinoma: (HCC); LSP1: Leukocyte specific protein 1; LSP1 KO: LSP1 knockout mice; LSP1 TG: LSP1 transgenic mice; WT: Wild Type, the control mice for each of the two strain in which TG and KO mice were derived from. PKCβ1: Protein Kinase C beta 1;Sorafenib; KSR (scaffold protein holding together the ERK activation complex); [RAF/MEK/ERK] (the kinase cascade functioning as the ERK activation complex); DEN: diethylnitrosamine.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The major goals were formulated in the two specific aims:
Specific Aim 1. Investigate the mechanisms by which LSP1expression attenuates response to Sorafenib and design diagnostic methodology to determine the appropriate use of Sorafenib based on LSP1 expression.

A. Demonstrate the effects of LSP1 on Sorafenib activity on normal hepatocytes.
B. Effects of Sorafenib on DEN induced liver carcinogenesis in LSP1 KO and LSP1 TG mice.
C. Investigate the molecular mechanism by which LSP1 interferes with the Sorafenib-RAF interaction
D. Does the amplified carboxy-terminal of LSP1 act as a dominant negative to inhibit LSP1 function
E. Development of fluorescent in situ hybridization (FISH) diagnostic tools for LSP1 C-terminal deletion or amplification using paraffin embedded archival material form human HCC.

Specific Aim 2. Phosphorylation of LSP1 inhibits its capacity to decrease hepatocyte proliferation. Investigate the effects of inhibition of LSP1 phosphorylation in HCC expressing intact LSP1 as a novel therapeutic approach for human HCC.

What was accomplished under these goals?

Specific Aim 1A. Demonstrate the effects of LSP1 on Sorafenib activity on normal hepatocytes.

For this major task, we wanted to study the impact of Sorafenib on regulation of growth of normal hepatocytes in primary culture and in whole liver during liver regeneration. We assessed the effects of LSP1 per se as a regulator of normal hepatocyte proliferation during liver regeneration of wild type mice (WT). transgenic mice (LSP1 TG) and mice with germ-line elimination of LSP1 (LSP1 KO). (Please note: the WT strains were different for the TG and the KO mice). We then assessed the effect of Sorafenib directly on hepatocytes during liver regeneration in KO and TG mice (using their WT as controls). We also assessed hepatocytes in primary culture. The growth of normal hepatocytes in culture from each of the four strains was assessed, to determine the effect of LSP1 on growth driven by growth factors (HGF and EGF) or spontaneous growth in culture medium not supplemented by growth factors.

Our results from these studies showed that hepatocytes of LSP1 KO had enhanced proliferation during liver regeneration after partial hepatectomy (PHx) as well in primary culture, with or without growth factors. We demonstrated that this correlated with the enhanced activity of the RAF/MEK/ ERK pathway, the target of Sorafenib. Figure 1, shows that there is enhanced liver regeneration in mice with germline elimination of LSP1 (LSP1 KO). This is most evident in days 2 and 4 after partial hepatectomy (PHx)., Figures 1E and 1F show

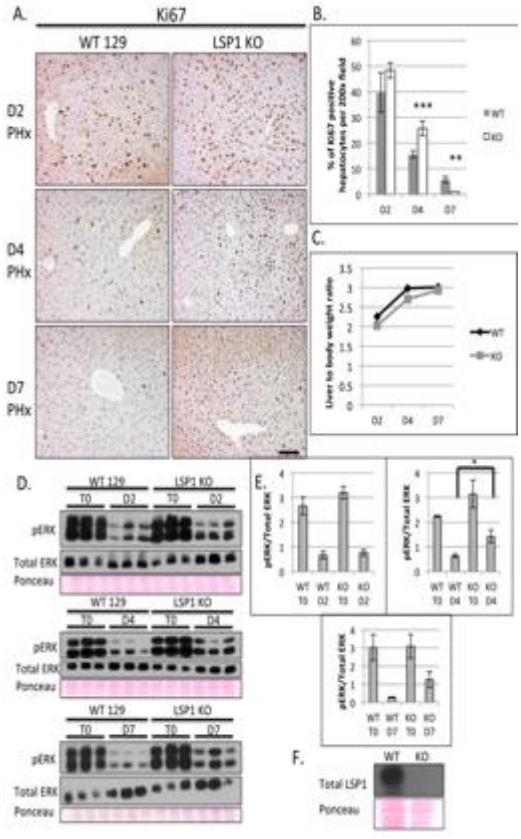


Figure 1: LSP1 KO livers display increased proliferation and pERK expression after PHx. A. Representative images of Ki67 immunohistochemistry of WT 129 (left) and LSP1 KO (right) livers at various time points after PHx. 200x magnification. Scale bar = 100μm. B. Quantification of the percentage of Ki67 positive hepatocytes C. Liver to body weight ratios of WT and KO mice following PHx. D. Western blots of pERK, and total ERK expression in WT and KO livers at day 2 (top panels), day 4 (middle panels) and day 7 (bottom panels) after PHx. Each lane represents a different animal. Ponceau S was utilized as a loading control. E. Quantification of the ratio of pERK/Total ERK from the western blots in D. *, $p<0.05$. F. Western blot of LSP1 expression in baseline WT and KO mouse livers. Ponceau S was utilized as a loading control.

the dramatic enhancement of ERK1 phosphorylation in the absence of LSP1. In Figure 2, LSP1 KO hepatocytes in culture in the absence of the hepatocyte growth factors HGF and EGF displayed spontaneous tendency to grow on their own, again documenting that LSP1 itself is a growth suppressor by regulating the ERK pathway.

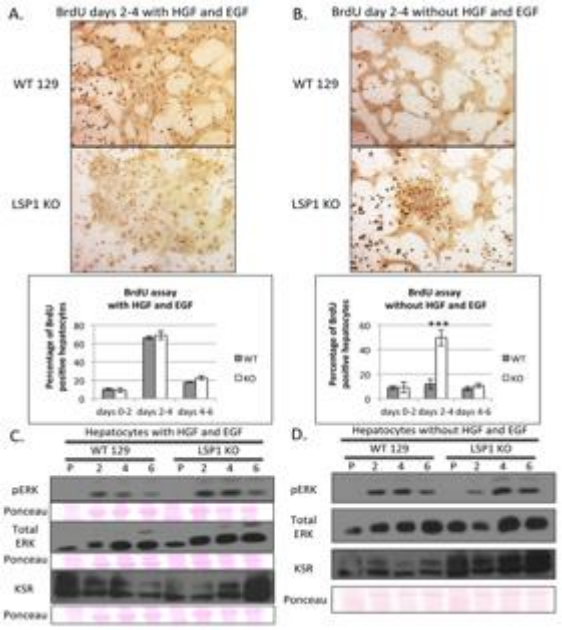


Figure 2: LSP1 KO hepatocytes exhibit enhanced proliferation and ERK phosphorylation in the absence of growth factors, HGF and EGF. Representative images of BrdU immunohistochemistry on days 2-4 and quantification of the percentage of BrdU positive WT and KO hepatocytes in culture A. with HGF and EGF and B. without HGF and EGF. Ponceau S was utilized as a loading control.

The opposite findings were seen in mice with transgenic over-expression of LSP1 (LSP1 TG), in Figures 3 and 4. **The overall results of figures 1-4 prove that LSP1 protein is indeed a growth suppressor for hepatocytes, both in culture and in the whole animal, and that it acts through the RAF/MEK/ERK signaling pathway.**

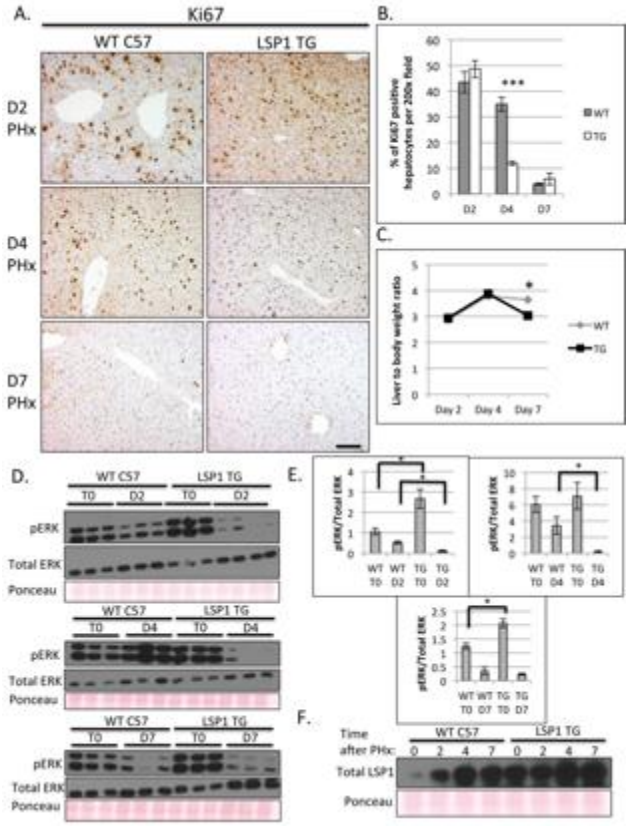


Figure 3: LSP1 overexpression in TG mice livers results in decreased proliferation and ERK phosphorylation following PHx. A. Representative images of Ki67 immunohistochemistry of WT C57 (left) and LSP1 TG (right) livers at various time points after PHx. 200x magnification. Scale bar = 100μm. B. Quantification of the percentage of Ki67 positive hepatocytes. C. Liver to body weight ratios of WT and TG mice following PHx, *, p<0.05. D. Western blots of pERK and total ERK expression in WT and TG livers at day 2 (top panels), day 4 (middle panels) and day 7 (bottom panels) after PHx. Each lane represents a different animal. Ponceau S was utilized as a loading control. E. Quantification of the ratio of pERK/Total ERK from the western blots in D, *, p<0.05. F. Western blot of LSP1 expression in WT and TG mouse livers at various time points after PHx. Ponceau S was utilized as a loading control.

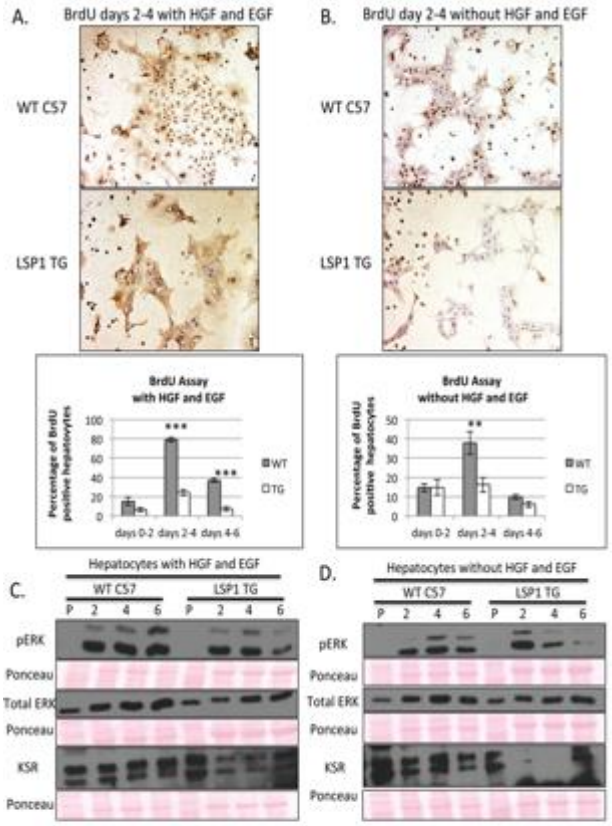


Figure 4: Hepatocytes from LSP1 TG mice displayed decreased cellular division and ERK activation in both the presence and absence of growth factors, HGF and EGF. Representative images of BrdU immunohistochemistry on days 2-4 and quantification of the percentage of BrdU positive WT and TG hepatocytes in culture A. with HGF and EGF and B. without HGF and EGF. Western blot analysis of pERK, total ERK, and KSR expression in WT and TG hepatocytes cultured C. in the presence and D. absence of growth factors. P= hepatocyte pellet. Ponceau S was utilized as a loading control

Having assessed the role of LSP1 in normal hepatocytes in Figs. 1-4, we then proceeded to assess the effect of LSP1 on efficacy of Sorafenib. The first component (RAF) of the LSP1 targeted and suppressed pathway, is the target of Sorafenib. The following two figures (FIG. 5 and 6) demonstrate that Sorafenib is more effective in the absence of LSP1. Fig. 5 compares the effects of Sorafenib on hepatocytes during liver regeneration, in WT mice and KO mice (germ-line absence of LSP1). As was shown in Figure 2, LSP1 KO mice actually have enhanced liver regeneration. Fig. 5, however, shows that in the absence of LSP1, regenerating proliferating hepatocytes become more sensitive to Sorafenib! The difference between the effects of Sorafenib on regenerating hepatocytes between WT and KO mice at the peak of hepatocyte proliferation (Day 2 after hepatectomy) is dramatic. In the absence of LSP1 in the KO mice, Sorafenib is much more effective, **The data shown above demonstrate clearly that LSP1 does control the RAF/MEK/ERK pathway during hepatocyte proliferation. In addition, and highly relevant to the projects funded by this award, they also show that LSP1 indeed blocks the effects of Sorafenib on cell proliferation**. The data in Figures 1-5 were all gathered with normal hepatocytes, from whole animals or from cell cultures. The data in Figure 6 were intended to directly assess the effect of LSP1 on the efficacy of Sorafenib in HCC cells (rat and human). Silencing RNA against LSP1 was used to "knock down" the LSP1 mRNA concentration, causing intense decrease in the expression of the LSP1 protein. ("Scrambled" ShRNA, with a completely random sequence, was used as control). The data demonstrate that in rat HCC cell line JM1, treatment with silencing RNA against LSP1 (LSP1 shRNA) renders the HCC cells much more sensitive to Sorafenib. Please notice the big difference in the number of cell killed at 40μm Sorafenib. The same results are seen in the human HCC cell line Hep3B (Fig. 5E). The counteractive effect of LSP1 on the cytocidal effects of Sorafenib was also seen (Fig. 5B) in the whole animal, by examining the effects of Sorafenib on liver regeneration after partial hepatectomy. **Based on the above, from the normal animal studies (Figure 5), we should expect that indeed Sorafenib would be more effective on HCC in which LSP1 (carboxyterminal) is deleted. This is verified by the findings of Figure 6.**

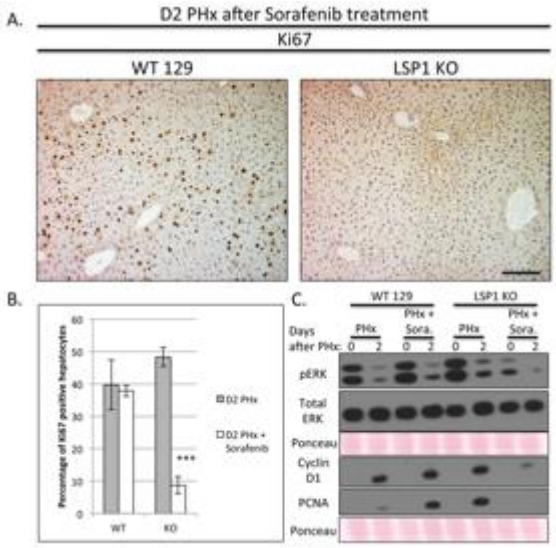


Figure 5: LSP1 KO mice treated with Sorafenib display decreased proliferation and pERK, cyclin D1 and PCNA expression on day 2 after PHx. A. Representative Ki67 immunohistochemistry images from WT129 and LSP1 KO livers on day 2 after PHx. 200x magnification. Scale bar= 100μM. B. Quantification of Ki67 positive hepatocytes in WT and LSP1 KO livers after PHx only and PHx with Sorafenib pre-treatment, ***, p<0.0001. 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 utilized as a loading control.

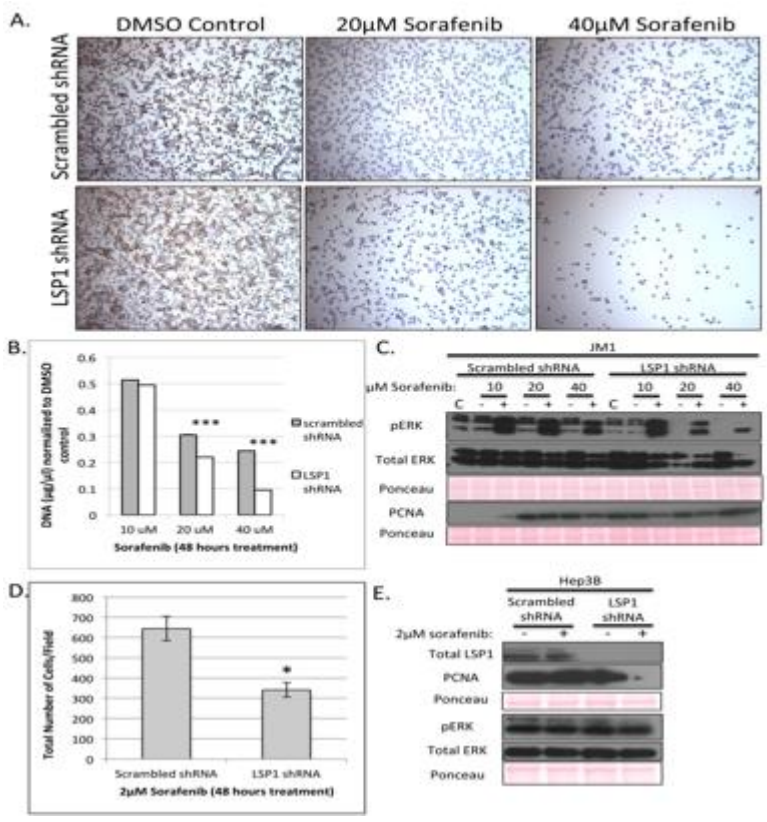


Figure 6: Loss of LSP1 expression in the JM1 rat hepatoma cell line leads to increased Sorafenib sensitivity with decreased cell numbers and pERK expression. A. Representative bright field images of scrambled shRNA (top row) and LSP1 shRNA (bottom row) JM1 hepatoma cells treated with DMSO (control, left column), 20μM Sorafenib (middle column), and 40μM Sorafenib (right column). 200x magnification. B. Quantification of the concentration of DNA per well normalized to the DMSO control, ***, p<0.0. C. Western blot analysis of pERK, total ERK, and PCNA expression in scrambled and LSP1 shRNA JM1 cells. C= untreated control, - = DMSO control, + = Sorafenib treated (10μM, 20μM, and 40μM). Ponceau S was utilized 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 2μM Sorafenib for 48 hours, *, p< 0.05. E. Western blot analysis of total LSP1, PCNA, pERK, and total ERK in the LSP1 and scrambled shRNA transfected Hep3B treated with 2μM Sorafenib or DMSO as control.

Based on the above, from the normal animal studies (Figure 5), we should expect that indeed Sorafenib would be more effective on HCC in which LSP1 (carboxyterminal) is deleted. This is verified by the findings of Figure 6.

Specific Aim 1B. Effects of Sorafenib on DEN induced liver carcinogenesis in LSP1 KO and LSP1 TG mice.

Each genetically modified mouse group in regards to LSP1 KO and LSP1 TG is derived from different mouse strains (129/svj for LSP1 KO and C57BL6 for LSP1 TG). Thus, the carcinogenesis experiments assessing the effects of Sorafenib and LSP1 on tumor growth were comprised of four groups (LSP1 KO and the WT 129/svj, and LSP1 TG and WT C57BL6). Given the fact that these mice were bred in our facility and births of new mice were coming in at a slow pace (especially for LSP1 TG), we kept very close calendar based timetables to ensure that the same chronology for tumor assessment and Sorafenib treatment was done for all mice in this study. We entered mice into this protocol in each of the four groups as they became available, and we administered DEN (20 mg/kg) to each mouse at day 14 after birth. We sacrificed mice when they reached the point when tumors in reliable size and numbers appear. We monitored the mice for tumor numbers and size at 6-7 months using ultrasound and did not visualize many tumors. Therefore, we decided to wait and sacrifice the animals at 11 months. We started administering Sorafenib (60mg/kg/day) or vehicle control at ten months after DEN injection, same for all mice, when tumors were reliably demonstrable, by ultrasound and direct inspection via opening the abdominal cavity in selective representative mice from each group.

Results: Following completion of the one-month treatment with Sorafenib (60mg/kg/day) or vehicle control, we harvested the livers from all of the animals and measured liver to body weight ratios as well as counted and measured tumors in all of the livers.

LSP1 KO mice: Average tumor diameter was significantly smaller in the vehicle treated LSP1 KO mice in comparison to the WT 129 controls and they were fewer tumors in the KO. LSP1 WT 129 mice displayed a significant decrease in tumor size when treated with Sorafenib, which provides more evidence that the WT mice responded better to Sorafenib in comparison to the LSP1 KO mice. Western blot analysis of whole liver lysates from the LSP1 KO and WT mice demonstrated that the WT129 mice displayed increased PCNA expression in comparison to the KO, indicating increased proliferation in the WT. We also hypothesized that the LSP1 KO mice would exhibit more and larger tumors in comparison to WT but our data indicate the opposite, which is that the KO mice seemed to be more resistant to DEN induced hepatocarcinogenesis. One explanation for the discrepancy in the results from previous experiments is that these LSP1 KO mice are a global LSP1 knockout, therefore loss of LSP1 in a cell type other than the hepatocyte (e.g immunology related cells such as lymphocytes and macrophages) may be more active in their anti-tumor effects. Previous studies on LSP1 when it was first discovered in leukocytes and lymphocytes has indicated that these cells had enhanced proliferation and migration compared to the wild type mice. This would make them more effective in their antitumor effects. The results are shown in Fig. 7, below.

Figure 7.

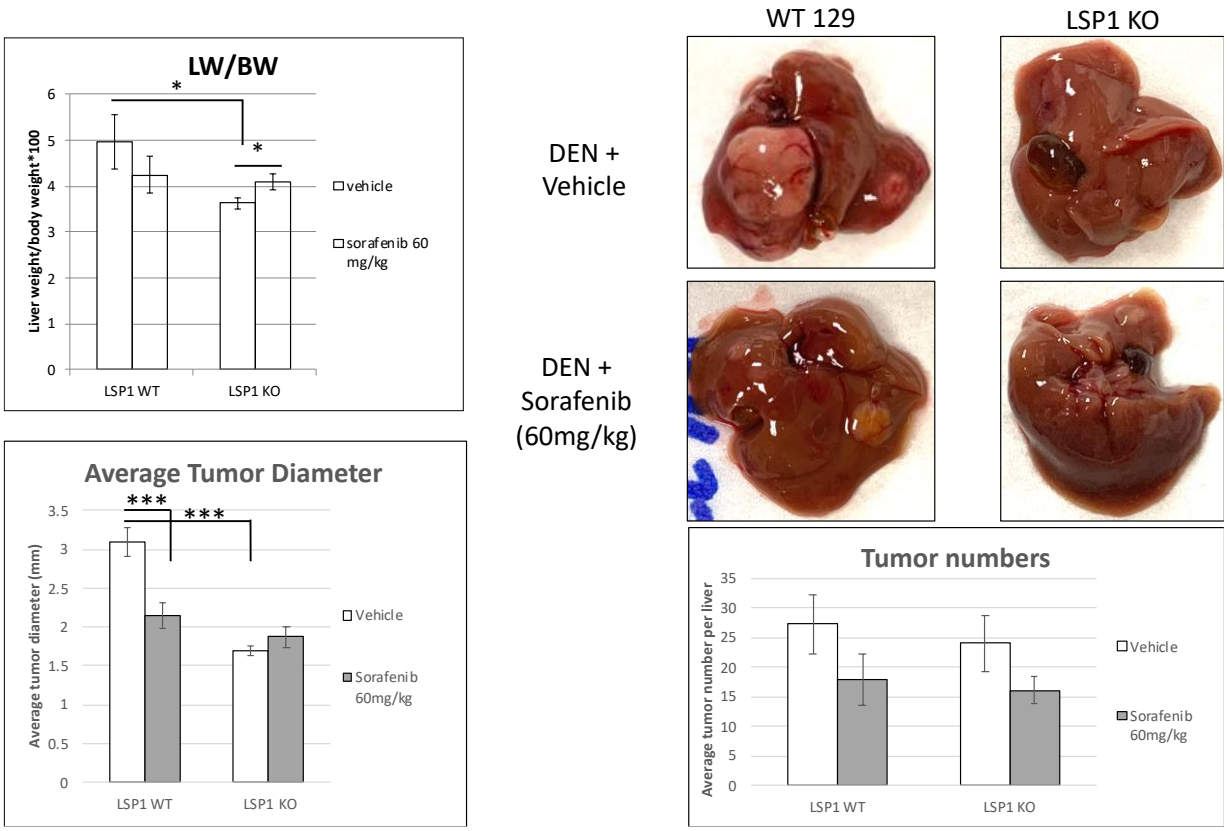


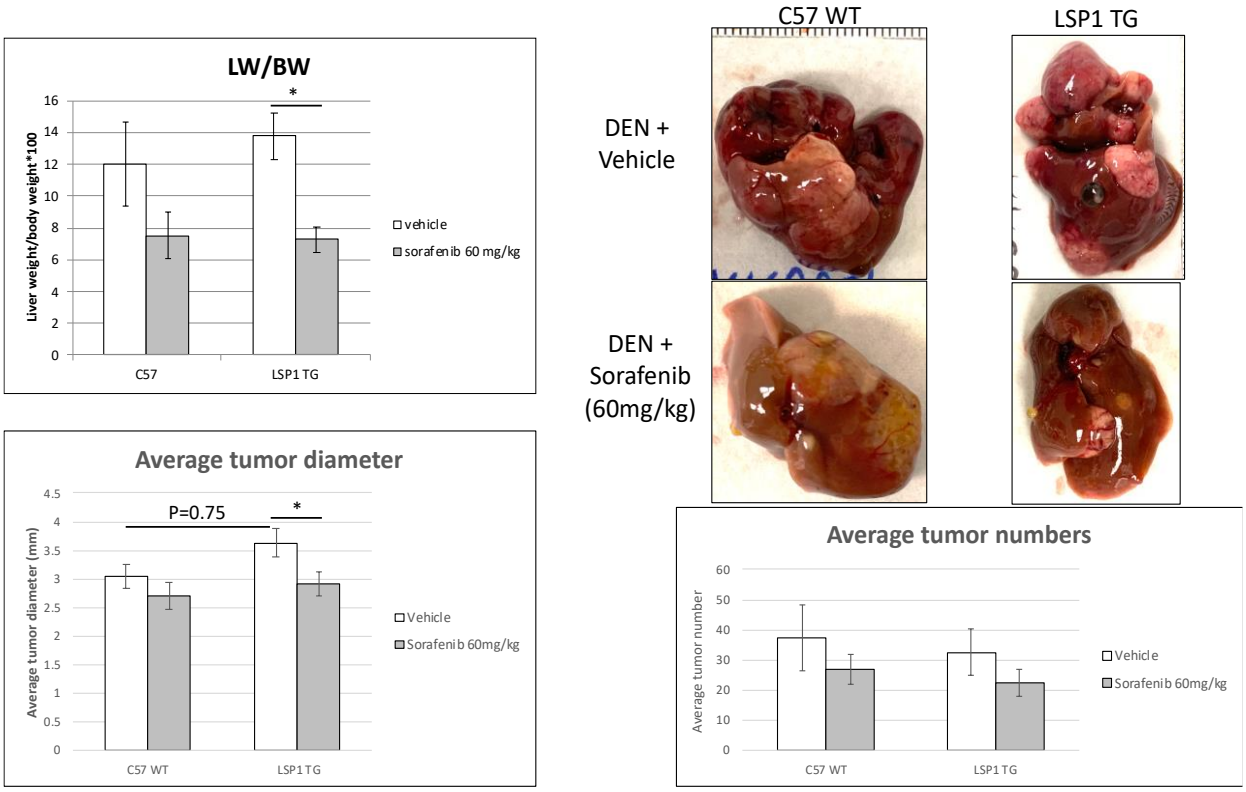
Figure 7. Immunoprecipitation (IP) of KSR scaffold protein from liver tissue lysates obtained from LSP1 KO and LSP TG mice (compared to the WT mice for their strains) at different times of liver regeneration after PHx. The immunoprecipitates were examined by western blot for activated ERK (pERK: activated by phosphorylation).

LSP1 TG mice: we found that the vehicle treated LSP1 TG mice displayed a nearly significant increase in the average tumor diameter. Following Sorafenib treatment, the LSP1 TG mice exhibited a significant decrease in tumor diameter, whereas with the WT control, we did not observe any significant differences in these parameters after treatment in comparison to the vehicle treated WT mice. Contrary to our hypothesis, it appears that there was better response in the LSP1 TG mice compared to the WT mice. The results are shown in Fig. 8, below.

An unexpected finding was that by western blot, the TG livers appeared to express less LSP1 than the WT controls. One explanation is that since these livers are from 10-11 months old mice that the hepatocytes lose expression of the transgene overtime. This is an often described phenomenon with progressive loss of growth inhibitory transgenes in liver, as hepatocytes losing the growth inhibitory transgene proliferate in higher numbers and outgrow the hepatocytes expressing the transgene, to the point that at 11 months of mouse age, the transgene expressing cells are becoming a small minority. These findings may account for why the TG mice displayed larger tumors and responded better to Sorafenib than the WT, if the transgenic are losing expression of the transgene. We also analyzed protein expression from the tumors and non-tumor adjacent tissues that had been formalin fixed. However, the results were mixed and inconclusive, with some tumors expressing LSP1 and others not from both the WT and LSP1 TG animals. To the extent

that tumors in LSP1 TG were themselves losing the TG transcript, they were any more bound to behave by the assumptions of our main hypothesis.

Figure 8.



Overall, contrary to our main hypotheses, our data demonstrate that the LSP1 KO mice develop less tumors and do not respond to Sorafenib as well as the WT and the LSP1 TG develop large tumors but respond better to Sorafenib than the WT controls. Another major possibility is the fact that DEN, the carcinogen used as standard in liver carcinogenesis experiments, causes many mutations in the Ras genes. The RAS proteins are immediately upstream and regulate the RAF/MEK/ERK complex, held together by KSR and influenced by LSP1. Alterations in the RAS protein due to mutations of the Ras genes may alter the way the mutant RAS proteins interact with RAF, thus potentially negating any effects of LSP1 on RAF.

Specifc Aim 1C. Investigate the molecular mechanism by which LSP1 interferes with the Sorafenib-RAF interaction.

The [RAF/MEK/ERK] complex is held together the scaffold protein KSR. We have all the evidence now that LSP1 interacts with this complex. Our underlying hypothesis was that LSP1binding is responsible for maintaining the KSR[RAF/MEK/ERK] complex. We postulated that in the absence of LSP1, the KSR[RAF/MEK/ERK] complex would disassemble.

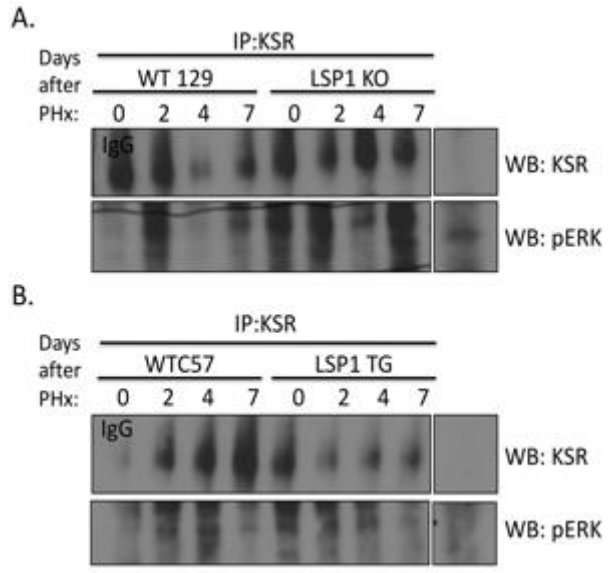


Figure 9. Immunoprecipitation (IP) of KSR scaffold protein from liver tissue lysates obtained from LSP1 KO and LSP TG mice (compared to the WT mice for their strains) at different times of liver regeneration after PHx. The immunoprecipitates were examined by western blot for activated ERK (pERK: activated by phosphorylation).

We performed these studies on already available tissue lysates of TG and KO and WT mice from other parts of the experiments related to the proposal. The RAF/MEK/ERK complex (the target of Sorafenib) is held together by the scaffold protein KSR. If the RAF/MEK/ERK complex is disassembled in the absence of LSP1, then ERK would not co-precipitate with KSR and, furthermore, ERK would not be activated by phosphorylation. We utilized tissues from LSP1 KO and LSP1 TG mice at different times after PHx (done to induced liver regeneration), obtained from the mice used in figures 1 and 2. We then generated tissue lysates and immunoprecipitated KSR. The results (Figure 7) show that activated ERK (pERK) is coprecipitating with KSR at all times during liver regeneration, regardless of whether LSP1 is absent (KO) or excessively present (TG) or normal levels (WT mouse strains). In addition, ERK is actually activated in all cases. These results are very important. **They indicate that absence of functional LSP1 does <u>not</u> result in disassembly of the RAF/MEK/ERK complex.** It remains intact, and, in agreement with the results of Figures 1 and 2, phosphorylation/activation of ERK actually increases in the absence of LSP1. T**hus, in the absence of LSP1, the complex is still active and can be targeted in Sorafenib chemotherapy.** The results of figure 5 indicate that the complex is even more sensitive to Sorafenib in the absence of LSP1.

Specific Aim 1D. Does the amplified carboxy-terminal of LSP1 act as a dominant negative to inhibit LSP1 function?

We made several efforts to construct a plasmid that would only include the amplified carboxyterminal of LSP1, but we were not able to transfect HCC cell lines or normal hepatocytes.

Specific Aim 1E. Development of fluorescent in situ hybridization (FISH) <u>diagnostic tools</u> for LSP1 C-terminal deletion or amplification using paraffin embedded archival material form human HCC.

We expended considerable time and effort in that regard. We had probes designed by ACD targeting the two exons of the carboxyterminal part of LSP1. We modified the probes by adding immunofluorescence and tested them towards several HCC cell lines, as well as frozen and paraffin sections of HCC tissue. We collaborated with the diagnostic FISH (Fluorescent in situ hybridization) lab of our department. They unfortunately were not able to label any targets in normal of HCC derived cells. The reasons were primarily technical and that often occurs with newly designed probes. The costs became prohibitive to further pursue this project.

Specific Aim 2. Phosphorylation of LSP1 inhibits its capacity to decrease hepatocyte proliferation. Investigate the effects of inhibition of LSP1 phosphorylation in HCC expressing intact LSP1 as a novel <u>therapeutic approach</u> for human HCC.

There are two enzymes implicated in phosphorylation of LSP1, namely PKCβ1 and MAPKAPK2 (a.k.a MK2). In the previous period, we had tried several documented inhibitors of each of the two enzymes, including Ruboxistaurin, Hispidin, Lipoxin A4 and Sotrastaurin. The results differed between the different human HCC cell lines. Ruboxistaurin reduced phosphorylation of LSP1 S252 only in the Hep3B cell line but not in the HepG2 or Huh7 HCC lines. The other inhibitors that were tested failed to prevent phosphorylation of LSP1 in culture. These differences are typical of weak inhibitors, especially those who may require metabolic activation to an active form. Access of the inhibitor to the inside of the cells can also be a problem. To go beyond the inconsistencies of the results, we decided to apply silencing RNA (ShRNA) against PKCβ1 and MK2. This approach decreases or eliminates the mRNAS for each enzyme and should eliminate the proteins following creation of stable cell lines expressing the ShRNA. This approach does not depend on peculiarities of cell metabolism, or access to the cell cytoplasm. The results of treating PKCβ1 and MK2 with ShRNA on the phosphorylation of LSP1 are shown in Figure 10. Overall, it appears that MAPKAPK2 (MK2) is the better target for pursuing to affect Ser252 LSP1 phosphorylation. As mentioned above, we have tried existing inhibitors for each of the two kinases and the results were variable. The lack of consistently effective chemical inhibitors for MK2 and PKCβ1 limits our capability to pursue using such inhibitors in a standard carcinogenesis experiment, to assess any therapeutic significance. We are very interested in this topic, however, as it would open new therapeutic modalities for HCC treatment. We intend to pursue this approach by using chemical inhibitors other than the existing ones as they become available in the future. Alternatively, ShRNAs are being used for therapeutic purposes for other conditions, in administration modes associated with nanoparticles, microdroplets, etc. This is something that needs be pursued in the future, using MK2 as a target. The overall cost of this experimental approach however is beyond the capability of being pursued within the limited budget of the no cost extension phase of the grant. In addition, the amount of time needed for the mice to develop HCC using DEN was 10-11 months, therefore, we did not have a sufficient amount of time to test the MK2 shRNAs on mouse HCC. In the future, we could utilize adeno-associated viruses to specifically express MK2 shRNA in hepatocytes to determine if loss of MK2 expression is sufficient to suppress DEN induced hepatocarcinogenesis through the inhibition of LSP1 phosphorylation.

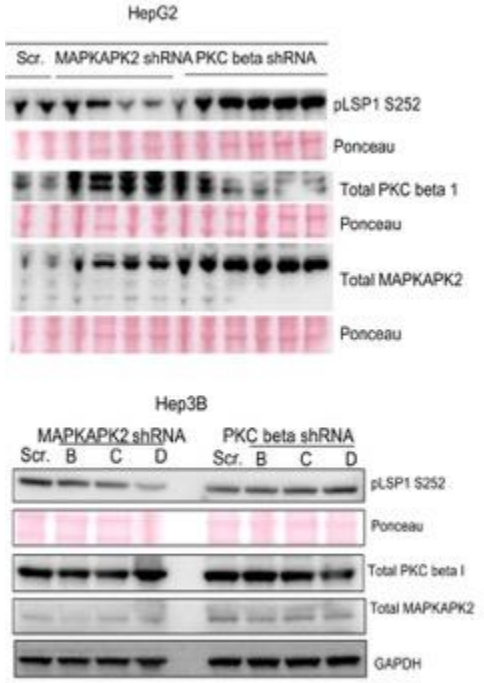


Fig. 10. Effects of ShRNA for MAPKAPK2 and PKCβ1 on LSP1 phosphorylation on Serine 252 in human HCC cell lines HepG2 and Hep3B.
<u>Upper panel</u>: Stable cell lines of HepG2 cells were generated that are expressing different ShRNA probes for MAPKAPK2 and PKCβ1 mRNAs. Three of the probes for MAPkAPK2 show decrease in phospho-LSP1 (Ser252), whereas the lines expressing the probes for PKCβ1 did not show any effect on phopshoLSP1.
<u>Lower panel:</u> We are in the process of testing different ShRNA probes in order to choose the effective ones and generate stable cell lines for Hep3B. Probes C and D for Sh. MAPKAPK2 mRNA appear effective in reducing

What opportunities for training and professional development has the project provided?

Dr. Kelly Koral is a senior postdoctoral fellow in our laboratory and she is receiving further training beyond her PhD, performing most of the tasks related to this proposal, including the DEN carcinogenesis, liver regeneration, and cell culture experiments of normal hepatocytes and human HCC cell lines. She has enhanced her experience beyond normal liver and hepatocytes, into analyzing carcinogenesis data and performing in depth analysis of cell signaling. She is now exploring pursuit of an academic career.

How were the results disseminated to communities of interest?

The results from our studies were presented in national meetings by Dr. Koral and myself. List of presentations at national society meetings:

1. **Koral K,** Tao J, Bhushan B, Stoops J, Orr A, Mars WM, Monga SP, Michalopoulos GK. Lymphocyte Specific Protein-1 Suppresses Hepatocarcinogenesis Driven by Mutant β-catenin and Met Overexpression. Oral Presentation 2019. Experimental Biology 2019, Orlando, FL. April 6-9, 2019.
2. **Koral K,** Tao J, Bhushan B, Stoops J, Orr A, Mars WM, Monga SP, Michalopoulos GK. Lymphocyte Specific Protein-1 Suppresses Hepatocarcinogenesis Driven by Mutant β-catenin and Met Overexpression. Oral and Poster Presentations 2019. University of Pittsburgh Department of Pathology Annual Retreat 2019, Pittsburgh PA. May 22, 2019.
3. **Koral K**, Bhushan B, Stoops JW, Orr A, Mars WM, Michalopoulos G. Lymphocyte Specific Protein-1 suppresses TCPOBOP induced hepatocellular proliferation. Oral and Poster Presentation 2020. Experimental Biology 2020. Cancelled due to Covid-19.
4. **Koral K**, Bhushan B, Stoops JW, Orr A, Mars WM, Michalopoulos G. TCPOBOP Induced Hepatocellular Proliferation Is Attenuated By Lymphocyte Specific protein-1. Poster of Distinction. AASLD The Liver Meeting Digital Experience 2020.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

The work published from our lab on LSP1 was referred to support work by other investigators in liver cancer and other cancers. Examples:

1. Zeng et al., The Phosphatidylinositol 3-Kinase Pathway as a Potential Therapeutic Target in Bladder Cancer. Clin. Cancer Research, 2017 Nov 1;23(21):6580-6591 (ref. 36).
2. Kwon et al., Regulation of tumor growth by leukocyte-specific protein 1 in T cells. J. Immunother. Cancer, 2020 Oct;8(2):e001180 (ref. 20)
3. Cong et al., MiR-920 and LSP1 co-regulate the growth and migration of glioblastoma cells by modulation of JAK2/STAT5 pathway. Journal of Bioenergetics and Biomembranes (2020) 52:311–320 (reference of Koral et al.).
4. Cao et al., Elevated lymphocyte specific protein 1 expression is involved in the regulation of leukocyte migration and immunosuppressive microenvironment in glioblastoma. Aging, 202, vol12, p. 1656-1684 (reference of Koral et al.).

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

A full publication of our studies with normal liver and the interaction between LSP1 and sensitivity to Sorafenib was published in the American Journal of Pathology (AJP), acknowledging support by funding from the Department of Defense current award.

Koral K, Haynes M, Bowen WC, Orr A, Mars W, Michalopoulos GK. Lymphocyte-Specific Protein-1 Controls Sorafenib Sensitivity and Hepatocellular Proliferation through Extracellular Signal-Regulated Kinase 1/2 Activation. Am J Pathol. 2018 Sep;188(9):2074-2086. doi: 10.1016/j.ajpath.2018.06.005. Epub 2018 Jul 3. PubMed PMID: 30126548.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Presentations in national society meetings:

1. **Koral K,** Tao J, Bhushan B, Stoops J, Orr A, Mars WM, Monga SP, Michalopoulos GK. Lymphocyte Specific Protein-1 Suppresses Hepatocarcinogenesis Driven by Mutant β-catenin and Met Overexpression. Oral Presentation 2019. Experimental Biology 2019, Orlando, FL. April 6-9, 2019.
2. **Koral K,** Tao J, Bhushan B, Stoops J, Orr A, Mars WM, Monga SP, Michalopoulos GK. Lymphocyte Specific Protein-1 Suppresses Hepatocarcinogenesis Driven by Mutant β-catenin and Met Overexpression. Oral and Poster Presentations 2019. University of Pittsburgh Department of Pathology Annual Retreat 2019, Pittsburgh PA. May 22, 2019.
3. **Koral K**, Bhushan B, Stoops JW, Orr A, Mars WM, Michalopoulos G. Lymphocyte Specific Protein-1 suppresses TCPOBOP induced hepatocellular proliferation. Oral and Poster Presentation 2020. Experimental Biology 2020. Cancelled due to Covid-19.
4. **Koral K**, Bhushan B, Stoops JW, Orr A, Mars WM, Michalopoulos G. TCPOBOP Induced Hepatocellular Proliferation Is Attenuated By Lymphocyte Specific protein-1. Poster of Distinction. AASLD The Liver Meeting Digital Experience 2020.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	George K. Michalopoulos
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-9922-6920
Nearest person month worked:	2
Contribution to Project:	As PI, Dr. Michalopoulos is responsible for overall project management and compliance
Funding Support:	*N/A*

Name:	Michael Nalesnik
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Generate probe
Funding Support:	*N/A*

Name:	William Bowen
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3.5
Contribution to Project:	In vivo with animal & cell culture analysis
Funding Support:	*N/A*

Name:	Kelly Koral
Project Role:	Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	DEN carcinogenesis, liver regeneration, and cell culture experiments of normal hepatocytes and human HCC cell lines.
Funding Support:	*N/A*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Appendix: Full publication:

Koral K, Haynes M, Bowen WC, Orr A, Mars W, Michalopoulos GK. Lymphocyte-Specific Protein-1 Controls Sorafenib Sensitivity and Hepatocellular Proliferation through Extracellular Signal-Regulated Kinase 1/2 Activation. Am J Pathol. 2018 Sep;188(9):2074-2086. doi: 10.1016/j.ajpath.2018.06.005. Epub 2018 Jul 3. PubMed PMID: 30126548.

The American Journal of Pathology, Vol. 188, No. 9, September 2018

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GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

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

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Accepted for publication June 11, 2018.

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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.

Supported by Department of Defense grant CA160119, the University of Pittsburgh Menten Endowment, and the Cleveland Foundation Morningstar Fund.

Disclosures: None declared.

https://doi.org/10.1016/j.ajpath.2018.06.005

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

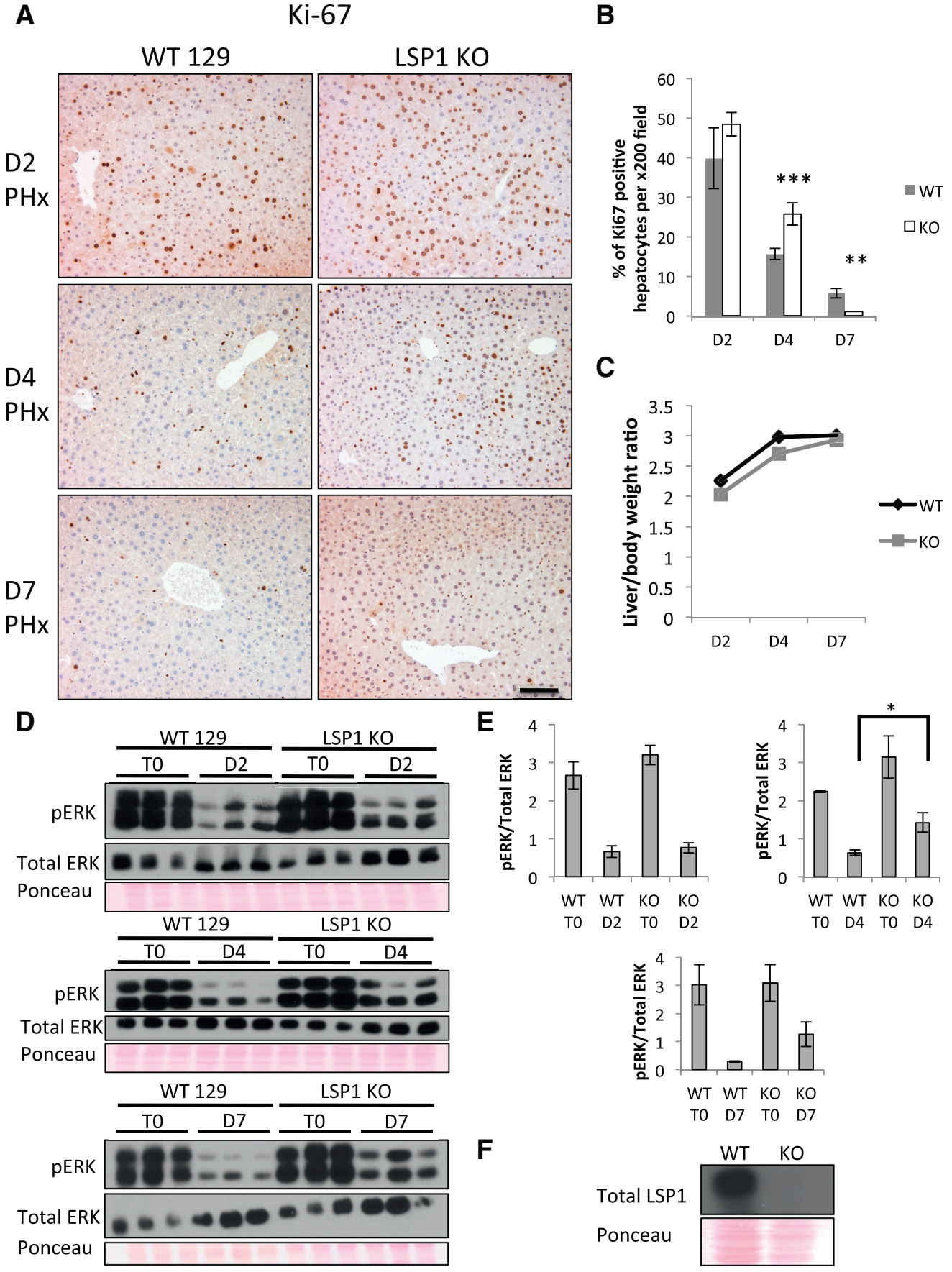
Materials and Reagents

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
D2 PHx
D4 PHx
D7 PHx
B
% of Ki67 positive hepatocytes per x200 field
60
50
40
30
20
10
0
***
**
D2
D4
D7
WT
KO
C
Liver/body weight ratio
3.5
3
2.5
2
1.5
1
0.5
0
D2
D4
D7
WT
KO
D
WT 129
LSP1 KO
T0
D2
pERK
Total ERK
Ponceau
WT 129
LSP1 KO
T0
D4
pERK
Total ERK
Ponceau
WT 129
LSP1 KO
T0
D7
pERK
Total ERK
Ponceau
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WT T0
WT D2
KO T0
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pERK/Total ERK
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WT T0
WT D4
KO T0
KO D4
pERK/Total ERK
WT T0
WT D7
KO T0
KO D7
F
WT
KO
Total LSP1
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.

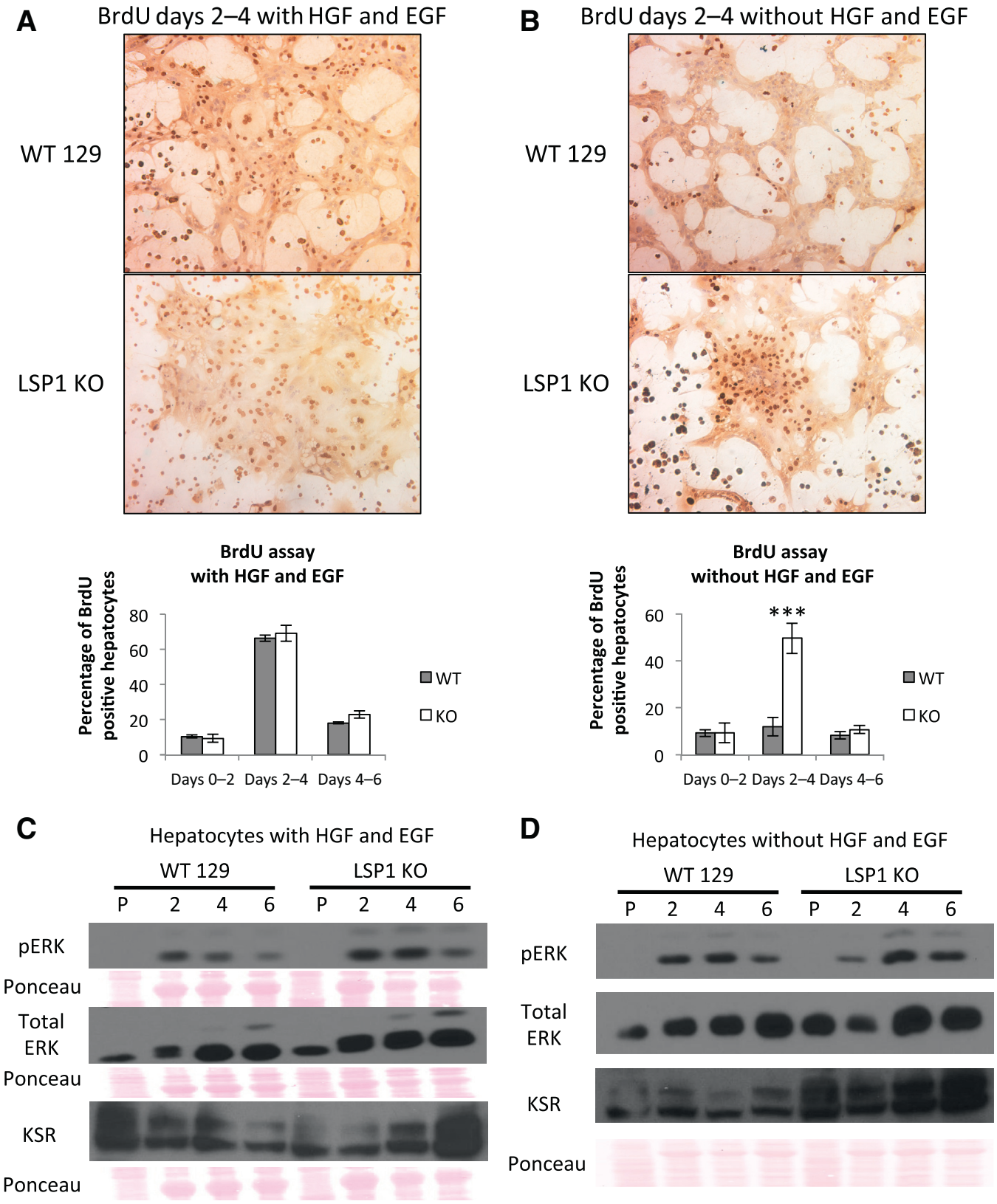


Figure 2 LSP1 knockout (KO) hepatocytes exhibit enhanced proliferation and extracellular signal-regulated kinase (ERK) phosphorylation in the absence of growth factors, hepatocyte growth factor (HGF), and epidermal growth factor (EGF). Representative images of bromodeoxyuridine (BrdU) immunohistochemistry 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

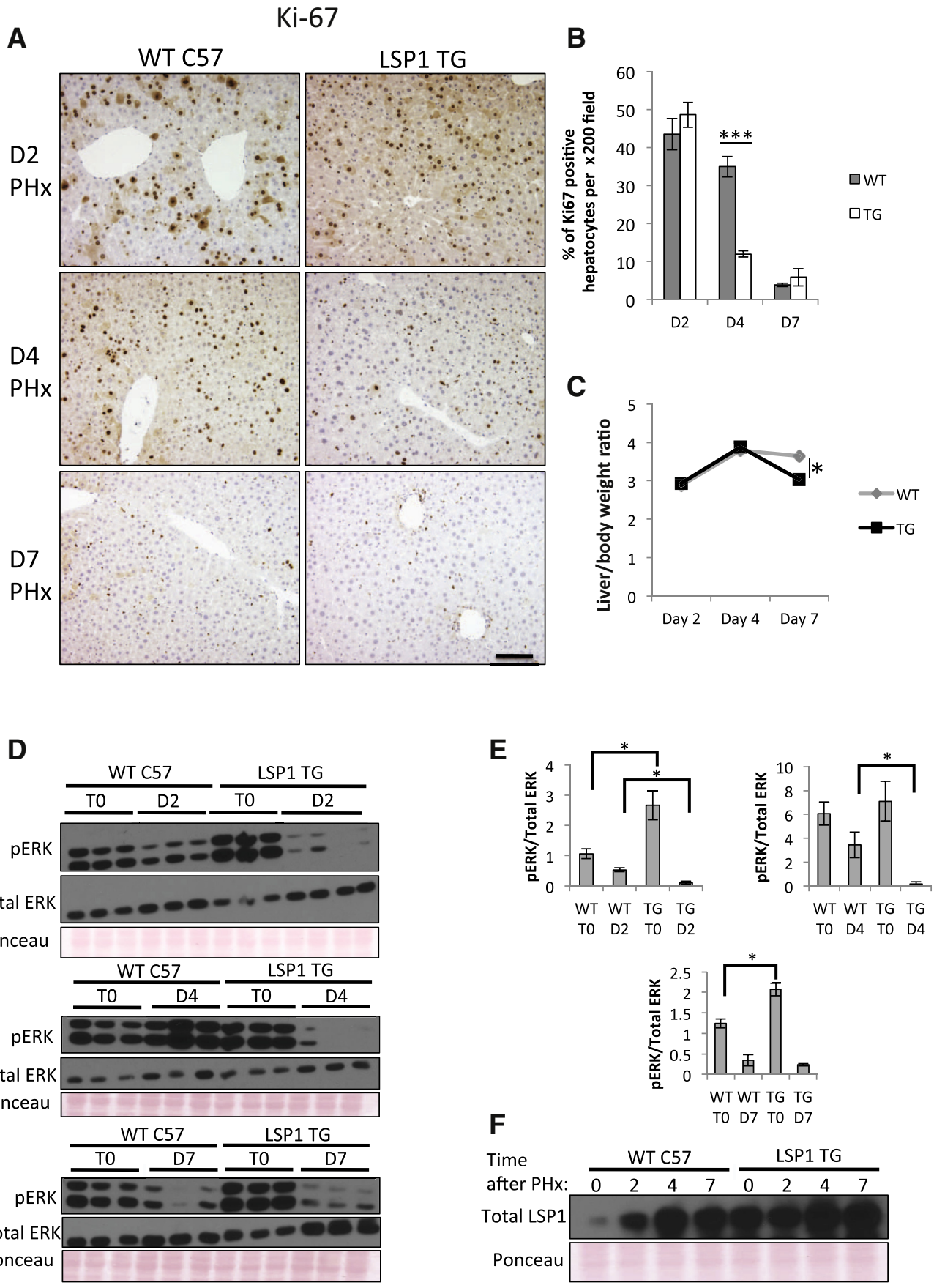
Our previous study demonstrated that LSP1 overexpression through hydrodynamic tail vein injection results in decreased proliferation after PHx[11]; therefore, in this study, it was determined how loss of LSP1 expression would affect liver regeneration after PHx. We hypothesized that the lack of LSP1 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
LSP1 TG
D2 PHx
D4 PHx
D7 PHx
B
% of Ki67 positive hepatocytes per x200 field
***
WT
TG
D2
D4
D7
C
Liver/body weight ratio
*
WT
TG
Day 2
Day 4
Day 7
D
WT C57
LSP1 TG
T0
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D4
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pERK
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Time after PHx:
0 2 4 7 0 2 4 7
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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.

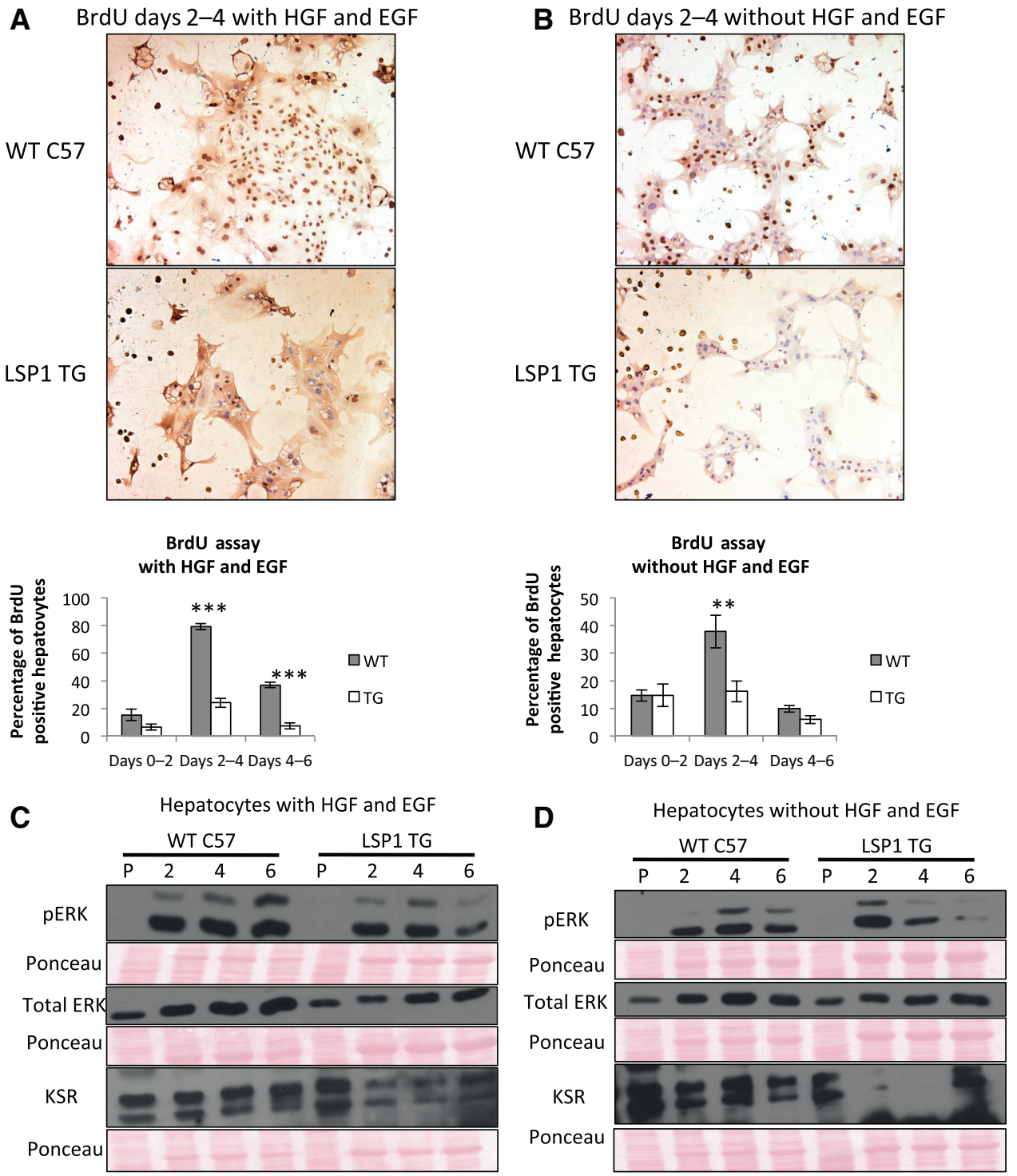

Figure 4 Hepatocytes from LSP1 transgenic (TG) mice display decreased cellular division and extracellular signal-regulated kinase (ERK) activation in both the presence and absence of growth factors, hepatocyte growth factor (HGF), and epidermal growth factor (EGF). Representative images of bromodeoxyuridine (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

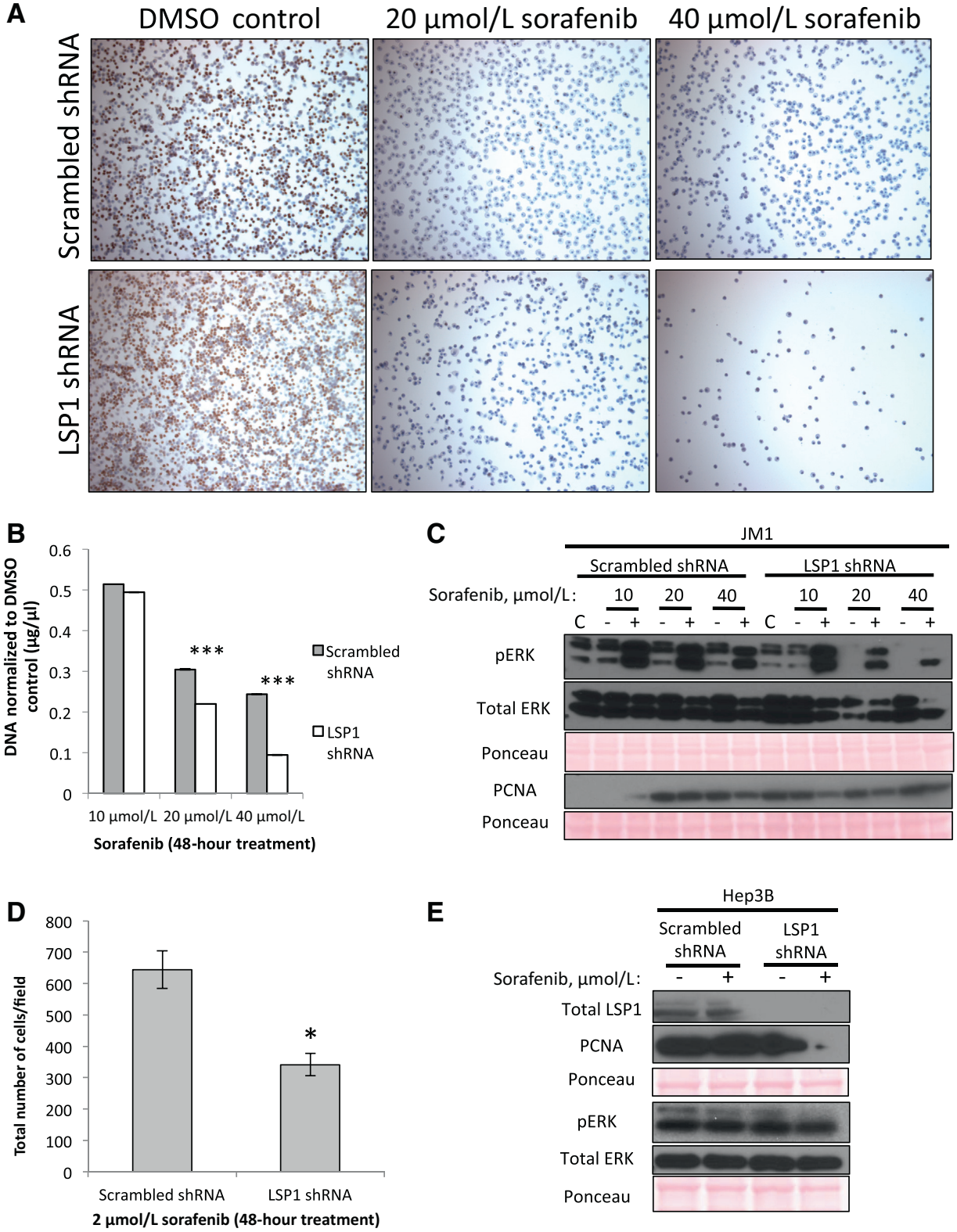

Figure 5 Loss of LSP1 expression in the JM1 rat hepatoma cell line leads to increased sorafenib sensitivity with decreased cell numbers and phosphorylated extracellular signal-regulated kinase (pERK) expression. **A:** Representative bright-field images of scrambled and LSP1 shRNA JM1 hepatoma cells 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).

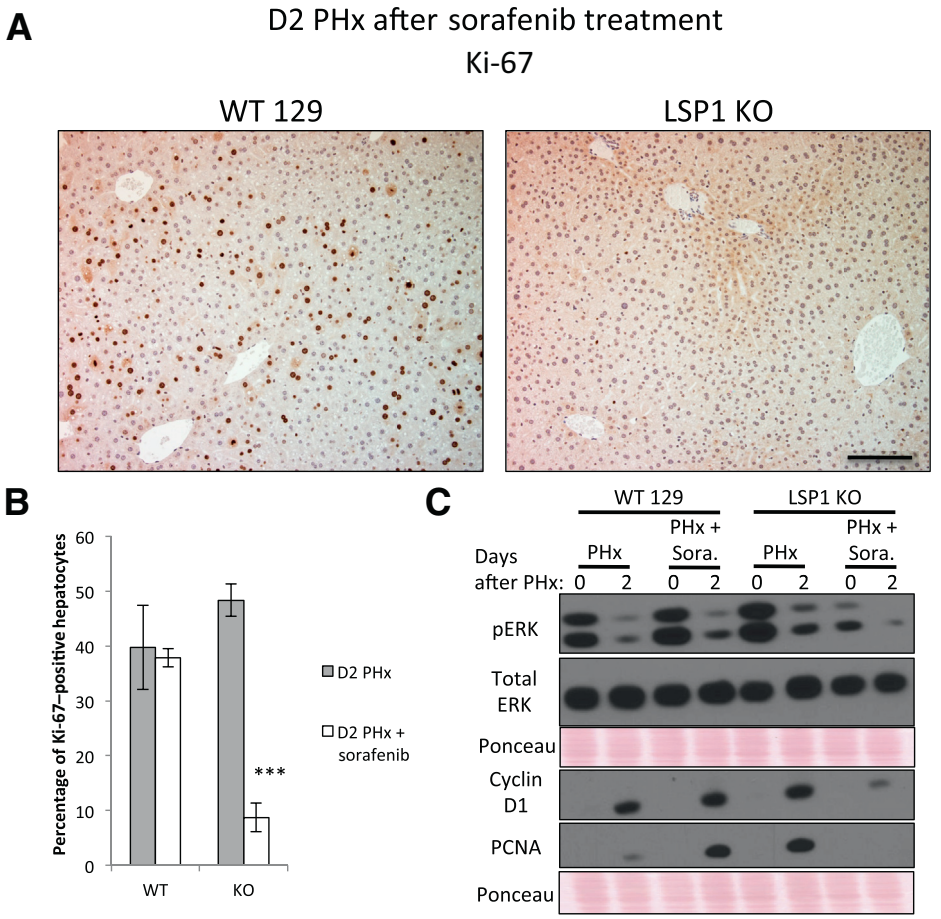

Figure 6 LSP1 knockout (KO) mice treated with sorafenib display decreased proliferation and phosphorylated extracellular signal-regulated kinase (pERK), cyclin D1, and proliferating cell nuclear antigen (PCNA) expression on day (D) 2 after PHx. **A:** Representative Ki-67 immunohistochemistry images 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.

Acknowledgment

We thank Dr. Jan Jongstra (University Health Network, Toronto, ON, Canada) for providing rabbit anti−lymphocyte-specific protein-1 primary antibody.

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