Gastrointestinal, Hepatobiliary and Pancreatic Pathology

The Critical Role of AKT2 in Hepatic Steatosis Induced by PTEN Loss

Lina He,* Xiaogang Hou,* Gary Kanel,[†] Ni Zeng,*
Vivian Galicia,* Ying Wang,[‡] Jian Yang,[§]
Hong Wu,[‡] Morris J. Birnbaum,[¶]
and Bangyan L. Stiles*

From the Departments of Molecular Pharmacology and Pharmaceutical Sciences,* University of Southern California, Los Angeles, California; the Department of Pathology,[†] Keck School of Medicine, University of Southern California, Los Angeles, California; the Department of Molecular Pharmacology and Toxicology,[‡] David Geffen School of Medicine, University of California, Los Angeles, California; the Department of Physiology,[§] University of South Alabama College of Medicine, Mobile, Alabama; and the Departments of Medicine, Cell and Developmental Biology, and Pharmacology,[¶] University of Pennsylvania, Philadelphia, Pennsylvania

Insulin signaling in the liver leads to accumulation of phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). Deletion of the phosphatase *Pten* (phosphatase and tensin homologue deleted on chromosome 10) reduces PIP₃ levels and leads to fatty liver development. The purpose of this study was to investigate the mechanisms underlying lipogenesis that result from PIP₃ accumulation using liver *Pten*-deletion mice. To explore the role of AKT2, the major liver AKT isoform in steatosis induced by deletion of *Pten*, we created mice lacking both *Pten* and *Akt2* in hepatocytes and compared the effect of deleting *Akt2* and *Pten* in the double mutants to the *Pten* deletion mice alone. Hepatic lipid accumulation was significantly reduced in mice lacking both PTEN and AKT2, as compared with *Pten* mutant mice alone. This effect was due to the role of AKT2 in maintaining expression of genes involved in *de novo* lipogenesis. We showed that lipid accumulation in the double mutant hepatocytes was partially reversed by expression of constitutive active FOXO1, a transcription factor downstream of AKT not dependent on inhibition of atypical protein kinase C. In summary, this study delineated regulation of lipid metabolism by PI3K signaling pathway by showing that AKT mediates PIP₃ accumulation (mimicked by PTEN loss) induced lipid deposition in the liver and provided an important molecular mechanism for in-

sulin-regulated hepatic lipogenesis. (Am J Pathol 2010, 176:2302–2308; DOI: 10.2353/ajpath.2010.090931)

Lipid phosphatase and tensin homologue deleted on chromosome 10 (PTEN) functions to convert phosphatidylinositol-3,4,5-trisphosphate (PIP₃) to phosphatidylinositol bisphosphate.¹ Thus, PTEN antagonizes the insulin-phosphoinositide 3-kinase (PI3K)/AKT pathway by reducing the levels of PIP₃. In the liver, insulin activates PI3K signal to drive hepatic lipid accumulation. Mice lacking all PI3K activities are hypolipidemic and exhibit reduced expression of lipogenic genes in the liver.² Previous studies reported a liver steatosis phenotype in mice when PTEN is lost in the hepatocytes.^{3,4} In hepatocyte-specific *Pten* deletion mice, liver becomes a lipogenic organ, and accumulates triglyceride (TG) even though there is systemic hypoglycemia.⁴ Both sterol regulatory element-binding proteins (SREBP-1c) and fatty acid synthase (FAS) are markedly elevated when PTEN is lost in the hepatocyte.^{3,4} This model, thus, is relevant to study how activation of the PI3K/AKT signaling pathway, specifically PIP₃ accumulation, induces lipogenesis.

In this study, we considered the role of AKT2 in mediating the lipogenic effect of insulin/PI3K signaling. AKT2 is the major isoform of AKT that is expressed in the liver. AKT plays a pivotal role in hepatic insulin action.⁵ Adenovirus expression of constitutively activated AKT *in vivo* led to a similar fatty liver phenotype as loss of PTEN.⁶ We hypothesized that AKT2 may mediate the metabolic phenotypes observed in the livers of *Pten* mutant animals. We investigated this hypothesis by crossing the hepatocyte-specific *Pten* mutant mice⁴ with the *Akt2*^{-/-} mice.⁷ Our results indicate that loss of AKT2 can effectively block the fatty deposition in the liver of the *Pten* mutant mice. Our

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Address reprint requests to Bangyan Stiles, Ph.D., Molecular Pharmacology and Pharmaceutical Sciences, University of Southern California, Los Angeles, CA 90089, E-mail: bstiles@usc.edu.

study shows that AKT2 is critical for the expression of lipogenic genes. This effect is independent of the atypical protein kinase C (aPKC) and may be partially dependent on the forkhead transcriptional factor FOXO1.

Materials and Methods

Animals

The *Pten*^{loxP/loxP}; *Alb-Cre*⁺ (*C*⁺; *A*^{+/+}, *Pten* null) mice⁴ were crossed with *Akt2*^{-/-} (*C*⁻; *A*^{-/-}, *Akt2* null) mice⁷ to generate the double mutant *Pten*^{loxP/loxP}; *Akt2*^{-/-}; *Alb-Cre*⁺ (*C*⁺; *A*^{-/-}, DM). Male mice were used for experiments. Controls are male *Pten*^{loxP/loxP}; *Alb-Cre*⁻ mice. Experiments are performed following protocols approved by the University of Southern California Institutional Animal Care and Use Committee.

Fasting blood glucose levels were determined using a Therasense glucometer after overnight fasting. Livers were perfused with cold PBS and collected in formalin for histology, optimal cutting temperature compound for frozen section and flash frozen for protein, RNA and lipid analysis. Tissue sections were stained with H&E and Oil Red O.

Cell Lines, Plasmids, and Transfections

Pten mutant and control hepatocyte cell lines were established from the *Pten* mutant and control mice as described previously.⁸ Plasmid for wild-type FOXO1-WT and FOXO1-AAA mutants are obtained from Add-gene. Plasmids were transfected into the *Pten* mutant and control hepatocytes using Lipofectamine 2000 Transfection Reagent (Invitrogen). Cells were harvested 48 hours after transfection.

Determination of Serum Metabolic Indexes

Serum was collected from 16-hour-fasted mice through cardiac puncture before sacrifice. Analysis for serum TG, cholesterol, non-esterified fatty acids, and 3-hydroxybutyrate were determined using kits provided by Wako chemical.

Determination of Liver TG

Liver lipid was extracted with chloroform/methanol (2/1) according to Folch method. The supernatant was used for TGs assay. The pellets were further lysed for DNA extraction. The TG content was determined using Triglyceride (GPO) Reagent Set (Thermo).

Oil Red O Staining

Frozen liver sections were briefly fixed in cold 10% formalin. Slides were stained in oil red O solution (3 mg/ml) and hematoxylin for counterstain.

Table 1. Real-Time Quantitative PCR Primers

Gene name	Primer sequence
<i>ACC1F</i>	5'-ACAGTGGAGCTAGAATTGGAC-3'
<i>ACC1R</i>	5'-ACTTCCCGACCAAGGACTTTG-3'
<i>FASF</i>	5'-AGCGGCCATTTCATTGCCC-3'
<i>FASR</i>	5'-CCATGCCAGAGGGTGGTTG-3'
<i>SREBP1F</i>	5'-AACGTCACTTCCAGCTAGAC-3'
<i>SREBP1R</i>	5'-CCACTAAGGTGCCTACAGAGC-3'
<i>GlucokinaseF</i>	5'-CCCTGAGTGGCTTACAGTTC-3'
<i>GlucokinaseR</i>	5'-ACGGATGTGGAGTGTGAAGC-3'
<i>SCD-1F</i>	5'-CCGGAGACCCCTTAGATCGA-3'
<i>SCD-1R</i>	5'-TAGCCTGTAAAAGATTTCTGCAACC-3'
<i>GAPDH</i>	5'-GTCGGTGTGAACGGATTGG-3'
<i>GAPDHR</i>	5'-GACTCCACGACATACTCAGC-3'

Glucose Tolerance Test

The glucose tolerance test was performed on mice fasted for 16 hours at 1 and 3 months of age.⁴ Mice were given a single dose (2g/kg body weight) of D-dextrose (Sigma) by i.p. injection after a baseline glucose check. Circulating glucose levels were measured at indicated time after injection.

Protein Electrophoresis

Protein lysates (75 μ g) were loaded for each sample for electrophoresis. Membranes were probed with antibodies for total PTEN, AKT (1 + 2 + 3), AKT2, p-AKT, acetyl-CoA carboxylase, p-FOXO1 (Ser 256), and p-aPKC (Thr410/403) from Cell Signaling, FAS (Upstate), and SREBP-1 Ab-1 (Clone 2A4) (Thermo). The membranes were also probed with vinculin and β -actin (Sigma) for loading control.

Quantitative PCR

Sequences used in generating qPCR are for acetyl-CoA carboxylase (ACC), FAS, SREBP-1c, Glucokinase (GK), and glyceraldehyde-3-phosphate dehydrogenase (primers listed in Table 1). Real-time PCR reactions were performed on Biorad iCycler. Relative fold changes were obtained by comparing the Δ Ct between mutant and control groups.

Statistical Analysis

All data are subjected to multivariate analysis of variance. Fischer's LSD tests were used to determine differences among the groups. $P \leq 0.05$ is considered to be statistically significant. Data presented as mean $\pm$ SEM.

Results

To investigate the function of AKT kinase in hepatic steatosis in *Pten* mutant mice, we crossed the *Pten*^{loxP/loxP}; *Alb-Cre*⁺ (*Pten* null) mice with the *Akt2*^{-/-} mice (*Akt2* null) to generate the double mutant *Pten*^{loxP/loxP}; *Akt2*^{-/-}; *Alb-Cre*⁺ (double mutant, DM). Figure 1A (left panel) shows the genotyping for *Pten*, *Akt2* and *Cre* for each group of mice. Genotypes were confirmed with Western blot analysis (Figure 1A, right panel). Loss of *Pten* in-

Figure 1. Generation of PTEN and AKT2 double mutant mice. **A:** Genotyping and Western blot confirmation of mice carrying various genotypes. **Left:** Representative PCR genotyping gel images of liver tissue lysates. **Right:** Representative Western blot images of lysates isolated from livers of various groups of mice. Blots were probed with PTEN (**top**) and AKT2 (**middle**). Actin was also blotted for loading controls (**bottom**). **B:** Representative Western blot analysis of phosphorylated AKTs. **Top panel,** phospho-Ser 473; **Bottom panel,** phospho-Thr308. Phosphorylation on both residues was robustly induced by loss of PTEN (*Pten* null) compared with the control groups (Con). β -actin was detected as a loading control. **C:** Body weight was measured in mice carrying various genotypes. Open bar, control (Con); Striped bar, *Pten* mutant only (*Pten* null); Solid bar, *Pten* and *Akt2* double mutant (DM); patterned bar, *Akt2* mutant only (*Akt2* null). $n = 3$ to 6.

duced robust phosphorylation on both Ser 473 and Thr 308 of AKT (Figure 1B). Phosphorylation of AKT was not visible when PTEN is intact in the control or AKT2 null mice. We observed that AKT (ser473) phosphorylation is decreased by approximately twofold in the DM group, as compared with the *Pten* null group (Figure 1B, top panel). Phosphorylation on Thr308 is more profoundly reduced to a level similar to controls when AKT2 is lost (Figure 1B, bottom panel). No compensatory increase of AKT1 was observed (data not shown). AKT3 level was not detectable in the liver lysates. The three mutant groups of mice are grossly morphologically normal with body weight comparable with the control mice, *Pten*^{loxP/loxP}; *Alb-Cre*⁻ (Con) (Figure 1C).

AKT2 Is Necessary for Lipid Accumulation in the Liver

Loss of PTEN led to increased liver to body weight ratio (Figure 2A). Deletion of *Akt2* in the DM group reduced liver weight, as compared with the *Pten* deletion mice. Loss of PTEN also led to accumulation of lipid in the hepatocytes.⁴ Loss of AKT2 led to a dramatic decrease in the severity of fatty liver (Figure 2B and Supplemental Figure 1 at <http://ajp.amjpathol.org>). Significantly less oil red O staining is observed in livers of the DM mice compared with the *Pten* mutant. At 1 month of age, only *Pten* null mice accumulated lipid in the liver (see Supplemental Figure 1 at <http://ajp.amjpathol.org>). At 3 months of age, the *Pten* null mice displayed an even greater degree of steatosis, which was suppressed to almost control levels in the DM mice (Figure 2B). The lipid contents in the DM mice are dramatically reduced compared with the *Pten* null mice (Figure 2B). The control and the *Akt2*^{-/-} mice (*Akt2* null) did not stain with oil red O, indicating little or no lipid deposition in the livers of these mice. Lipid accumulation occurs in both central vein (Figure 2B and

Figure 1sA) and portal triad area (Supplemental Figure 1B at <http://ajp.amjpathol.org>).

Loss of PTEN led to a two- and four fold increase of TG content in the liver of 1- and 3-month-old mice, respectively, as compared with the respective age matched controls (Figure 2C). Loss of AKT2 significantly blocks this effect of PTEN loss. The TG content in the DM mice is reduced to a level similar to the controls in 3-month-old mice, and even less than controls in the 1-month-old mice (Figure 2C). A reduction of approximately 2.5-fold is observed in 3-month old DM mice vs. age matched *Pten* null mice. Deletion of *Akt2* alone also led to >two fold decrease in liver TG, as compared with the control mice (Figure 2C). Thus, loss of AKT2 not only affected lipid accumulation induced by PTEN loss in the hepatocyte but also reduced lipid storage when not compounded with other factors that induces insulin signal.

AKT2 Is Important for Lipogenesis in the Liver

The accumulation of lipids in the liver of *Pten* mutant mice is associated with an increase in *de novo* lipogenesis.⁴ Serum index from the DM mice and *Pten* null groups suggests that there is no change in serum TG and non-esterified fatty acid levels (Table 2), two neutral lipids that can decrease if hepatic lipid export is inhibited. Cholesterol and 3-hydroxybutyrate levels were also not significantly different between the two groups, although AKT2 loss alone did lead to a significant reduction in 3-hydroxybutyrate (Table 2).

To investigate whether the PTEN loss-associated lipogenic effect is mediated by AKT2, we determined the levels of lipogenic enzymes ACC and FAS, enzymes that catalyze the two initial steps in *de novo* lipid synthesis. Both ACC and FAS mRNA levels are increased in the *Pten* null liver (significantly reduced Δ Ct in the *Pten* null group), consistent with the severe fatty liver phenotypes

Figure 2. Loss of AKT2 reduces the lipogenic ability of liver. **A:** Loss of AKT2 (patterned bar) reduced the liver to body weight ratio, as compared with the control mice (open bar). The size of the liver is also reduced in the double mutant mice (solid bar) compared with *Pten* mutant only (stripped bar) mice. *n* = 8. **B:** Liver tissues sections from 3-month-old mice carrying various genotypes. **Left panel,** control; **Second from the left,** *Pten* null only; **Third from the left,** *Pten* and *Akt2* double mutant (DM); **Right panel,** *Akt2* null only. **Top panels,** H&E staining; **Bottom panels,** oil red O staining. Magnification $\times 20$. **C:** Quantification of liver triglyceride (TG) demonstrated loss of lipid accumulation in the *Akt2* null (patterned bar) and double mutant mice (solid bar), as compared with controls (open bar) and *Pten* mutant only (stripped bar). *n* = 3 to 6. **P* $\leq$ 0.05 considered statistically significant difference. Data presented as mean $\pm$ SEM.

observed in these mice (Figure 3A, left two panels and Supplemental Figure 2 at <http://ajp.amjpathol.org>). Given the dramatic reduction in liver TG content in the DM mice, we expected that the DM mice would have the same level of ACC and FAS as control or even *Akt* null mice. Figure 3A shows that expression of ACC is dramatically reduced (higher ΔCt) in the DM group compared with the *Pten* null mice. The level of ACC expression in the DM mice is comparable with the controls or less. Loss of AKT2 also increased the ΔCt for amplifying ACC and calculated to be fivefold reduction in level of ACC compared with the controls (Figure 3A, left panel and Supplemental Figure 2 <http://ajp.amjpathol.org>). This lowered ACC is accounted for by decrease in both ACC1 and ACC2 expression (Table 3). Levels of FAS were reduced to a similar extent (Figure 3A, middle panels and Supplemental Figure 2A at <http://ajp.amjpathol.org>). AKT2 loss reduced FAS expression in the *Pten* null mice to the same level as the controls (Figure 3A, middle panels and Supplemental Figure 2A at <http://ajp.amjpathol.org>). These data confirmed that AKT2 is critical for the expression of liver lipogenic genes in the unstimulated (compare *Akt2* null mice with controls) and

stimulated (compare DM with *Pten* null) states. We also observed significant down-regulation of other genes involved in lipogenesis. Stearoyl-CoA desaturase 1, a key enzyme involved in lipogenesis is sensitive to changes in insulin signaling. We found that stearoyl-CoA desaturase 1 levels are dramatically induced as a result of PTEN loss (threefold) and are reduced back to the levels of control mice when AKT2 is concurrently lost (Table 3). AKT2 loss alone led to a >fivefold reduction in stearoyl-CoA desaturase 1. Together, our data supports a primary role of AKT2 in PTEN loss-induced lipogenesis.

We investigated the status of SREBP-1c, a critical transcriptional factor regulating lipogenesis in the liver and found that the expression of SREBP-1c is dependent on AKT2 (Figure 3A, right panels). When AKT2 is lost in the *Akt2* null or DM mice, SREBP-1c expression is dramatically reduced. In the 1-month-old *Akt2* null and DM mice, SREBP-1c levels were 50% and 25% of the controls, respectively (Figure 3A, right panels). When calculated to fold changes, loss of *Pten* dramatically induced SREBP-1c by fourfold, as compared with the controls, leading to a 16- and eightfold increase, comparing to the

Table 2. Serum Measurements of *Pten* and *Akt* Mutant Mice

Measurements	Con	<i>Pten</i> null	DM	<i>Akt2</i> null
Triglycerides (mg/dl)	102.6 $\pm$ 10.6	87.4 $\pm$ 7.9	96.5 $\pm$ 13.4	94.1 $\pm$ 5.4
Cholesterol (mg/dl)	111.4 $\pm$ 9.34	100.0 $\pm$ 13.3	140.1 $\pm$ 13.6	144.7 $\pm$ 5.3
NEFA (mM)	1.41 $\pm$ 0.03	1.22 $\pm$ 0.13	1.27 $\pm$ 0.09	1.22 $\pm$ 0.14
3-HB (mM)	0.69 $\pm$ 0.02	0.84 $\pm$ 0.07	0.69 $\pm$ 0.05	0.40 $\pm$ 0.03***

Data reported as mean $\pm$ SEM. *n* = 3 to 6, of 3-month-old mice.
 ***Different from all other groups.

Figure 3. Loss of AKT2 attenuates liver lipogenesis. **A:** qPCR analysis of key lipogenic enzymes and factors: acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), and sterol regulatory element binding protein-1c (SREBP-1c). $n = 3$. Relative ΔCt represents the Ct needed to reach threshold level subtracting the Ct needed for glyceraldehyde-3-phosphate dehydrogenase to reach the threshold as loading control. $P \leq 0.05$ is considered to be significant. *, different from control; **, different from *Pten* null; ***, different from DM. Data presented as mean $\pm$ SEM. **B:** Western analysis of ACC, FAS, and nuclear form of SREBP-1c levels in livers of various mouse models. **Left**, 1-month-old mice. **Right**, 3-month-old mice. Vinculin (Vin) and actin is detected as controls for loading.

Akt2 null and DM mice, respectively (Figure 3A, right panels). SREBP-1c levels in 3-month-old mice are consistently threefold lower in the two groups of mice lacking AKT2 than the controls (Supplemental Figure 2 at <http://ajp.amjpathol.org>). Loss of PTEN led to moderate but significant change in the expression of SREBP-1c (Supplemental Figure 2 at <http://ajp.amjpathol.org>). Loss of AKT2 alone or in DM mice resulted in an approximately four fold reduction in SREBP-1c and 2 expressions, as compared with their respective controls (Figure 3, Supplemental Figure 2 at <http://ajp.amjpathol.org> and Table 3). No changes were observed with PGC-1 in the *Pten* mutant mice (data not shown).

Similar to the gene expression increase, nuclear SREBP-1c, FAS, and ACC protein levels increased dramatically in the *Pten* null group (Figure 3B). This increase is blocked by loss of AKT2 in the DM mice. FAS protein levels in the DM group is similar to that of the controls,

Table 3. Loss of AKT2 Reduces the Expression of Lipogenic Genes

Gene of interest		Genotype			
		Con	<i>Pten</i> null	DM	<i>Akt2</i> null
ACC1	1	1.11 $\pm$ 0.11	0.26 $\pm$ 0.03**	0.40 $\pm$ 0.04***	
ACC2	1	1.04 $\pm$ 0.17	0.34 $\pm$ 0.08**	0.35 $\pm$ 0.02**	
SCD1	1	3.2 $\pm$ 0.13*	1.06 $\pm$ 0.09**	0.17 $\pm$ 0.01***	
SREBP-2	1	0.90 $\pm$ 0.16	0.35 $\pm$ 0.04**	0.21 $\pm$ 0.02***	

Data reported as mean $\pm$ SEM with controls arbitrarily set as 1. The relative expression of the gene of interest in other groups are compared to the control levels and reported as relative expression. $n = 3$ of 3-month-old mice.

*Different from control and *Pten* null groups.

**Different from *Pten* null group.

***Different from all other groups.

especially in the 3-month-old mice. Further reduction in FAS protein levels were observed in the *Akt2* null mice, as compared with the DM and the control groups, indicating that other factors than AKT2 may also play roles in the protein levels of FAS in these mice.

Loss of AKT2 Abrogates the Effect of *Pten* Deletion on Glucose Tolerance

The hepatocyte-specific, PTEN-deficient mice displayed improved glucose tolerance.⁴ This was completely reversed by concomitant removal of *Akt2* such that the glucose excursion was indistinguishable from *Akt2*^{-/-} mice. Both *Akt2*^{-/-} and DM mice displayed hyperglyce-

Figure 4. AKT2 loss reduces glucose tolerance of hepatocyte *Pten* mutant mice. **A:** Plasma glucose levels were determined in mice fasted overnight. $n = 8$. Open bar, control (Con); Stripped bar, *Pten* mutant (PTEN null); Solid bar, double mutant (DM); Patterned bar, *Akt2* mutant (AKT2 null). Data presented as mean $\pm$ SEM. **B:** Glucose tolerant test (GTT) shows intolerance to clear glucose load in *Akt2* null (dashed line with triangle) or DM (solid line with square), as compared with the hepatocyte *Pten* null (solid line circle) mice. Controls, dashed line with open circle. **Top panel**, 1-month-old mice. **Bottom panel**, 3-month-old mice. $n = 8$ to 9. *, different from control; **, different from *Pten* null; ***, different from double mutant (DM). Data presented as mean $\pm$ SEM. $P \leq 0.05$ considered statistically significant difference. Con, open bar; *Pten* null, stripped bar; double mutant (DM) mice, solid bar; and *Akt2* null, patterned bar. Data presented as mean $\pm$ SEM. $n = 3$ to 6.

Figure 5. AKT2 not atypical PKC activity is associated with the reduced lipid accumulation in the liver of double mutant mice. **A:** Representative Western blot analysis of phosphorylated atypical PKC. **B:** Representative Western blot analysis of phosphorylated FOXO1. **C:** Phospho-FOXO levels of *Pten* control and mutant cells transfected with wild-type (wt) FOXO or nuclear localized mutant FOXO (FOXO-AAA). Actin is used as loading control. **D:** FAS levels in cells transfected with the indicated FOXO constructs. Vinculin (Vinc) is used as loading control.

mia and glucose intolerance, as compared with *Pten* mutant groups (Figure 4). Loss of AKT2 led to an approximately 25 to 30% increase in fasting glucose levels in the DM and *Akt2* null groups, as compared with the control and *Pten* null groups at 1 month of age (Figure 4A). This trend continued at 3 months of age. Indeed, the glucose tolerance test curve of the DM is at the same level as the control mice whereas PTEN loss induced a downshift of the curve (Figure 4B, bottom panel). The glucose tolerance test curve indicates that the *Akt2* mutant mice are deficient in clearing glucose as previously reported.⁷ No significant differences were observed with fasting insulin levels among any of the groups (data not shown).

The Reduction of Lipogenesis and Glucose Sensitivity Is Associated with AKT, not PKC, Activation

Signals downstream of PI3K may diverge in their functions during insulin signal transduction. Atypical PKC (aPKC) expression has been shown to induce the expression of lipogenic genes in livers lacking PI3K enzymes.² We found that the levels of phospho-aPKC are not decreased in the DM group, as compared with the *Pten* null group (Figure 5A). If any change is seen, there are moderate increases in phospho-aPKC in the two groups lacking AKT2, a possible consequence of feedback activation in PI3K signaling upstream of AKT due to lack of AKT2.

Increasing evidence has shown that the forkhead transcriptional factor FOXO1 may play a role in the regulation of lipogenesis.^{9,10} Phosphorylation of FOXO1 by AKT leads to its nuclear exclusion and inability to assume its transcriptional activity. When PTEN is lost, the level of phosphorylated FOXO1 becomes detectable (Figure 5B). Its level is reduced in DM groups and further reduced when AKT2 is lost by itself (Figure 5B). These data indicate that FOXO1 phosphorylation by AKT2 may mediate the effect of AKT2-regulated lipogenesis.

To evaluate whether phosphorylation of FOXO led to the increased lipogenesis effect seen with *Pten* mutation, we introduced wild-type FOXO (wtFOXO) and a non-phosphorylatable mutant of FOXO (FOXO-AAA) to the hepatocyte cell lines that we established from the control and mutant mice.⁸ When introduced to the control mice, neither wtFOXO nor mutant FOXO-AAA are phosphory-

lated, indicated by the lack of phospho-FOXO in the transfected *Pten* control cell lines (Figure 5C). When introduced to the *Pten* mutant cell line, the wtFOXO is readily phosphorylated (Figure 5C), consistent with the phosphorylation status of AKT in the cells. The FOXO-AAA mutant, however, is not phosphorylated, as indicated by the lack of phospho-FOXO band in this sample group (Figure 5C). We investigated the effects of introducing FOXO-AAA mutant to the *Pten* mutant cells and compared it to the cells that received wtFOXO. The FOXO-AAA mutant transfected cells showed reduced levels of FAS compared with the wt-FOXO transfected cells (Figure 5D). Interestingly, the FOXO-AAA induced FAS protein levels in the *Pten* control cells. The reason for this is not clear and may not be dependent on the signals from PI3K/AKT as this signal axis are not active, as indicated by the lack of FOXO phosphorylation in the wtFOXO-transfected control cells.

Discussion

PTEN is a lipid phosphatase that negatively regulates insulin signaling. Loss of PTEN leads to lipid accumulation in hepatocytes and steatosis in the liver. How PTEN loss results in this metabolic phenotype is not clear. Our present study using a combination of *Pten* and *Akt2* deletion specifically evaluated the function of AKT2 in liver metabolism. Loss of AKT2 alone or in combination with AKT3 renders the mice glucose and insulin intolerant.^{7,11,12} Studies using cells isolated from the AKT2 mice and cultured cell lines have shown that AKT2 is required for insulin-dependent uptake of glucose in adipocytes.^{6,13,14} Our results show that germline loss of AKT2 can efficiently block both the lipid accumulation and the improved glucose tolerance induced by hepatocyte-specific *Pten* deletion. AKT2 loss alone also reduced lipogenesis in addition to rendering the mice mildly glucose intolerant as reported previously.⁷ Loss of PTEN mimics the constitutive engagement of the insulin receptor and accumulation of PIP₃ in the cells. Under this condition, the increased lipogenesis and expression of lipogenic genes, such as FAS, SREBP-1c, and ACC, depend on AKT2 to be present. However, our study does not exclude the involvement of other factors in hepatic lipogenesis, especially given the fact that slightly more reduction of lipogenic expression is observed with the

Akt2^{-/-} mice than in DM mice. The effect of AKT2 loss, however, is much more significant, and thus, is likely an essential regulator for the *Pten* loss induced lipid accumulation in hepatocytes. Our observation supports a pivotal role for AKT2 in lipogenesis.

These observations appear to be in disagreement with analysis in mice lacking two isoforms of PI3K.² In the PI3K-deficient mice, the reduction of FAS and SREBP-1c mRNA expression was rescued by adenovirus expression of ca-PKC λ but not myr-AKT. Several explanations may account for these disagreements. First, the use of a nonspecified isoform of myr-AKT in the PI3K-deficient mice study makes it difficult to directly compare our data with the atypical PKC data. How AKT2 and AKT1 differ functionally on the molecular level is not clear. If AKT1 is the isoform used for myristylation, it may not have similar effect on srebp-1c and FAS expression as AKT2 activation. Secondly, direct targeting of AKT to the membrane with the attachment of myristylation signal may not mimic the endogenous activation of AKT. Finally, the PI3K-deficient mice lack the function of lipogenesis, whereas PTEN-deficient mice are functionally enhanced in lipogenesis.^{2,4} The differential effects seen with the two studies may be a difference between stimulated and basal levels of lipid homeostasis regulation. Loss of PKC¹⁵ or AKT2 by themselves both led to reduced lipid accumulation in the liver, suggesting that both may maintain the basal lipid homeostasis. Our study showed that AKT2 is also critical for liver lipid accumulation in the stimulated stage of PTEN loss. Without AKT2, the expression of lipogenic genes such as ACC, FAS, and the lipogenic transcriptional factor SREBP-1c cannot be induced by activation of PI3K signaling through PTEN loss. While expression of PKC λ is sufficient to drive the expression of these genes, the requirement of PKC λ in the stimulated stage remains to be tested. In the stimulated stage mimicked by loss of PTEN, lipogenesis in hepatocytes is partially reversed by expression of a nuclear localized constitutively active FOXO. This transcriptional factor has been shown to play a role in lipogenesis previously.^{9,10} Our data support the idea that FOXO may be partially responsible for lipogenesis occurring in hepatocytes when PIP₃ levels are accumulated. In conditions where AKT is not activated (as in the case of control cells), inhibition of FOXO activity may not have a significant effect.

In summary, we have explored the relationship between AKT2 and PTEN regulated metabolic changes in the liver. Our data strongly support the indispensable function of AKT2 in liver lipid accumulation and regulation of glucose tolerance. While this manuscript was in the final stage of review, Leavens et al reported that AKT2 is also necessary for hepatic lipid accumulation in insulin resistance models.¹⁶ We further observed that this change in metabolic signal led to a 3-month delay in tumor development (data not shown). How this branch of the signaling downstream of PTEN may contribute to the development of liver cancer in this model^{3,8} remains to be investigated.

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