PIK3IP1, a Negative Regulator of PI3K, Suppresses the Development of Hepatocellular Carcinoma

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Abstract

Phosphatidylinositol-3-kinase (PI3K) is a well-known regulator of cell division, motility, and survival in most cell types. Recently, we characterized a novel protein that we call PI3K Interacting Protein 1 (PIK3IP1), which binds to the p110 catalytic subunit of PI3K and reduces its activity *in vitro*. Little is known about the role of PIK3IP1 in normal and neoplastic growth *in vivo*. Proper liver function and development depend on intact PI3K signal transduction; when dysregulated, the PI3K pathway is linked to the development of liver cancer. To begin to dissect the contribution of PIK3IP1 to hepatic PI3K signaling *in vivo* and to liver tumorigenesis in particular, we formulated the following hypothesis: because PIK3IP1 down-regulates PI3K signaling and uncontrolled PI3K signaling is associated with liver cancer, then PIK3IP1-mediated down-regulation of the PI3K pathway should inhibit hepatocellular carcinoma (HCC) development. To test this idea, we generated transgenic mice overexpressing PIK3IP1 in hepatocytes in a mouse strain prone to develop HCC. Isolated PIK3IP1 transgenic mouse hepatocytes showed blunted PI3K signaling, DNA synthetic activity, motility, and survival compared with controls. *In vivo*, spontaneous liver tumorigenesis was significantly dampened in the transgenic animals. This was accompanied by decreased hepatic PI3K activity and reduced hepatocyte proliferation in the transgenics compared with controls. We also observed that human HCC expressed less PIK3IP1 protein than adjacent matched liver tissue. Our data show that PIK3IP1 is an important regulator of PI3K *in vivo*, and its dysregulation can contribute to liver carcinogenesis. [Cancer Res 2008;68(14):5591–8]

Introduction

The class IA phosphoinositol-3-kinases (PI3K) regulate important cellular processes such as proliferation, growth, survival, motility, and metabolism. PI3K is stimulated by association of the p85 regulatory subunit with tyrosine-phosphorylated proteins at the plasma membrane, which leads to activation of the p110 catalytic subunit and generation of the second messenger phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P_3] from phosphatidylinositol-4,5-bisphosphate. The appearance of PI(3,4,5)P_3 attracts Akt (also known as Protein Kinase B) and other signal transduction molecules to the membrane, the association of which triggers typical PI3K-dependent cellular responses (1). We recently identified and characterized a new negative regulator of PI3K we named Phosphatidylinositol-3-kinase Interacting Protein 1 (PIK3IP1) that binds to the p110 catalytic PI3K subunit by virtue of a region sharing homology with the p85 regulatory subunit. When overexpressed in cultured cells, PIK3IP1 suppresses PI3K activity, whereas PI3K activity increases when PIK3IP1 levels are experimentally reduced (2). However, the consequences of PIK3IP1 on PI3K signaling *in vivo* remain unknown.

A variety of normal tissues such as the liver use the PI3K cascade to convey extracellular signals inside the cell leading to changes in gene and protein expression. Abnormal activation of the PI3K pathway, however, can cause unchecked growth and increased cell survival (1). Several human malignancies including ovarian, breast, and liver cancer display dysregulation of the PI3K signal transduction pathway (1, 3, 4). In human hepatocellular carcinoma (HCC), for example, Lee and colleagues (5) detected mutations in *pik3ca* (the gene for p110 α). Of the various tumor types tested, liver cancer shows the highest percentage of cases with *pik3ca* mutations (36%; ref. 1). Another PI3K pathway constituent that is targeted in liver cancer is phosphatase and tensin homologue (PTEN). PTEN is the product of a well-known tumor suppressor gene and acts as a lipid and protein phosphatase that regulates the relative cellular concentration of PI(3,4,5)P_3 (6). PTEN protein abundance is down-regulated in human HCC (7, 8). Mutations and loss of heterozygosity of the *PTEN* gene (9) have been identified as well. Experimentally, liver tumors developed in mice lacking functional PTEN in hepatocytes (10). Given the emerging importance of PI3K to tumorigenesis in tissues such as liver, we chose to explore the possibility that PIK3IP1 plays a role in normal and cancerous hepatic cell growth by regulating PI3K signaling. To do so, we used a mouse model of PIK3IP1 gain-of-function in hepatocytes.

Materials and Methods

Chemicals and reagents. The following reagents were described previously: polyclonal antibody against PIK3IP1, anti-p110 antibodies, anti–phospho-Akt, and anti-Akt antibodies as well as Protein-A agarose and IgG control antibody (2). Anti–phospho-p85 antibody was obtained from Cell Signaling. Mouse epidermal growth factor (EGF) was purchased from BD Biosciences. Hepatocyte growth factor (HGF; five amino acid–deleted isoform) was a kind gift from Snow Brand Milk Products, Ltd. Ly294002 (LY) was purchased from LC Laboratories. All other chemical reagents were obtained from Sigma Company unless otherwise noted.

Assurances. Appropriate University of Pittsburgh Institutional Review Board, Institutional Animal Care and Use Committee, and Recombinant DNA approvals were obtained before commencing these studies.

Human tumor tissues. Archived paraffin-embedded and frozen human HCC and adjacent liver tissues were obtained through the Health Systems Tissue Bank at University of Pittsburgh Medical Center.

Generation and characterization of liver-specific PIK3IP1 transgenic mice. A 0.8-kb mouse *pik3ip1* cDNA containing the entire coding

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region of PIK3IP1 was derived from a mouse mammary gland. PCR primers (forward primer, 5′-TTAGGATCCCATTTGGACACTGGCTG-3′; reverse primer, 5′-GATCCTAGGCCTGTACCAGTGTTTAC-3′) were prepared that incorporated BamHI sites in the 5′ and 3′ noncoding regions of the cDNA near the translation stop and start codons, respectively. The amplified region was inserted into the BamHI site of an albumin promoter–driven expression vector, which was kindly provided by Dr. Richard Palmiter (University of Washington), as described by Bell and colleagues (11). This transgene was used to generate transgenic (TG) mice on a B6SJL background at the Duke University Medical Center Transgenic Mouse Facility. TG mice were identified by Southern blot analysis (using standard procedures) of BamHI-digested genomic tail DNA using a ^{32}P-labeled 0.8-kb mouse *pik3ip1* cDNA probe. To confirm the results, PCR analysis of blood DNA was performed using oligonucleotide primers designed from the *pik3ip1* gene as follows: forward primer: 5′-GACGTGAGTTGCCCAGAGACC-3′; reverse primer: 5′-TTACCTGCAGCCATTGCCGCTAGTGAG-3′. TG mice were propagated by breeding with wild-type C3H mice for greater than four generations. Livers of young mice (≤6 mo) were harvested, and liver and body weights were recorded to calculate the liver to body weight ratio. Grossly, the livers seemed to be normal in the TG and non-TG (NTG) animals, and the liver to body weight ratios were similar between the two groups. Histologically, major architectural changes attributable to the presence of the *pik3ip1* transgene were not observed.

For tumor studies, 8 male TG and 8 male NTG littermates (average age, 14 mo) were sacrificed by cervical dislocation and necropsied. Gross examination of the liver for tumors was performed. The presence of grossly visible tumor was documented. A portion of each liver lobe was placed in formalin, embedded, sectioned, and stained with H&E. Tissue sections were examined histologically to confirm the presence of hepatocellular carcinoma. The remaining liver tissue was snap frozen for additional experimentation.

Assessment of proliferation and apoptosis. Ki67 or proliferating cell nuclear antigen (PCNA) immunostaining and terminal deoxynucleotidyl-transferase–mediated dUTP nick-end (TUNEL) staining of formalin-fixed liver using standard procedures was performed to assess hepatocyte proliferation and apoptosis, respectively. The Ki67 antibody was purchased from Abcam and used at a concentration of 1:50. The PCNA antibody was purchased from Dako and used at a concentration of 1:4,000. The DeadEnd Colorimetric TUNEL System (Promega Corporation) was used to evaluate apoptosis. Three to five thousand hepatocyte nuclei in nontumorous tissues were examined per mouse after Ki67 or TUNEL staining. About 600 hepatocytic nuclei were assessed in tumorous and nontumorous human tissues after PCNA. The formula used to determine the proliferation or apoptotis indices was as follows: number of Ki67, PCNA, or TUNEL-positive hepatocyte nuclei, respectively, divided by the total number of hepatocyte nuclei counted.

Extraction of total RNA and quantitative reverse transcription-PCR. Total RNA was extracted according to the TRIzol manufacturer's protocol (Invitrogen) from TG (n = 8) and NTG (n = 11) mouse liver tissue. Animals ranged in age from 1 to 6 mo. The RNA was reverse transcribed by standard procedures and then subjected to quantitative reverse transcription-PCR (qRT-PCR) analysis for 40 cycles using the ABI Prism 7000 Sequence Detection System with Taq Man Gene Expression Assays reagents as well as primers specific for mouse PIK3IP1 purchased from Applied Biosystems. 18S rRNA primer (Applied Biosystems) was used as an internal control for sample quality, and diethylpyrocarbonate water was used as a negative control for product contamination.

Protein isolation, SDS-PAGE, and Western blot analyses. Human and mouse liver tissues as well as isolated mouse hepatocytes were subjected to Western blot (WB) analysis as described previously (2). Commercially available antibodies were used at the dilutions recommended by their manufacturers. Western Lightning Chemiluminescence Reagent PLUS (PerkinElmer, Inc.) and Biomax film (Eastman Kodak Company) were used to detect the WB signals, the intensities of which were measured using Scion Image 1.63 software (Scion Corporation).

***In vitro* PI3K activity assay.** PI3K activity assays were performed as previously described (2). Briefly, protein lysates (1 mg) were immunoprecipitated with anti-p110 antibodies, washed 3X with radioimmunoprecipitation assay buffer, followed by three washes with PI3K reaction buffer [PI3K-RB; 20 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, and 0.5 mmol/L EGTA]. Immunoprecipitates were suspended in 50 μL of PI3K-RB containing 0.2 mg/mL phosphatidylinositol (Sigma) followed by incubation for 10 min at room temperature. After incubation at room temperature, 440 μL of PI3K-RB containing 30 μCi of ^{32}P-γATP, 0.88 mmol/L ATP, and 20 mmol/L $MgCl_2$ wered added to the resuspended immunoprecipitates, which were then incubated for 10 min at room temperature. Chloroform:methanol:HCl (100 μL; 200:100:2) was used to halt the reaction. TLC was used to separate the products in chloroform:methanol:ammonium hydroxide:water (86:76:10:14) running buffer. Lysates were routinely assessed for p110 abundance by immuniprecipitation and WB analysis.

Isolation and culture of primary mouse hepatocytes. Mouse hepatocytes were isolated from male PIK3IP1 TG and NTG littermates by the *in situ* two-step collagenase perfusion technique described previously (11). Hepatocytes were pelleted by centrifugation, assessed for viability by Trypan Dye exclusion, which typically ranged from 70% to 90%, and cultured in Eagle's Minimal Essential Medium (EMEM; Cellgro) containing 10% fetal bovine serum. The medium was then changed to serum free after 3 h. For some experiments, hepatocytes were plated in hepatocyte growth medium (HGM; ref. 12) with slight modification as follows: (*a*) EMEM was used in place of Dulbecco's Modified Essential Medium; (*b*) nicotinamide was omitted; and (*c*) dexamethasone was added at a final concentration of 10^{-8} mol/L.

^{3}H-thymidine incorporation assay. Mouse hepatocytes from either TG or NTG littermates were seeded at 250,000 cells per well in 6-well plates in triplicate in HGM. After 18 h under serum-free conditions, the cells were changed to HGM medium containing 2.5 μCi ^{3}H-thymidine with or without a combination of HGF (30 ng/mL) and EGF (25 ng/mL) and with either LY (10 μmol/L in DMSO) or DMSO alone as a vehicle control. After 48 h, radioactivity was measured by scintillation counting as described (13).

Transwell migration assay. Transwell migration assays were performed on hepatocytes as described previously (14). Briefly, TG or NTG hepatocytes were seeded onto the upper part of a 12-μm pore Transwell chamber (collagen I coated) at a density of 50,000/cm^2 and allowed to attach for 3 h. Unattached cells were washed; adherent cells were maintained in serum-free EMEM for 12 h before transfer of the Transwell to a fresh 12-well plate containing EGF (20 ng/mL) or control medium with either LY (10 μmol/L in DMSO) or DMSO alone as a vehicle control. Cells were then allowed to migrate for 24 h before fixing with 4% paraformaldehyde in PBS for 30 min. Cells were then stained with 0.1% Coomassie blue in 10% methanol/10% acetic acid for 1 h. Transwells were washed; stationary cells were removed from the filter top with a cotton-tipped applicator; cell debris was washed away; then, cells that had migrated to the bottom of the Transwell filter were enumerated by counting 10 fields for each Transwell at ×200 magnification.

***In vitro* scratch assay.** One million TG or NTG hepatocytes were seeded in 6-well plates to obtain confluent monolayers. After 12 h, a scratch to dislodge cells was made in the monolayers using a blunted Pasteur pipette, and hepatocytes were washed several times to remove floating cells and debris. Cultures were treated with or without EGF (20 ng/mL) and with either LY (10 μmol/L in DMSO) or DMSO alone as a vehicle control for up to 5 d. Daily, the medium was changed and reconstitution of the scratched surface was monitored and photographed.

UV-induced apoptosis assay. One hundred thousand TG or NTG hepatocytes were cultured for 12 h on single-well chamber slides (9.4 cm^2 per well; Lab-Tek). Some slides were treated with a dose of 50 J/m^2 UV irradiation without medium. Mock treatment consisted of medium removal for an equivalent time period but no UV exposure. Serum-free medium was replaced, and after 24 h, slides were fixed with 4% paraformaldehyde and TUNEL stained for detection of apoptotic cells. Cleaved caspase 3 was assessed by WB using cell lysates harvested from some cultures.

Statistical analysis. Statistical analysis was carried out using a two-tailed Student's t test unless otherwise specified. Results were considered to be statistically significant when P values were determined to be <0.05.

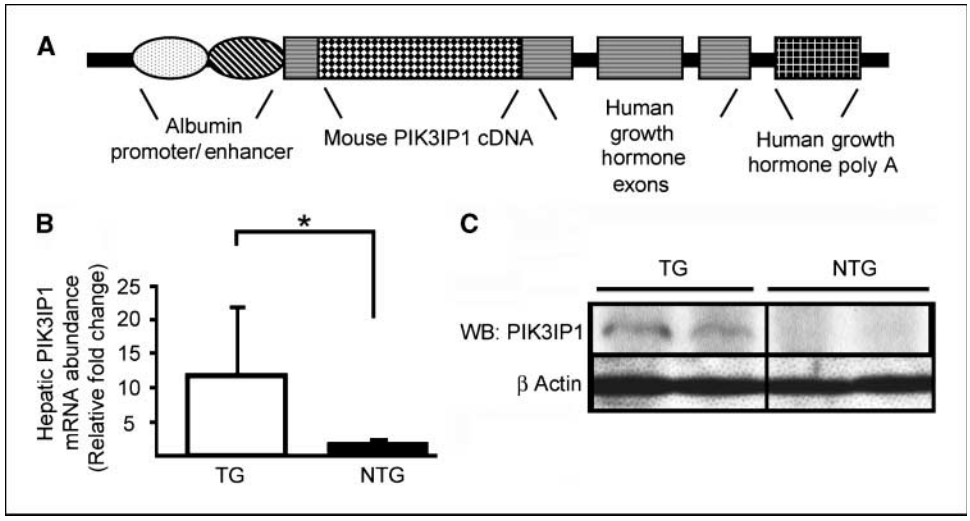

Figure 1. Generation and characterization of TG mice expressing PIK3IP1 in hepatocytes. *A,* PIK3IP1 transgene construct. Full-length mouse *pik3ip1* cDNA was subcloned into an albumin promoter/enhancer growth hormone expression construct. *B, pik3ip1* mRNA is overexpressed in PIK3IP1 TG mouse liver. Total liver RNA from TG (n = 8) and NTG (n = 11) mice was subjected to qRT-PCR. Data were normalized to 18S rRNA; the relative fold change in *pik3ip1* mRNA abundance was calculated and graphed. *, P = 0.0006. *C,* PIK3IP1 protein is overexpressed in PIK3IP1 TG mouse liver. Liver protein lysate from TG (n = 2) and NTG (n = 2) mice was subjected to WB for PIK3IP1 protein. β Actin was used as a loading control.

Results

Generation and characterization of hepatocyte-specific PIK3IP1 overexpressing mice. We subcloned the full-length mouse *pik3ip1* cDNA into a construct containing the human growth hormone gene under the transcriptional control of the mouse albumin promoter/enhancer (Fig. 1*A*) as we described previously for human HGF (11) and generated TG mice. We used this particular promoter/enhancer cassette because albumin promoter activity is specific for hepatocytes, increases after birth, and plateaus within ~1 month (15).

PIK3IP1 expression reduces PI3K activity and phospho-Akt levels in mouse liver. We first ascertained that TG mice overexpress PIK3IP1 in the liver by qRT-PCR (Fig. 1*B*) and Western blot (Fig. 1*C*) compared with controls. We noted no differences in p110 α or β protein levels between the livers of TG and NTG mice (data not shown). However, we did observe a significant diminution in PI3K activity by ~50% (Fig. 2*A* and *B*) and a marked decrease in phospho-Akt levels by WB (Fig. 2*C*) in the TG livers compared with controls, suggesting that PIK3IP1 regulates p110 activity and signaling in liver.

PIK3IP1 down-regulates Akt in isolated mouse hepatocytes. To further investigate the effects of PIK3IP1 on liver cell biology and the PI3K pathway, we isolated hepatocytes from TG and NTG animals. We confirmed that PIK3IP1 is overexpressed in the TG hepatocytes as compared controls by qRT-PCR (data not shown). Next, we examined the hepatocytes for basal Akt activation by WB and detected decreased levels of endogenous phospho-Akt in cultured TG hepatocytes compared with control cells (Fig. 2*D*). This indicates that PIK3IP1 expression does indeed down-regulate the PI3K pathway in TG hepatocytes.

PIK3IP1 suppresses mouse hepatocyte DNA synthesis and motility in culture. Because we determined that PIK3IP1 reduces signaling through the PI3K pathway in TG hepatocytes and because PI3K activity is linked to increased cell motility and

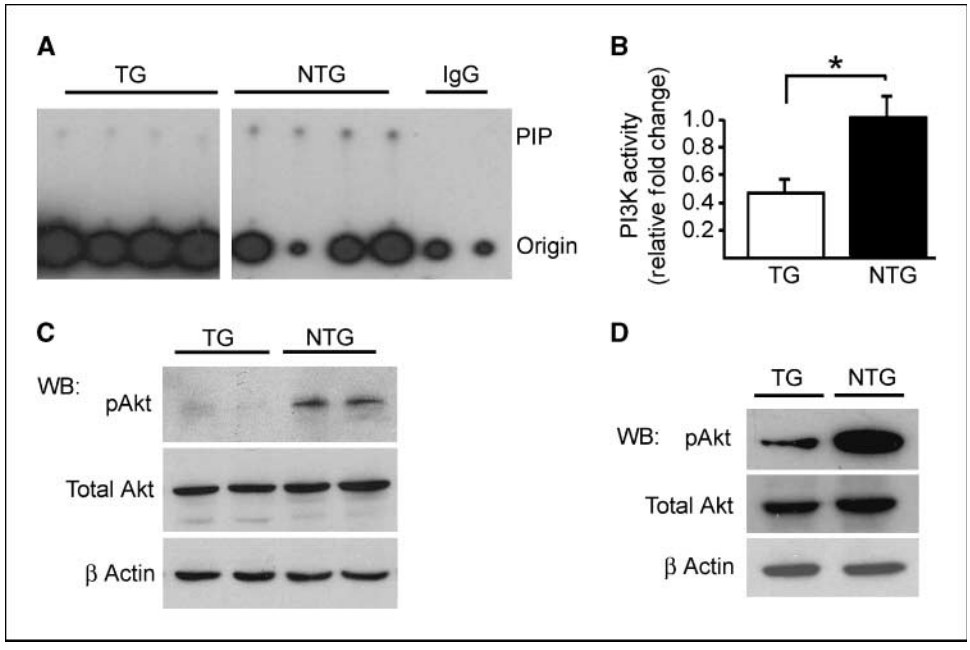

Figure 2. PIK3IP1 expression suppresses PI3K activity and phospho-Akt abundance in TG mouse liver and hepatocytes. *A* and *B,* PI3K activity is reduced in PIK3IP1 TG mouse liver. PI3K activity in the livers of TG (n = 2) and NTG (n = 2) littermates was assessed in duplicate by standard PI3K assay. Production of phosphatidylinositol-3-phosphate (PIP) is shown. The relative fold change in PIP production was calculated after densitometric analysis of the PIP signals and is graphed in *B*. *, P < 0.003. *C,* phospho-Akt is down-regulated in PIK3IP1 TG mouse liver. WB analysis of liver protein lysates from TG (n = 2) and NTG (n = 2) littermates was carried out using antibodies to phospho-Akt and total Akt. β Actin was used as a loading control. *D,* phospho-Akt is dampened in PIK3IP1 TG mouse hepatocytes. TG and NTG-cultured mouse hepatocytes were subjected to WB analysis using anti–phospho-Akt and total Akt antibodies. β Actin was used as a loading control.

proliferation in a wide variety of cell types, we next explored the functional consequences of PIK3IP1 expression and PI3K down-regulation on DNA synthesis and motility in the TG and NTG hepatocytes. When cultured hepatocytes were induced to undergo DNA synthesis by addition of known hepatic mitogens (HGF and EGF; ref. 16), the DNA synthetic response in TG hepatocytes measured by fold increase in ^{3}H-thymidine incorporation over control cultures was strongly impaired (Fig. 3*A*). We also added LY, a known PI3K inhibitor (17), to some cultures to determine the effect of PI3K inhibition on hepatocyte DNA synthesis; LY treatment also blunted growth factor–induced hepatocyte DNA synthesis (data not shown). Overall, our results show that PIK3IP1 expression diminishes mouse hepatocyte DNA synthetic activity likely through inhibition of PI3K signaling.

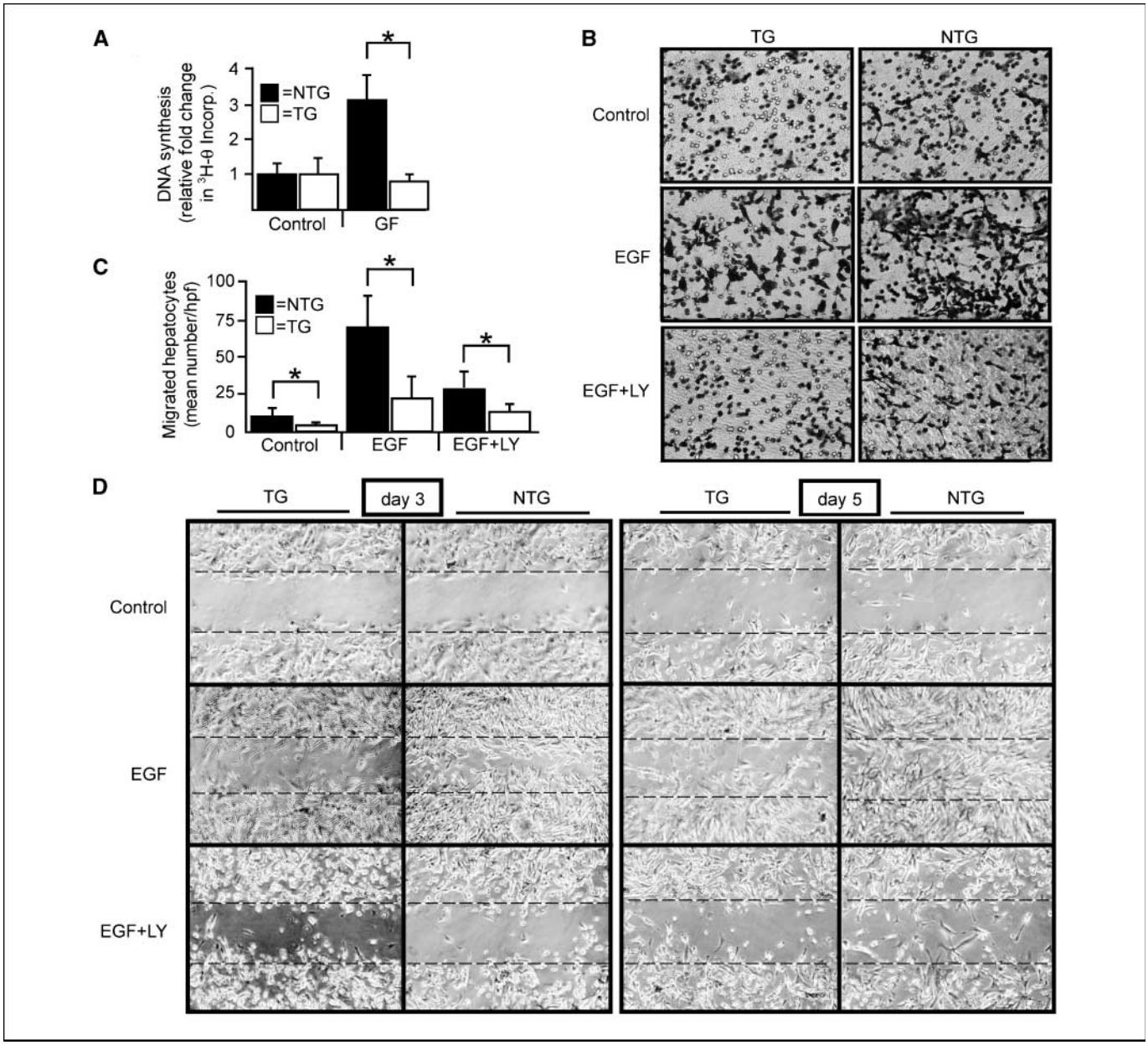

Figure 3. PIK3IP1 reduces hepatocyte DNA synthesis and migration. *A,* DNA synthesis is blunted in PIK3IP1 TG mouse hepatocytes. TG and NTG-cultured mouse hepatocytes were stimulated to undergo DNA synthesis by addition of HGF and EGF (*GF*; *control,* no growth factor added). A standard ^{3}H-thymidine incorporation (^{3}*H-θ incorp*) assay was performed in triplicate. The relative fold change in average ^{3}H-thymidine incorporation over control cultures was assessed. *, $P = 0.01$. This assay was repeated with hepatocytes isolated from a different set of animals and had a similar outcome. *B* and *C,* hepatocyte migration is reduced by PIK3IP1: transwell migration assay. A transwell migration assay was performed using TG and NTG plated at a density of 50,000/cm^2. Migration was stimulated by addition of EGF. Some cultures were also treated with LY (a PI3K inhibitor). Hepatocytes that migrated to the underside of the transwell insert were stained with Coomassie blue, enumerated, and photographed; the images are shown in *B*. The mean number of migrated hepatocytes per hpf is graphed in *C*. *, $P < 0.01$. *Control,* no growth factor added. This assay was repeated with hepatocytes isolated from a different set of animals and had a similar outcome. *D,* hepatocyte migration is reduced by PIK3IP1: *in vitro* scratch assay. An *in vitro* scratch assay was carried out using TG and NTG-cultured mouse hepatocytes. Migration was stimulated by addition of EGF. Some cultures were also treated with LY (a PI3K inhibitor). Restitution of the scratched surface was observed >5 d. The photographs shown were taken at days 3 and 5 postscratch. *Control,* no growth factor added. This assay was repeated with hepatocytes isolated from a different set of animals and had a similar outcome.

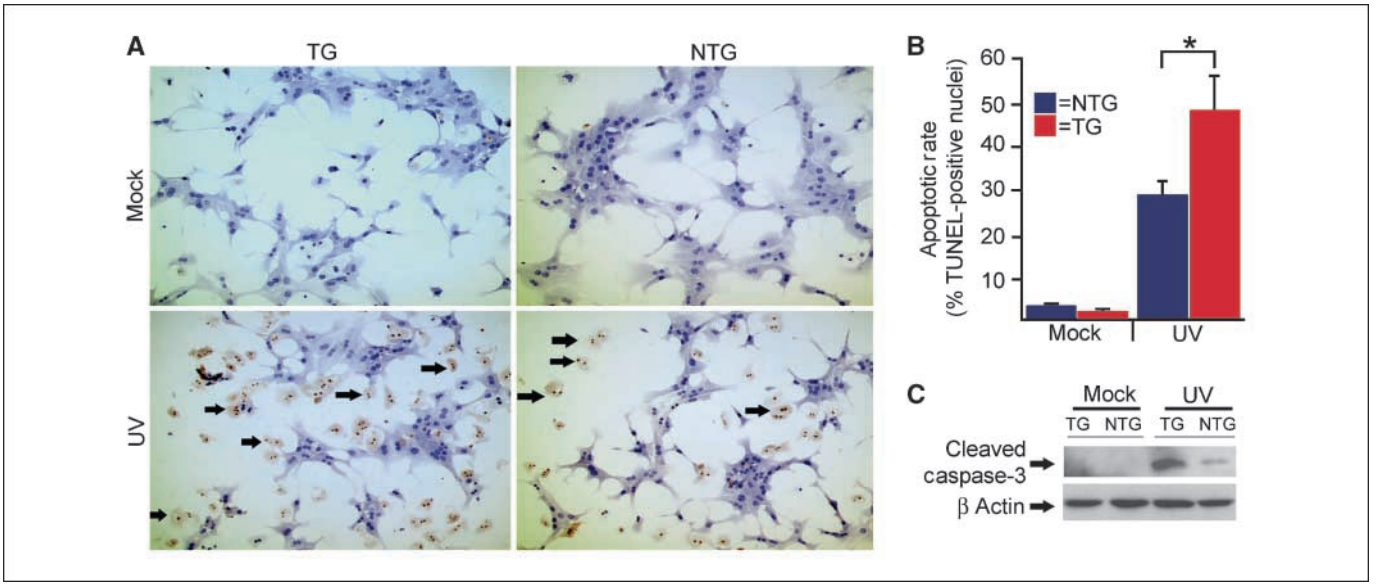

Figure 4. PIK3IP1 augments hepatocyte apoptosis. TG and NTG-cultured mouse hepatocytes were induced to undergo apoptosis with UV light (UV, 50 J/m^2) or mock treatment. *A,* TUNEL assay. The cells were fixed, immunostained for TUNEL, and photographed. *Arrows,* TUNEL-positive hepatocyte nuclei. The apoptotic rate was calculated and graphed (*B*). This assay was repeated with hepatocytes isolated from a different set of animals and had a similar outcome. *C,* measurement of cleaved caspase-3 by WB. TG and NTG-cultured mouse hepatocytes were induced to undergo UV-induced apoptosis as above. Protein lysates were prepared and subjected to WB for cleaved caspase 3. β Actin was used as a loading control.

To examine the effect of PIK3IP1 on cell motility, another cellular function ascribed to PI3K (18), we subjected TG and NTG hepatocytes to a transwell migration assay. In response to EGF, a well-characterized hepatocyte motogen (14), 3-fold fewer TG hepatocytes than NTG hepatocytes migrated across the transwell insert [mean number of migrated hepatocytes per high power field (hpf), 23.8 ± 13.8 versus 70.3 ± 22.5, respectively; Fig. 3*B* and *C*]. This difference was found to be statistically significant (P = 0.00003). As a control for PI3K inhibition, LY was added to some EGF-treated TG and NTG cultures; LY reduced migration of both cell types by >2-fold (Fig. 3*B* and *C*).

In parallel, we performed a second type of motility assay (i.e., an *in vitro* scratch assay; ref. 19) that compares the ability of TG and NTG hepatocytes to reepithelialize a scratched surface. As shown in Fig. 3*D*, reepithelialization was significantly delayed in TG hepatocyte cultures compared with controls that typically reconstituted the void in 5 days in our assay. We added LY to some EGF-treated cultures; it halted reepithelialization by both TG and NTG hepatocytes. Taken together, our data indicate that PIK3IP1 impedes mouse hepatocyte motility, a response that is likely to be at least in part PI3K dependent.

PIK3IP1 promotes apoptosis in isolated mouse hepatocytes. Considering the key role of PI3K in cell survival (20), we next assessed if PIK3IP1 spurs hepatocyte cell death. To do so, we induced apoptosis in TG and NTG hepatocytes with UV exposure and examined the cells for apoptosis by TUNEL staining and by WB for cleaved caspase 3. Normal cultured hepatocytes are known to undergo apoptosis upon UV treatment (21). In our experiments, we observed that 47.4 ± 6.3% of TG hepatocytes and only 28.5 ± 3.3% of control cells became TUNEL positive 48 h after UV irradiation (Fig. 4*A* and *B*), which was determined to be a statistically significant difference (P = 0.00005). TG hepatocytes also showed significantly more cleaved caspase 3 by WB at this time point when compared with NTG cells (Fig. 4*C*). Taken together, these data support the idea that PIK3IP1 promotes apoptosis in isolated hepatocytes, an observation that is in agreement with our previous finding that PIK3IP1 enhanced apoptotic cell death induced by staurosporine addition to C33A human endometrial cancer cells (2).

PIK3IP1 suppresses hepatocyte proliferation and spontaneous HCC development *in vivo*. Once we established that PIK3IP1 overexpression alters PI3K activity in mouse liver and in isolated hepatocytes, we wanted to determine whether its presence affects the appearance of liver tumors *in vivo*. Our TGs were crossed onto a C3H background. Male mice of the C3H strain spontaneously develop HCC without carcinogen treatment: the incidence of liver tumors is estimated to be 72% to 91% in males at age 14 months.[4] On necropsy, we found that 62.5% of NTG male mice (average age, 14 months) had histologically confirmed HCC in their livers, whereas only 12.5% of PIK3IP1-matched male TG littermates developed HCC (Fig. 5*A* and *B*). This comparison was statistically different (P = 0.0387) between the groups using χ^2 analysis.

To begin to decipher how PIK3IP1 modulates liver tumorigenesis in our animals, we examined the hepatocyte proliferation rate in the adjacent normal liver tissue of TG and NTG mice used in the tumor study. We immunostained their livers for Ki67, a nuclear antigen which appears in G_1-G_M. We observed that ~1.00 ± 0.52% of NTG hepatocyte nuclei stained for Ki67, whereas only 0.36 ± 0.21% of TG hepatocyte nuclei were Ki67 positive (Fig. 5*C* and *D*). This difference was found to be statistically significant between the two groups using the Mann-Whitney test (P < 0.0039). The percentage of apoptotic hepatocytes as determined by TUNEL staining was not statistically different between the NTG and TG groups (0.27 ± 0.15% versus 0.44 ± 0.32%, respectively) using the Mann-Whitney test. Collectively, these data indicate that the presence of PIK3IP1 in hepatocytes suppresses hepatocyte proliferation *in vivo* and spontaneous liver tumorigenesis.

[4] http://jaxmice.jax.org/strain/000659.html

PIK3IP1 protein expression is reduced in human HCC. Lastly, to gain insight as to whether PIK3IP1 plays a role in human liver tumorigenesis, we examined PIK3IP1 expression in human HCC and adjacent nontumor tissue by WB and found that PIK3IP1 protein abundance was diminished in the majority of HCC cases (9 of 12) compared with matched adjacent liver (Fig. 6*A* and *B*). On average, tumors expressed 22% less PIK3IP1 protein than control tissues (P = 0.0025). However, 3 of the 12 cases expressed only half the amount of PIK3IP1 protein compared with adjacent tissue (cases 2, 6, and 9; Fig. 6*A* and *B*). We then carried out analysis of human HCC and adjacent liver tissues for PI3K activity as measured by the abundance of tyrosine phosphorylated p85 (which is a surrogate of PI3K activation; ref. 22) relative to total p85 in WB and for proliferation activity (as determined by PCNA nuclear labeling). We next compared these findings to PIK3IP1 abundance in the same tissues. As expected, we observed a high proliferative index (average labeling index, 30% in tumors versus 2.3% in the corresponding adjacent livers) and readily detectable PI3K activity in tumors, which were associated with reduced PIK3IP1 abundance in several HCC cases tested (data not shown).

Discussion

Unregulated signaling through the PI3K pathway is linked to the development of certain types of cancer including HCC (1, 5). Although others have shown that hepatocyte-specific gene deletion of the PI3K signaling regulator *PTEN* results in the appearance of liver tumors in a mouse model (10), no studies of which we are aware have examined the effects of directly suppressing PI3K activity on liver tumor development. Our laboratory discovered and characterized a novel negative regulator of Class Ia PI3Ks called PIK3IP1. PIK3IP1 is a transmembrane protein that possesses a region in its intracellular domain that shares homology with the p85 regulatory subunit of PI3K. Previously, we showed through a variety of *in vitro* experiments that PIK3IP1 binds to the p110 catalytic subunit of PI3K through the intracellular p85–like domain of PIK3IP1 to down-regulate PI3K activity (2). However, we had not elucidated the role of PIK3IP1 in controlling PI3K activity *in vivo*. The liver requires sufficient PI3K signaling to function appropriately. For example, mice lacking all isoforms of p85 α (thus showing substantially reduced PI3K activity because of a destabilizing effect on p110) die perinatally partly due to liver necrosis (23). As mentioned, unfettered PI3K signaling also alters liver homeostasis. Aberrant PI3K signal transduction is implicated in liver tumorigenesis in both humans and rodents (5, 7, 8, 10). Given all of this information as well as our own published observations that PIK3IP1 negatively regulates the PI3K pathway *in vitro*, we wanted to ascertain whether PIK3IP1 is involved in normal liver growth and hepatic tumorigenesis. We hypothesized that PIK3IP1 expression in hepatocytes suppresses PI3K activity in these cells and thereby inhibits hepatocyte growth, motility, and liver tumorigenesis.

To test this hypothesis, we took a gain-of-function approach and generated TG mice in which PIK3IP1 expression is directed to the hepatocytes. Our decision to target PIK3IP1 expression to hepatocytes is supported by physiologically relevant evidence summarized as follows: (*a*) the liver has high endogenous PI3K activity compared with most other tissues (i.e., relative mouse

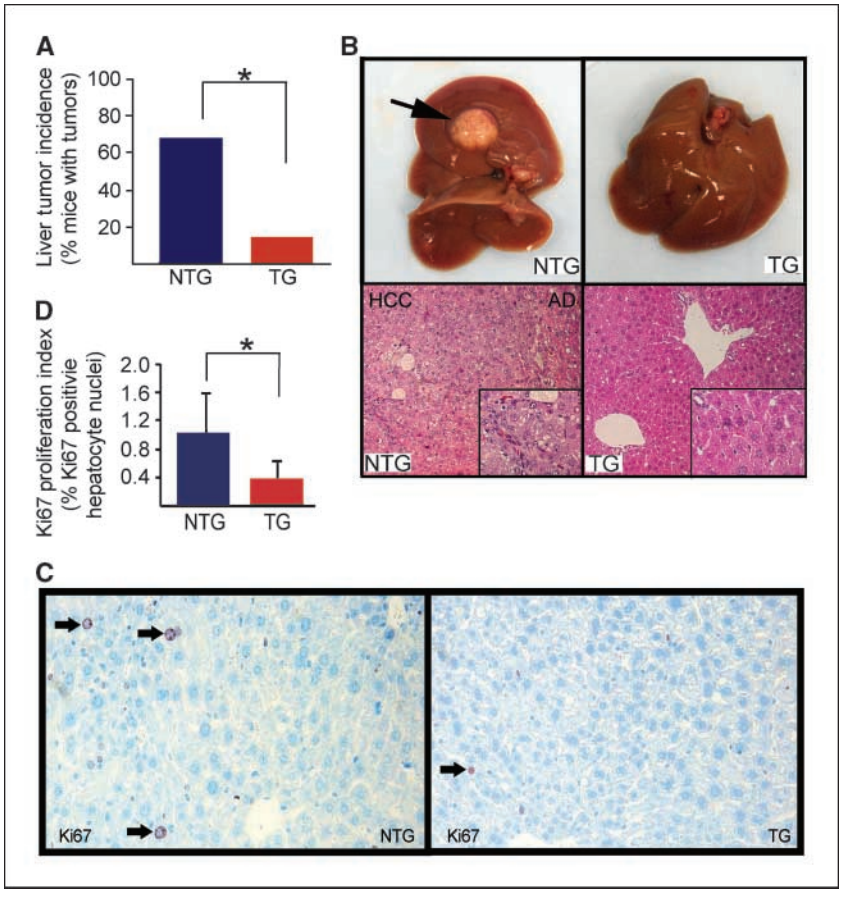

Figure 5. PIK3IP1 expression suppresses spontaneous tumorigenesis and hepatocyte proliferation in mouse liver. *A* and *B,* spontaneous liver tumor development is inhibited in PIK3IP1 TG mice. Graphic representation (*A*) showing the percentage of PIK3IP1 TG (n = 8) and NTG (n = 8) control mice with liver tumors (average age, 14 mo). *, P = 0.0387. Gross (*top*) and histologic (*bottom*; ×200) images of representative livers and liver tumors are shown in *B*. For the NTG animal, the liver tumor is indicated by an arrow in the gross image and by *HCC* in the histologic image. *AD,* adjacent liver tissue. *Bottom insets,* ×400 magnification of tumor or normal liver for NTG and TG, respectively. *C* and *D,* hepatocyte proliferation is reduced in PIK3IP1 TG mouse liver. Adjacent nontumorous liver tissue from the TG and NTG mice described in *A* and *B* was subjected to immunostaining for the Ki67 proliferation marker. Representative histologic images are shown in *C*. The Ki67-positive (*arrows*) and Ki67-negative hepatocyte nuclei were enumerated, and the Ki67 proliferation index was calculated and graphed (*D*). *, P = 0.0039.

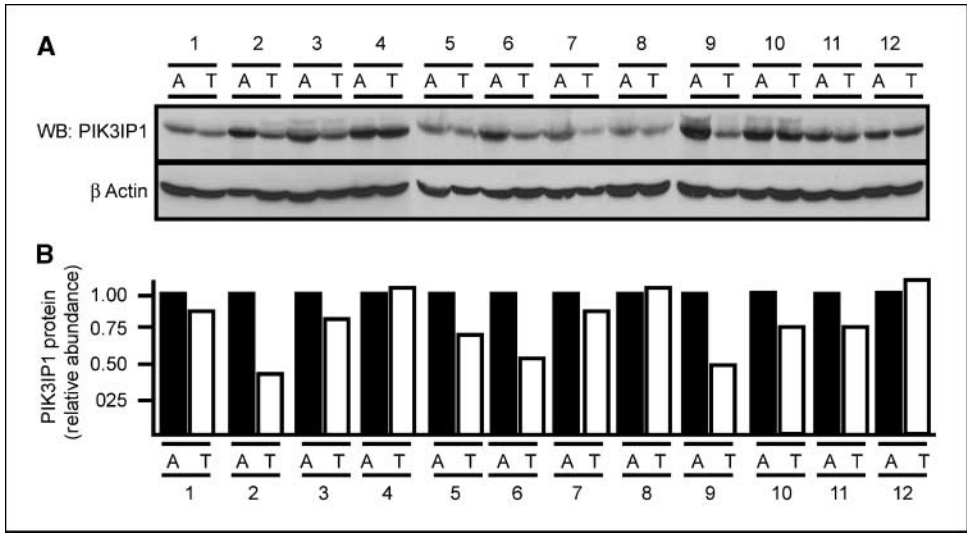

Figure 6. *A,* PIK3IP1 protein expression is down-regulated in human HCC. *A* and *B,* 12 cases of human HCC (*T*) and matched adjacent (*A*) liver tissue were probed for PIK3IP1 protein expression by WB. β Actin was used as a loading control. The average relative PIK3IP1 abundance in each tumor versus its matched adjacent tissue was calculated after densitometric analysis and graphed (*B*).

tissue PI3K activity measured by phospho-/total AKT WB: brain, 0.4; heart, 1.0, testis, 1.2; spleen, 1.5; kidney, 1.6; lung, 1.8; and liver, 2.9); (*b*) liver survival is dependent on a functioning PI3K pathway (23); (*c*) PIK3IP1 mRNA and protein are expressed by liver (2) and hepatocytes (data not shown); and (*d*) the expression levels of PI3K pathway constituents (5, 7, 8) are altered in human liver tumors compared with adjacent liver.

Analysis of our PIK3IP1-expressing mice revealed that hepatic PI3K activity is reduced compared with control animals supporting an *in vivo* role for PIK3IP1 as a PI3K regulatory molecule. Hepatocytes isolated from these animals showed a blunted response to growth factor–induced DNA synthesis. In addition, we found that LY, a known PI3K inhibitor, staunched DNA synthesis in isolated mouse hepatocytes, a finding that is in agreement with studies by others showing that PI3K blockade by wortmannin (another well-studied PI3K inhibitor; ref. 24) cuts HGF-induced DNA synthesis in cultures of rat hepatocytes by about half compared with HGF treatment alone (25). Given the fact that the hepatocyte DNA synthetic response stimulated by growth factors is sensitive to PI3K inhibition, it is reasonable to postulate that the suppressive effect of PIK3IP1 on this process is mediated by its ability to down-regulate PI3K activity.

Growth factors such as EGF or HGF stimulate hepatocyte motility in culture. Nakanishi and colleagues (26) reported that blocking PI3K signaling with wortmannin mitigated HGF-induced motility of human liver cancer cell lines in culture; however, it is unknown whether the motility of *normal* hepatocytes depends on signal transduction through the PI3K pathway. Our experiments are the first to show that normal mouse hepatocytes treated with LY are significantly less motile than controls in transwell and scratch assays, suggesting that hepatocyte motility does indeed rely, at least in part, on intact PI3K signaling. In the same types of studies, we found that, akin to our observations in LY-treated hepatocytes, motility in PIK3IP1-overexpressing TG hepatocytes was reduced compared with controls, which may well be a consequence of its ability to suppress PI3K.

Tumor development requires the activation of a set of cellular functions such as division, proliferation, migration, survival, and tissue remodeling, many of which are influenced by PI3K. In our TG animal model, we observed a significant reduction in spontaneous liver tumorigenesis in PIK3IP1-overexpressing TG male mice compared with controls. Because our studies indicated that PIK3IP1 expression in hepatocytes suppressed hepatic PI3K activity as well as hepatocyte DNA synthesis in culture, we chose to examine hepatocyte replication *in situ* in the livers of the TG and NTG animals. Typically, the vast majority of hepatocytes in the adult are in a state of quiescence (i.e., in G_0) *in vivo*. For example, Counts and colleagues (27) pulsed mice of two different strains (B6C3F1 and C57B/6) with BrdUrd for 1 week, sacrificed the animals, and determined the hepatocyte BrdUrd labeling index as a measure of proliferation. They noted that only 2% to 4% of hepatocytes labeled with BrdUrd, indicating that a minor proportion of normal hepatoyctes were engaged in the cell cycle. We observed that hepatocyte replication in our TG animals was reduced: the percentage of hepatocytes staining with the proliferation marker Ki67 was approximately two-thirds less in TG mice than that observed in control animals. Thus, one mechanism by which PIK3IP1 suppresses liver tumorigenesis in mice may be through inhibition of innate hepatocyte replication, a process that we show is likely to be at least partially dependent on PI3K signaling.

As noted, we observed PIK3IP1-induced inhibition of hepatocyte proliferation as well as motility, two functions crucial not only to hepatic tumor development and growth but to governing the ability of the liver to physiologically replenish itself or regenerate after insult. Liver regeneration is a remarkable process whereby the liver parenchyma regrows in an orderly fashion after limited hepatic damage (by exposure to toxins or anoxia, for example) or surgical excision (i.e., two-thirds partial hepatectomy). Proper liver regrowth requires a combination of controlled cell division, migration, and tissue remodeling and is stimulated and regulated by a variety of endocrine and paracrine growth factors, cytokines, and hormones, some of which are known to signal through PI3K such as HGF (16). It seems likely then that the PI3K signaling network plays an instrumental part of the normal regenerative response in hepatic tissue. Despite the obvious link between PI3K and liver regeneration, little has been published elucidating the role of PI3K in regrowth of the liver. A recent report by Jackson and colleagues (28) shows that treatment of mice with wortmannin slowed liver regeneration at 2 days after partial hepatectomy as measured by the percentage of liver remnant to body mass as well as by BrdUrd incorporation into hepatocyte nuclei. When the mice

were treated with siRNA to either p85 α or p110 α, a more robust inhibition of liver regeneration was observed. We are currently assessing the role of PIK3IP1 in liver regeneration after surgical resection in our PIK3IP1-overexpressing mice.

Altogether, our *in vivo* and *in vitro* data demonstrating that hepatic PIK3IP1 expression negatively regulates PI3K activity in this tissue and suppresses the development of HCC coupled with our findings that PIK3IP1 protein expression is reduced in most cases of human HCC point to a tumor suppressor–like function for PIK3IP1 and suggest that down-regulating PI3K may well have an inhibitory effect on liver tumorigenesis, a notion that deserves further attention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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