Published in final edited form as:

J Gastroenterol. 2009 ; 44(8): 847–855. doi:10.1007/s00535-009-0073-3.

The tumor suppressor protein PTEN inhibits rat hepatic stellate cell activation

Motoki Takashima,

Division of Gastroenterology and Hepatology, Department of Medicine, University of North Carolina, CB #7032, Medical Biomolecular Research Building, Room 7340B, Chapel Hill, NC 27599-7032, USA

Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan

Christopher J. Parsons,

Division of Gastroenterology and Hepatology, Department of Medicine, University of North Carolina, CB #7032, Medical Biomolecular Research Building, Room 7340B, Chapel Hill, NC 27599-7032, USA

Kenichi Ikejima,

Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan

Sumio Watanabe,

Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan

Eric S. White, and

Division of Pulmonary and Critical Care Medicine, University of Michigan Medical School, Ann Arbor, Michigan, USA

Richard A. Rippe

Division of Gastroenterology and Hepatology, Department of Medicine, University of North Carolina, CB #7032, Medical Biomolecular Research Building, Room 7340B, Chapel Hill, NC 27599-7032, USA

Motoki Takashima: ; Christopher J. Parsons: ; Kenichi Ikejima: ; Sumio Watanabe: ; Eric S. White: ; Richard A. Rippe: raripe@med.unc.edu

Abstract

Background—Following a fibrogenic stimulus, the hepatic stellate cell (HSC) transforms from a quiescent to an activated cell type associated with increased proliferation, collagen and smooth muscle α -actin (α SMA) expression. Phosphatase and Tensin Homolog Deleted on Chromosome Ten (PTEN), a tumor suppressor phosphatase, has been shown to play a role in several nonmalignant diseases. Here, we investigated the role of PTEN during HSC activation.

Methods—Rat HSCs 2 days after isolation were transduced with adenoviruses expressing either the wild-type (WT) or a dominant negative form of PTEN, and culture-associated activation of HSCs, including morphological changes, expression of α SMA and α 1(I) collagen, and cell proliferation, were evaluated. Apoptosis of HSCs was detected by measuring activity of caspase 3/7. Phosphorylation status of Akt, p70^{S6K}, and Erk was detected by Western blotting.

Results—Overexpression of WT-PTEN inhibited phenotypic changes were associated with HSC activation, including morphological changes, expression of α SMA and α 1(I) collagen, and HSC

proliferation, including cyclin D1 expression. WT-PTEN expression also induced apoptosis in HSCs with increased caspase 3/7 activity. Expression of WT-PTEN also caused decreased activation of Akt, p70^{S6K}, and Erk signaling pathways.

Conclusions—Taken together, these findings show that PTEN represents an important negative regulator for transactivation of HSCs. This may have important implications for the design of therapeutic strategies to prevent the progression of liver fibrosis.

Keywords

Hepatic stellate cells; Phosphatase and Tensin Homolog Deleted on Chromosome Ten (PTEN); Liver fibrosis; Smooth muscle α -actin (α SMA); Collagen

Introduction

Liver fibrosis, and its end stage disease liver cirrhosis, represents a major medical problem worldwide. The hepatic stellate cell (HSC) plays a critical role in the development and maintenance of liver fibrosis. In the normal liver, HSCs reside in a quiescent state characterized by vitamin A storage, a low proliferative rate, and trace production of ECM components. However, following a fibrogenic stimulus, HSCs undergo a complex activation process associated with morphological changes from a quiescent vitamin A-storing cell to that of an activated myofibroblast-like cell [1,2]. HSC activation is also associated with a dramatic increase in the synthesis and deposition of ECM components, of which type I collagen predominates, the appearance of the characteristic activation marker smooth muscle α -actin (α SMA), and an increase in cellular proliferation.

The tumor suppressor protein Phosphatase and Tensin Homolog Deleted on Chromosome Ten (PTEN) is a dual specificity protein and lipid phosphatase [3]. Deletions or mutations of PTEN have been found to occur in a wide range of advanced cancers including glioblastoma, melanoma, endometrial carcinoma, prostate, breast, kidney, and small cell lung cancer [3,4]. Altered PTEN expression has also been associated with nonmalignant diseases characterized by tissue destruction and remodeling, such as pulmonary fibrosis, bronchial asthma, and rheumatoid arthritis [5–7]. Hepatocyte specific deletion of PTEN showed increased steatosis as well as increased risk for the development of hepatocellular carcinoma [8,9]. A number of studies have indicated that PTEN is effective at dephosphorylating proteins, and that 3,4,5-trisphosphate (PIP₃), a product of phosphatidylinositol-3-kinase (PI3K), is the primary physiologic substrate of PTEN [10,11]. PIP₃ is necessary for phosphorylation and subsequent activation of the downstream target Akt, a kinase that promotes cell survival and growth in various cell types [12–14]. Furthermore, it has been reported that PTEN induces apoptosis, and inhibits proliferation and migration in some normal cell types [15]; however, the fundamental roles of PTEN in normal cells remain largely unknown.

The PI3K-Akt signaling pathway is activated in HSCs by platelet-derived growth factor (PDGF) and promotes cellular proliferation and collagen gene expression [16–18]. Inhibiting PI3K activity suppresses cell proliferation and type I collagen gene expression in activated HSCs [17]. Therefore, the PI3K/Akt signaling pathway represents an important intracellular signaling pathway associated with the fibrogenic nature of HSC activation. The role of PTEN in HSC activation has not been investigated; however, given the role of PTEN in mediating Akt activation, we hypothesized that PTEN plays an important role in regulating cellular functions associated with the development of HSC phenotype. Here, we investigated whether overexpression of PTEN inhibits activation, proliferation, and survival of HSCs.

Materials and methods

Hepatic stellate cell isolation

HSCs were purified from Sprague-Dawley rats (>400 g, Charles River Laboratory, Cambridge, MA, USA) by sequential digestion of the liver with pronase and collagenase, followed by Nycodenz gradient centrifugation as previously described [19]. Cell purity, assessed by examining the autofluorescence properties of the stored retinoids in HSCs, was typically between 90 and 95%. HSCs were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), and 2 mM L-glutamine, and cultured in a 95% air–5% CO₂ humidified atmosphere at 37°C. The growth medium was changed every other day. HSCs were activated by culturing on plastic for 7 days [20]. All animal procedures were performed under the guidelines set by the University of North Carolina Institutional Animal Care and Use Committee and are in accordance with those set by the National Institutes of Health.

Adenoviral transduction

Ad5-βgal, which contains the β-galactosidase gene driven by the cytomegalovirus promoter, was used as a control virus throughout this study. Ad5-wild-type PTEN (Ad5-WT-PTEN) expresses the active form of PTEN, while Ad5-C124S PTEN expresses a dominant negative form of PTEN; both viruses were kindly provided by Dr. C. Kontos (Department of Medicine, Duke University Medical Center, Durham, NC, USA). The C124S mutation results in a phosphatase-dead protein, which possesses neither lipid nor protein phosphatase activity [21]. Viral amplification was performed in 293 cells and the virus purified by cesium chloride centrifugation by standard methodology [22]. HSCs, 2 days after isolation (day 2), were transduced at a multiplicity of infection (MOI) of 150 for 16 h in Dulbecco's Modified Eagle's Medium containing 2% fetal bovine serum (FBS). After 16 h, the transduction medium was changed to fresh growth medium containing 10% FBS. In some of the experiments, HSCs were cultured for 48 h in medium without FBS supplementation to synchronize the cells. Afterwards, cells were treated with 10% FBS. Viral transduction efficiencies were typically between 85 and 95% as assessed by the percentage of GFP-positive cells (data not shown).

Western blot analysis

Cultured HSCs were washed with phosphate-buffered saline, and the cells were lysed using protein sample buffer (100 mM Tris–HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Western blot analysis was performed as described previously [23]. Anti-phospho-Akt (Ser⁴⁷³), anti-Akt, anti-phospho-Erk, anti-PTEN or anti-cleaved-caspase 3 (Cell Signaling, Beverly, MA, USA) antibodies were incubated for 16 h at 4°C followed by 1 h incubation at room temperature with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), each diluted 1:3000; primary anti-phospho-p70^{S6K}, anti-cyclin D1, anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-αSMA, or anti-PCNA antibody (Dako, Carpinteria, CA, USA) incubated 1 h at room temperature followed by 1 h incubation at room temperature with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody each diluted 1:3000. Signals were quantitated by Alpha Imager analysis (Alpha Innotech, San Leandro, CA, USA).

Cell proliferation studies

Isolated HSCs were seeded at a density of 3×10^5 cells/plate in 60 mm tissue culture dishes. HSCs were transduced with the adenoviruses on day 2 after cell isolation (average cell number; 1.57×10^5) as described above. HSCs were counted using a hemocytometer, and cell viability assessed by Trypan blue staining every other day for 10 days.

RNase protection assay

Total RNA was isolated from rat HSCs cultured for 7 days, and RNase protection assays performed as described previously [24]. Radiolabeled probes were prepared for rat $\alpha 1(I)$ collagen [24] and glyceraldehyde-3-phosphate dehydrogenase (pTRI-GAPDH-Rat, Ambion Inc., Austin, TX, USA) then were hybridized with 5 μ g of total HSC RNA. Protected fragments were separated using standard 6% acrylamide/urea sequencing gels. Following electrophoresis, bands were visualized by autoradiography and quantitated by PhosphorImager analysis (Amersham Biosciences, Piscataway, NJ, USA).

Caspase 3/7 Assay

Caspase 3/7 activity was measured using a Caspase 3/7 assay kit (Promega, Madison, WI, USA). HSCs were harvested 48 h after viral transduction and protein extracts prepared following the manufacturers' instructions. Caspase activity was measured in 96-well plate by product absorbance at excitation: 485 nm—emission: 525 nm every 15 min for 16 h at 37°C using a fluorescent micro-plate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Results were analyzed for statistical significance according to the Student's *t* test. Statistical values of $p < 0.05$ were considered to be significant. Data are presented as means $\pm$ SEM.

Results

Overexpression of PTEN inhibits HSC activation

To investigate endogenous PTEN expression during HSC activation, HSCs were isolated and cultured for 0, 2, and 7 days. PTEN expression was not detected in freshly isolated, quiescent HSCs (day 0); however, after 2 days in culture PTEN expression was weakly detected and was prominent after 7 days in culture (Fig. 1a). Therefore, HSC activation is associated with the induction of PTEN expression.

To determine the effect of PTEN overexpression on morphological changes associated with HSC activation, HSCs were transduced on day 2 with Ad5-WT-PTEN, dominant negative Ad5-C124S PTEN, or Ad5- β gal as a control virus. HSCs transduced with Ad5- β gal and Ad5-C124S and cultured for 7 days appeared to undergo the typical activation process of HSCs when untreated and cultured on plastic (Fig. 1b). The cells showed reduced lipid droplets after 4 and 7 days in culture, and they exhibited enlarged cellular bodies with increased cell numbers. In contrast, HSCs transduced with Ad5-WT-PTEN maintained their star-like processes and cytoplasmic lipid droplets. In addition, the cells did not appear to proliferate during the 7 day culture period (Fig. 1b). These results demonstrate that overexpression of PTEN in HSCs prevents morphological changes typically associated with activation, including cell spreading and loss of the cytoplasmic lipid droplets, and that its phosphatase activity is responsible for this inhibitory effect.

To investigate the effect of PTEN on α SMA expression, a characteristic biomarker for HSC activation, HSCs were transduced with Ad5-WT-PTEN, Ad5-C124S, or Ad5- β gal. Both Ad5-WT-PTEN and Ad5-C124S viruses were shown to overexpress immunodetectable PTEN (Fig. 2). Ad5-WT-PTEN reduced α SMA expression 66% in HSCs compared to untransduced control cells (Fig. 2). Transduction of HSCs with either Ad5-C124S or Ad5- β gal showed no effect on α SMA expression. Therefore, PTEN expression inhibits the induction of α SMA expression typically associated with HSC activation in HSCs.

The activated HSC is the predominant hepatic cell type in the liver responsible for the increased synthesis and deposition of type I collagen during fibrosis. To assess the role of PTEN on $\alpha 1$

(I) collagen mRNA steady state expression, day 2 HSCs were transduced with Ad5-WT-PTEN, Ad5-C124S, or Ad5- β gal and 72 h later α 1(I) collagen gene expression assessed. Ad5-WT-PTEN reduced α 1(I) collagen mRNA steady state levels by 65%, while Ad5-C124S and Ad5- β gal had no effect on α 1(I) collagen expression compared to the control (Fig. 3). These findings indicate that PTEN overexpression is able to decrease α 1(I) collagen mRNA expression in HSCs, which is dependent on its phosphatase activity.

Overexpression of PTEN inhibits proliferation of HSCs with reduced cyclin D1 and PCNA expression

Since the morphological studies suggested that PTEN expression inhibited HSC proliferation during the activation process, we further investigated the mechanism by which PTEN reduces HSC proliferation. HSCs, cultured for 2 days, were either left untreated or transduced with Ad5-WT-PTEN, Ad5-C124S, or Ad5- β gal. HSCs transduced with Ad5-WT-PTEN began to show reduced cell numbers 2 days following WT-PTEN overexpression showing a 24% reduction in cell numbers from the starting number of cells. After days 6 and 10 (4 and 6 days following viral transduction) cell numbers decreased 48 and 70%, respectively. Cells untreated or transduced with Ad5-C124S or Ad- β gal showed increased cell numbers and proliferated at similar rates throughout the 8 day culture period as the untreated cells (Fig. 4a).

To investigate the mechanism by which PTEN inhibited proliferation of HSCs we investigated the effect of PTEN expression on cyclin D1 and PCNA expression. Overexpression of PTEN reduced cyclin D1 expression by 96% and PCNA expression by 93%, compared to untreated control cells while transduction with either Ad5-C124S or Ad5- β gal had no effect on the expression of either cyclin D1 or PCNA (Fig. 4b). Together, these results indicate that PTEN expression inhibits cellular proliferation which is associated with reduced expression of both cyclin D1 and PCNA in HSCs.

PTEN expression induced apoptosis in HSCs

Our results suggest that PTEN expression may actually promote cell death in HSCs. To further investigate this possibility we examined the potential role of apoptosis by examining cleavage of caspase 3, a marker of apoptosis, in HSCs overexpressing PTEN. Transduction of HSCs with Ad5-WT-PTEN induced caspase 3 cleavage, whereas cells transduced with either Ad5- β gal or Ad5-C124S did not result in caspase 3 cleavage (Fig. 5a). To quantitate this apoptotic effect, we measured caspase 3/7 activity. PTEN overexpression increased caspase 3/7 activity 4.4-fold compared to the control cells (Fig. 5b). However, transduction of HSCs with either Ad5-C124S or Ad5- β gal showed no significant increase in caspase 3/7 activity (Fig. 5b). These findings show that PTEN overexpression in HSCs blocks HSC proliferation, which is mediated by reduced cyclin D1 and PCNA expression, and also induces apoptosis, which is associated with increased caspase3/7 activity.

Increased PTEN expression inhibits serum-induced phosphorylation of Akt, p70^{S6K}, and Erk in HSCs

Akt is a downstream target of PI3K and an important cell survival factor in various cell types, particularly cancer cells [25]. We have previously shown that serum and PDGF both stimulate Akt phosphorylation at Ser⁴⁷³ in HSCs [17]. We used serum stimulation to assess the role of PTEN on Akt phosphorylation. HSCs transduced with the control adenovirus, Ad5- β gal, showed increased Ser⁴⁷³ phosphorylation of Akt following serum stimulation which peaked at 30 min that gradually decreased over the 120 min time period. However, serum-induced Akt phosphorylation was nearly completely blocked in HSCs transduced with Ad5-WT-PTEN (Fig. 6a).

As a downstream target of Akt, p70^{S6K} has been shown to regulate proliferation and cell survival in several cell types including HSCs [16,26–28]. To investigate the effect of PTEN on p70^{S6K} activation by assessing its phosphorylation status, quiescent HSCs were transduced with Ad5-WT-PTEN or Ad5-βgal. In control HSCs, p70^{S6K} phosphorylation was induced and reached maximal levels within 30 min after serum treatment which remained elevated for at least 120 min (Fig. 6b). In contrast, transduction of HSCs with Ad5-WT-PTEN completely blocked phosphorylation of p70^{S6K} at all time points. Extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase (MAPK) family. In HSCs, PDGF-induced ERK activation is a result of the sequential activation of Ras-Raf-MEK signaling [29]. In addition, inhibition of ERK phosphorylation has been shown to block HSC proliferation [30]. To investigate the role of PTEN on ERK phosphorylation, HSCs were transduced with Ad5-WT-PTEN, Ad5-C124S, or Ad5-βgal, and the effects on ERK phosphorylation assessed following serum stimulation. Following serum treatment, HSCs transduced with Ad5-βgal exhibited a transient increase in ERK phosphorylation which reached peaked after 30 min and was diminished after 120 min. However, transduction with Ad5-WT-PTEN efficiently blocked serum-induced ERK phosphorylation at all time points assessed (Fig. 6c).

Discussion

Activation of PI3K signaling is an important event during HSC activation where we and others have shown it regulates cell proliferation and collagen gene expression, two critical aspects for activated HSCs in the fibrogenic process [17,31]. In this study, we investigated the potential of PTEN to negatively regulate PI3K activity and prevent the activation of HSCs into fibrogenic cells. Here, we showed that PTEN has a significant role in regulating HSC activation. HSCs, when cultured on plastic become activated, which is associated with a loss of their stored retinoids, they express type I collagen and αSMA, and the cells proliferate. When day 2 cultured HSCs were transduced with WT-PTEN, the cells failed to activate. This was associated with the cells remaining small in size, retaining their stored retinoids (Fig. 1b), even after 7 days in culture, and the cells failing to proliferate (Fig. 1b, Fig. 4a). In addition, overexpression of PTEN inhibited the induction of both αSMA and α1(I) collagen gene expression, both markers of activated HSCs (Fig. 2). In contrast, day 2 HSCs transduced with Ad5-βgal or Ad5-C124S, a dominant negative PTEN which lacks lipid and protein phosphatase activity, activated in a normal manner where they lost their stored retinoid droplets within 4 days in culture, and by day 7 they exhibited enlarged cell bodies and transformed into myofibroblast-like cells associated with the expression of αSMA and α1(I) collagen, classical markers for activated HSCs. These observations are consistent with the previous reports, in terms of down-regulation of α1(I) collagen gene expression through inhibition of the PI3K-Akt pathway [16–18]. On the other hand, the role of PTEN in αSMA expression has not been fully clarified. Our findings suggested that PTEN also plays a regulatory role on expression of αSMA upon transactivation of HSCs.

PTEN is a negative regulator of PI3K signaling. PI3K activity phosphorylates PIP₂ to generate PIP₃. PIP₃ binds to the pleckstrin homology domain of Akt, directing it to the cell membrane where it becomes activated by phosphorylation events to initiate cell survival mechanisms. Thus, Akt represents a downstream target of PI3K, mediating cell survival by phosphorylating and inactivating several proapoptotic targets, including Bad, GSK-3β, and forkhead family proteins [31]. We have previously shown that p70^{S6K}, a downstream target of Akt, plays an important role in PDGF-induced HSC proliferation and cell cycle control [16]. Overexpression of a constitutively active form of Akt stimulates p70^{S6K} and promotes cell proliferation and cell survival [26–28]. PI3K/Akt signaling is activated in HSCs following PDGF or serum stimulation of HSCs [18]. A role for PI3K in HSC proliferation was supported in an in vivo study in rats which demonstrated that CCl₄ treatment leads to autophosphorylation of the PDGF

receptor and increased PI3K activity [18]. Activation of PI3K is also important for HSC proliferation and chemo-taxis in activated HSCs [18]. Furthermore, blocking PI3K with the chemical inhibitor LY294002 inhibits HSC proliferation [17,32]. PDGF also activates ERK in HSCs by sequential activation of Ras-Raf-MEK signaling [29]. A role of ERK in HSC proliferation was shown when inhibition of ERK phosphorylation blocked cell proliferation in activated HSCs [30].

The molecular mechanism of PTEN's effect on PI3K-Akt-p70^{S6K} and Erk signaling pathways has not been clarified in HSCs. Here we showed that overexpression of PTEN blocked serum-induced phosphorylation of Akt, p70^{S6K}, and Erk in HSCs (Fig. 6). These findings suggest that both PI3K-Akt and Erk signaling pathways are targets of PTEN in HSCs. p70^{S6K} is required for G1 cell-cycle progression and cell growth [33]. It phosphorylates the S6 protein of the 40S ribosomal subunit and is involved in translational control of 5'-oligopyrimidine tract mRNAs [33,34]. Rapamycin, an inhibitor of mTOR and thus the downstream kinase p70^{S6K}, blocks protein synthesis and inhibits cell cycle progression at the G1/S transition [35]. Previous studies have shown that D-type cyclins play an important role in cell cycle progression, and that expression of cyclin D1, D2, and E correlates with cellular proliferation [36,37]. Furthermore, cyclin D expression is post-transcriptionally regulated via the PI3K/Akt pathway [38], and inhibition of PI3K is able to block cyclin D1 expression in rat HSCs [39]. We assessed the role of PTEN on HSC proliferation and showed that increased PTEN expression dramatically reduces expression of cyclin D1 and PCNA in HSCs (Fig. 4b). This may potentially be a result of PTEN inhibition of p70^{S6K} phosphorylation and may explain the mechanism by which PTEN inhibits proliferation of HSCs.

We also observed that PTEN reduced cell numbers when overexpressed in HSCs (Fig. 4a). This was likely mediated in part by an increase in apoptosis since the number of Ad5-WT-PTEN-transduced HSCs was significantly lower than that of control cells. In addition, over-expressing PTEN induced cleavage of the proapoptotic caspase 3 and induced caspase3/7 activity. PTEN is not expressed in quiescent HSCs, but it does become induced following HSC activation (Fig. 1a). We showed that when PTEN overexpressed early during the activation process, the cells fail to activate and undergo apoptosis-mediated cell death. Since PTEN is a negative regulator of PI3K signaling, our results indicate that PI3K signaling is pivotal for the development of the activated state in HSCs, as overexpressing PTEN during the early stages of activation induced apoptotic-mediated cell death. We believe that PI3K signaling provides cell survival signals during HSC activation, probably mediated through cell survival signals via Akt activation as well as proliferative signaling mediated by p70^{S6K} signaling. Together, these findings have important ramifications for the design of therapeutic strategies to treat liver fibrosis.

Regarding the therapeutic implication, experimental approaches using overexpression of PTEN have been evaluated in other types of cells; for instance, it has been reported that overexpression of PTEN using adenovirus inhibits the growth of human prostate cancer xenografts in mice through induction of apoptosis and inhibition of angiogenesis and cellular proliferation [40]. In addition, adenoviral overexpression of PTEN has been demonstrated to reduce the symptoms of asthma by inhibiting VEGF expression in mice [41]. The gene manipulation of PTEN, however, needs attention since there might be serious adverse effects relating to development, tissue repair, and metabolism, especially in case targeting is insufficient [42–44]. To establish the PTEN-directed gene therapy for hepatic fibrosis, development of cell-specific gene modulation for HSCs is essential. In conclusion, our results would suggest that inducing PTEN expression during fibrosis would limit the progression of fibrosis and may potentially promote the resolution of liver fibrosis.

Acknowledgments

This work was partially funded by: NIHDK065972 (R.A.R.) and NIH-HL070990 (E.S.W.).

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Fig. 1.

Expression of PTEN and morphological changes in HSCs during culture-activation. **a** HSC activation increases endogenous PTEN expression. Cell extracts were prepared from freshly isolated HSCs (day 0) or from cells cultured for 2 or 7 days. PTEN expression was assessed by Western blot analysis. GAPDH was used as an internal control. The data presented is representative of three independent experiments. **b** PTEN overexpression inhibits activation-associated morphological changes. HSCs (day 2 in culture) were transduced with Ad5-βgal, Ad5-WT-PTEN, or Ad5-C124S-PTEN at a MOI of 150. After 16 h, the transduction medium was changed to fresh growth medium and subsequently changed every 48 h. Cell morphology was monitored at days 2, 4, and 7 by phase contrast microscopy ($\times 100$)

Fig. 2. PTEN overexpression inhibits α SMA expression in HSCs. HSCs were transduced with Ad5-WT-PTEN, Ad5-C124S, or Ad5- β gal, as a control. Cell extracts were prepared after 48 h following viral transduction and Western blot analysis performed to assess α SMA expression. GAPDH was used as an internal control. Adenoviral expression of WT-PTEN and DN-PTEN (Ad5-C124S) was confirmed by assessing PTEN expression. Graphical analyses from three independent experiments are shown. *Error bars* represent SEM. * $p < 0.05$ versus no virus and Ad5- β gal

Fig. 3.

PTEN overexpression suppresses $\alpha 1(I)$ collagen mRNA expression in HSCs. Day 2 HSCs were transduced with Ad5-WT-PTEN, Ad5-C124S, or Ad5- β gal. Cells were serum-starved for 48 h then stimulated with 10% serum for 24 h, before total RNA was isolated at day 7. Total RNA, 5 μ g, was hybridized with radiolabeled probes for either $\alpha 1(I)$ collagen or GAPDH, used as an internal control. tRNA was used as a negative control RNA sample; M 100 bp ladder. Graphical analysis is representative of three individual RNase protection assays. Expression of $\alpha 1(I)$ collagen mRNA was normalized to GAPDH levels. Error bars represent SEM. * $p < 0.05$ versus no virus and Ad5- β gal

Fig. 4.

PTEN overexpression inhibits HSC proliferation. **a** Overexpression of PTEN inhibits HSC proliferation. Day 2 HSCs were transduced with Ad5-WT-PTEN, Ad5-C124S, or Ad5-βgal and cell numbers assessed by manual cell counts using a hemocytometer every 48 h until day 10. Results are representative of three independent experiments. Error bars represent SEM.

* $p < 0.05$ versus no virus and Ad5-βgal. **b** PTEN inhibits expression of cyclin D1 and PCNA in HSCs. Day 2 HSCs were transduced with Ad5-WT-PTEN, Ad5-C124S, or Ad5-βgal. Cell extracts were prepared 48 h following viral transduction and Western blot analysis performed for cyclin D1 or PCNA expression. GAPDH was used as an internal control. Graphical analyses

from three separate experiments are shown. *Error bars* represent SEM. $*p < 0.05$ versus no virus and Ad5- β gal

Fig. 5. PTEN overexpression induces apoptosis in HSCs. **a** Day 2 HSCs were transduced with Ad5-WT-PTEN, Ad5-C124S, or Ad5-βgal. Total cell extracts (20 μg) were prepared 48 h after viral transduction and Western blot analysis performed using a cleaved-caspase3 antibody. GAPDH was used as an internal control. **b** Caspase3/7 activities were measured in control and Ad5-WT-PTEN, Ad5-C124S, or Ad5-βgal transduced HSCs. Product absorbance was read at excitation: 485 nm/emission: 525 nm every 15 min for 16 h at 37°C using a fluorescent microplate reader. Data are representative of three independent studies. *Error bars* represent SEM. * $p < 0.05$; versus no virus and Ad5-βgal

Fig. 6.

PTEN inhibits serum-induced phosphorylation of Akt, p70^{S6K}, and Erk in HSCs. HSCs were transduced with Ad5-WT-PTEN or Ad5-βgal on day 2, then serum starved for 48 h. Cellular proteins were harvested 0, 30, 60, and 120 min following stimulation with 10% serum. Western blot analysis was performed using anti-phospho-Akt (Ser⁴⁷³) (a), anti-phospho-p70^{S6K} (b), or anti-phospho-Erk (c). GAPDH and total Akt were examined as internal controls. Results are representative of three independent experiments