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Hedgehog acyltransferase as a target in pancreatic ductal adenocarcinoma

E Petrova[1,2,5], **A Matevossian**[1,3], and **MD Resh**[1,3,4]

[1]Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

[2]Graduate Program in Pharmacology, Weill Graduate School of Medical Sciences of Cornell University, New York, NY, USA

[3]Gerstner Sloan-Kettering Graduate School of Biomedical Sciences, New York, NY, USA

[4]Graduate Programs in Cell Biology and Biochemistry, Weill Graduate School of Medical Sciences of Cornell University, New York, NY, USA

Abstract

Sonic Hedgehog (Shh) is abnormally expressed in pancreatic cancer and is associated with disease onset and progression. Inhibition of Shh signaling is thus an attractive clinical target for therapeutic intervention. Most efforts to block Shh signaling have focused on inhibitors of Smoothened, which target the canonical Shh signaling pathway. These approaches have met with limited success, in part due to development of resistance-conferring mutations and contributions from non-canonical signaling pathways. Here, we show that Hedgehog acyltransferase (Hhat), the enzyme responsible for the attachment of palmitate onto Shh, is a novel target for inhibition of Shh signaling in pancreatic cancer cells. Depletion of Hhat with lentivirally delivered small hairpin RNA decreased both anchorage-dependent and independent proliferation of human pancreatic cancer cells. *In vivo*, Hhat knockdown led to reduction of tumor growth in a mouse xenograft model of pancreatic cancer. RU-SKI 43, a small molecule inhibitor of Hhat recently developed by our group, reduced pancreatic cancer cell proliferation and Gli-1 activation through Smoothened-independent non-canonical signaling. In addition, RU-SKI 43 treatment inhibited two key proliferative pathways regulated by Akt and mTOR. This work demonstrates that Hhat has a critical role in pancreatic cancer and that a small molecule inhibitor of Hhat can successfully block pancreatic cancer cell proliferation. It also highlights the importance of developing optimized Hhat inhibitors to be used as therapeutics in pancreatic cancer, as well as in other malignancies characterized by Shh overexpression.

Keywords

hedgehog; palmitoylation; pancreatic cancer

Correspondence: Dr MD Resh, Cell Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 143, New York, NY 10065, USA. reshm@mskcc.org.

[5]Current address: Bayer HealthCare Pharmaceuticals, Global Drug Discovery, Muellerstrasse 178, 13342 Berlin, Germany.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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INTRODUCTION

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related mortality in the United States.[1] The low response to standard therapy and high recurrence rates following treatment necessitate a better understanding of the molecular basis of the disease, as well as the development of novel therapies. Abnormal expression of the secreted signaling protein Sonic Hedgehog (Shh) is characteristic of both early- and late-stage pancreatic cancer.[2] Shh has been extensively studied for its role in development, where it is critical for proper embryonic patterning.[3] However, the exact signaling mechanism of Shh in pancreatic cancer remains unclear, and both canonical and non-canonical signaling pathways may be operative. Shh produced by pancreatic cancer cells may act through an autocrine signaling mechanism, but as activation of the Shh transducer Smoothened (Smo) has not been detected in pancreatic cancer cells[2,4–6] this likely occurs via a Smo-independent branch of the Shh pathway. In addition, Shh produced by tumor cells can act through a selective activation of a subset of cancer-initiating stem cells[7] and/or through paracrine signaling to the tumor microenvironment.[5]

Shh is a member of the mammalian family of hedgehog proteins, which includes Indian Hedgehog and Desert Hedgehog.[3] Binding of hedgehog proteins to the transmembrane receptor Patched activates Smo, leading to nuclear translocation of Gli transcription factors and expression of downstream target genes including Gli-1 and Patched-1.[8] The mature Shh signaling protein is formed via a series of post-translational processing reactions. Following removal of the signal peptide, Shh undergoes autocleavage to produce a 19-kDa N-terminal product, ShhN, which is modified with cholesterol at its C-terminus.[9] In a separate reaction, Hedgehog acyltransferase (Hhat), a member of the membrane-bound O-acyl transferase family of proteins, catalyzes attachment of palmitate to the N-terminal cysteine of ShhN.[9,10] Palmitoylation of Shh by Hhat is essential for Shh function both *in vitro* and *in vivo* during development.[11,12]

In this study, we demonstrate a key role for Hhat in pancreatic adenocarcinoma. We targeted Hhat using small hairpin RNA (shRNA)-mediated knockdown or a small molecule inhibitor of Hhat—RU-SKI 43. This compound was recently identified by our laboratory from a target-oriented high-throughput screen.[13] RU-SKI 43 is a potent and specific inhibitor of Hhat *in vitro* (IC_{50} = 0.85 μM) and in cells; it does not affect fatty acylation by other acyltransferases and blocks Shh signaling in cells.[13] In pancreatic cancer cells, Hhat inhibition by RU-SKI 43 or Hhat knockdown resulted in attenuation of Gli-1 activation through Smo-independent non-canonical signaling, decreased Akt and mTOR pathway activity, reduced cell proliferation *in vitro* and decreased tumor growth in a xenograft model of pancreatic cancer *in vivo*. These findings demonstrate that Hhat is a critical regulator in pancreatic adenocarcinoma and thus a novel target for blocking pancreatic cancer cell growth.

RESULTS AND DISCUSSION

To identify appropriate model systems with which to target Hhat, we measured Hhat expression in multiple human pancreatic cancer cell lines. Hhat mRNA was detected in AsPC-1, Panc-1 and Panc 05.04 cells (Figure 1a). The same cell lines also expressed Shh and Gli-1 (Figures 1b and c), consistent with other reports.[14] The pancreatic cancer cell lines BxPC-3 and Hs766t, as well as HeLa, a cervical cancer cell line used as control, did not exhibit upregulated production of Shh and Gli-1 (Figures 1b and c). In these three cell lines, levels of Hhat were barely detectable above background (Figure 1a).

We next used lentiviral-based shRNA to deplete Hhat in pancreatic cancer cells. Stable expression of Hhat-targeting shRNA reduced Hhat levels by 92% in AsPC-1 cells, compared with cells expressing a control, scrambled shRNA sequence (Figure 1d). A corresponding 65% decrease in Gli-1 mRNA levels was detected in AsPC-1cells (Figure 1e). Similar results were observed in Panc-1 and Panc 05.04 cells. Hhat depletion resulted in a 60% decrease in Gli-1 levels in both Panc-1 cells and Panc 05.04 cells (Supplementary Figure S1). Knockdown of Shh also decreased Gli-1 mRNA levels in AsPC-1 cells (Figures 1f and g), suggesting that an Hhat and Shh-dependent signaling pathway is operative in these cells. To further validate the requirement for Hhat, we used RU-SKI 43, a small molecule inhibitor of Hhat that specifically blocks Hhat-mediated palmitoylation of Shh.[13] Treatment of AsPC-1 cells with 10 μM RU-SKI 43 caused a 40% decrease in Gli-1 levels (Figure 1h).

To test whether Hhat depletion or inhibition can alter paracrine Shh signaling, a mouse fibroblast cell line, C3H10T1/2, was used as a Shh signaling reporter system. In the presence of Shh, these cells differentiate into osteoblasts and produce alkaline phosphatase.[15] When C3H10T1/2 cells were co-cultured with the Shh-secreting Panc-1 cells expressing control scrambled shRNA, a strong increase in alkaline phosphatase production was detected, compared with the signal produced by C3H10T1/2 cells alone (Figure 1i). The signal was reduced to baseline when C3H10T1/2 cells were co-cultured with Panc-1 cells treated with Shh or Hhat shRNAs (Figure 1i, Supplementary Figures S1a and S2), consistent with inhibition of Shh signaling. Treatment of co-cultured Panc-1 and C3H10T1/2 cells with RU-SKI 43 also blocked production of alkaline phosphatase, compared with co-cultured cells treated with DMSO (Figure 1j). These data indicate that Hhat inhibition decreases both Gli-1 levels and Gli-1 function.

To test whether Hhat is required for pancreatic cancer cell growth, we monitored cell proliferation following Hhat depletion or inhibition. Knockdown of Hhat (Figure 1d) decreased proliferation of AsPC-1 cells by 77% (Figure 2a) and that of Panc-1 and Panc 05.04 cells by 95% and 65%, respectively (Supplementary Figure S3). Treatment of AsPC-1 and Panc-1 cells with RU-SKI 43 strongly decreased cell proliferation (83% in AsPC-1 cells), whereas treatment with C-2, a compound that is structurally related to RU-SKI 43 but does not inhibit Hhat,[13] had no effect (Figure 2b, Supplementary Figure S4a and b). RU-SKI 43 had no effect on the proliferation of Hs766t pancreatic cancer cells (Figure 2c), which do not express Shh or Hhat (Figure 1). Treatment of AsPC-1 cells with increasing concentrations of RU-SKI 43 led to a dose-dependent decrease in cell proliferation that was paralleled by decreases in Gli-1 expression (Figures 2d and e). Moreover, the inhibitory

effect of RU-SKI 43 on cell proliferation was rescued by overexpressing Hhat (Figure 2f). These data strongly suggest that the proliferative defect induced by RU-SKI 43 in pancreatic cancer cells is specific to Hhat inhibition.

We next examined the role of Hhat in anchorage-independent growth. Stable knockdown of Hhat resulted in a 60% decrease in anchorage-independent proliferation of AsPC-1 and Panc-1 cells (Figures 3a and b). Similar results were obtained for cells with Shh knockdown (Figures 3a and b). Furthermore, treatment of AsPC-1 and Panc-1 cells with RU-SKI 43 decreased anchorage-independent proliferation by 80% and 90%, respectively (Figures 3c and d).

Several studies have concluded that pancreatic cancer cells do not respond to the Shh ligand via autocrine signaling, based on the finding that addition of exogenous Shh does not alter Gli-1 expression or cell growth.[4,6] However, we recently documented that the Shh signaling pathway is saturated in cells that overexpress Shh and cannot be further stimulated by addition of either exogenous Shh or a Smo agonist.[13] We hypothesized that dependence on Shh would only be revealed in pancreatic cancer cells when endogenous Shh levels were depleted. As predicted, addition of exogenous, recombinant Shh(C24II) had no effect on the growth of AsPC-1 cells treated with control siRNA, but stimulated the proliferation of cells in which Shh had been depleted (Figure 2g). These findings are consistent with the work of others, showing that when cells were first treated with 5E1, an antibody that blocks binding of endogenous Shh to Patched, subsequent addition of exogenous Shh stimulated tumor cell growth.[16] Moreover, consistent with the finding that signaling through Smo is not active in the pancreatic epithelium,[4–6] the Smo inhibitor LDE-225 had no effect on Panc-1 and AsPC-1 cell proliferation or Gli-1 expression levels when used at 0.1 μM, a concentration 100× higher than the IC_{50} for binding of LDE-225 to Smo[17] (Supplementary Figure S4). Of note, 10 μM LDE-225 resulted in the inhibition of cell proliferation and Gli-1 expression, but this likely represents a Smo-independent, off-target effect (Supplementary Figure S4). Taken together, these data reconcile previous studies with our findings on the roles of Shh and Hhat and support a model that involves autocrine, non-canonical signaling by Hhat and Shh in pancreatic cancer cells.

To identify other pathways that might be affected by Hhat inhibition in pancreatic cancer cells, Panc-1 cells were treated with DMSO or RU-SKI 43, and cell lysates were analyzed for phosphorylation or cleavage of 18 well-characterized signaling molecules involved in cell proliferation, survival or apoptosis (Supplementary Table S1). Treatment with RU-SKI 43 resulted in decreased phosphorylation (47–67%) of four proteins in the Akt pathway, including Akt (phosphorylation at both Thr307 and Ser473), PRAS40, Bad and GSK-3β (Figure 4a). RU-SKI 43 treatment also decreased phosphorylation of mTOR and S6, members of the mTOR signaling pathway (Figure 4b). The compound had no effect on Caspase 3 and PARP (Supplementary Table S1), suggesting that RU-SKI 43 reduces the proliferation of pancreatic cancer cells primarily by inhibiting proliferative pathways regulated by Akt and mTOR, rather than by affecting cell death. Combination treatment with RU-SKI 43 and the mTOR inhibitor rapamycin resulted in further decreases in cell proliferation compared with each agent alone (Figure 4c).

To determine the role of Hhat *in vivo*, we utilized a Panc-1 xenograft mouse model of pancreatic cancer. The half-life of RU-SKI 43 in mouse plasma is only 17 min and is thus too short for *in vivo* studies.[13] Instead, Panc-1 cells stably expressing Hhat shRNA, Shh shRNA or a control scrambled sequence (Supplementary Figure S5) were injected into immuno-compromised mice, and tumor formation was monitored for 72 days. Depletion of Shh resulted in 70% reduction of tumor growth (Figure 4d), confirming the importance of Shh signaling in pancreatic cancer observed by others in xenograft mouse models.[14] Notably, Hhat knockdown produced the same effect as Shh knockdown; tumor growth was inhibited by 70% over the course of the experiment (Figure 4d). Taken together, these findings provide proof of concept for a critical role of Hhat in pancreatic cancer cell growth *in vitro* and *in vivo*.

In this study, we describe for the first time the importance of Hhat as a novel target in pancreatic cancer. Most efforts to block Shh signaling have focused on downstream components of the canonical pathway, especially Smo,[18–20] but have met with limited success. Here, we highlight an upstream event in pancreatic cancer—palmitoylation of the Shh ligand by Hhat. Hhat-mediated Shh palmitoylation is critical for Shh function during development.[11,12] The studies in this report establish that this paradigm holds true in pancreatic cancer. Inhibition of Hhat function, by genetic or pharmacologic tools, resulted in decreased autocrine and paracrine Shh signaling as well as decreased anchorage-dependent and independent proliferation of pancreatic cancer cells. Furthermore, use of a xenograft mouse model of pancreatic cancer demonstrated the importance of Hhat *in vivo*. The findings reported in this study validate the importance of Hhat as a new target in pancreatic cancer.

This is the first report demonstrating the efficacy of the Hhat inhibitor RU-SKI 43 in pancreatic cancer. RU-SKI 43 was identified in a high-throughput screen for small molecule inhibitors of Hhat, and has been shown to specifically block palmitoylation of Shh and Shh signaling.[13] Here, we show that in pancreatic cancer cells RU-SKI 43 blocked Gli-1 activation and inhibited anchorage-dependent and independent cell proliferation. These effects were dose dependent and were rescued by Hhat overexpression. C-2, an inactive analog of RU-SKI 43, had no effect on pancreatic cancer cell growth. Moreover, RU-SKI 43 did not affect pancreatic cancer cells that do not express detectable levels of Hhat. Studies in other cell types established that RU-SKI 43 does not inhibit palmitoylation of H-Ras, Fyn or Wnt3a, or myristoylation of c-Src.[13] These data strongly suggest that the primary target for RU-SKI 43 in pancreatic cancer cells is Hhat.

Our studies show that Hhat regulates pancreatic cancer cells via Shh-dependent and independent mechanisms. Knockdown of Shh decreased Gli-1 mRNA levels (Figure 1g), anchorage-dependent and independent proliferation (Figures 2a, g, 3a and b) and tumor growth *in vivo* (Figure 4d). In addition, the proliferative defect following Shh depletion was partially rescued by exogenous Shh addition (Figure 2g). However, Gli-1 mRNA levels and pancreatic cancer cell proliferation were not affected by the Smo inhibitor LDE-225 (Supplementary Figure S4). These findings support an autocrine role for Shh in pancreatic cancer that is performed by a non-canonical Shh signaling mechanism. In addition, we present evidence for a role for Hhat in addition to palmitoylating and regulating Shh activity.

Hhat inhibition by RU-SKI 43 reduced the activation of Akt and mTOR signaling pathways in pancreatic cancer cells (Figures 4a and b). Notably, depletion of Shh had no effect on Akt or mTOR signaling (Supplementary Figure S6). This is consistent with the notion that Hhat may have other targets in mammalian cells, in addition to hedgehog proteins.[21] The Hhat ortholog Rasp is known to palmitoylate other substrates in flies in addition to Hh[22] and thus it is possible that Hhat inhibition affects proteins involved in non-Shh signaling pathways, directly or indirectly. Likely candidates are pathways regulated by activated K-Ras, which has been shown to induce Gli-1 independently of Shh.[4–6] Cell lines that contain mutant K-Ras also express Hhat and Gli-1, whereas those with wild-type K-Ras do not (Figure 1). Moreover, Hhat inhibition affects Akt and mTOR, downstream effectors of activated Ras. Our finding that RU-SKI 43 in combination with an mTOR inhibitor achieved a more effective inhibition than either drug alone suggests that inhibiting Hhat and mTOR together might have a synergistic effect on pancreatic cancer cell proliferation.

The current standard of care for pancreatic cancer, gemcitabine, is largely ineffective, with overall survival of 5–6 months. Thus, there is an unmet need for new ways to attack this cancer. Shh signaling is an attractive target in pancreatic cancer and in other malignancies.[23] Several Shh pathway inhibitors are currently in clinical trials[24] but these target the pathway downstream of Shh by inhibiting Smo or Gli proteins. Of note, the Smo inhibitor vismodegib (GDC-0449) has failed in the clinical setting because of the development of mutations in Smo that confer drug resistance.[25] Inhibiting Hhat offers a novel target and approach—by attacking upstream at the level of the Shh ligand. Cancers characterized by Shh overexpression, such as pancreatic, gastric, and lung cancers, and a subset of sarcomas,[23] would be appropriate settings for intervention with an Hhat inhibitor, and evidence documenting the importance of Hhat in one of these malignancies has recently emerged.[26] Inhibiting Hhat could potentially overcome the limitations of treatment with Smo inhibitors. Hhat inhibition would prevent activation of all signaling downstream of Shh, including non-canonical, Smo-independent signaling pathways that operate within the tumor cells.[27,28] Point mutations throughout the protein have been shown to compromise Hhat activity,[29] reducing the likelihood that drug resistance could develop within Hhat. Combination therapy approaches that attack both Shh production and the Shh response could therefore be designed, which might prove more efficacious than single agent therapy and might slow the development of drug resistance.[25] Finally, Hhat inhibition might allow for downregulation of not only Shh signaling in cells but also of Akt and mTOR, and thus have increased efficacy. Given the importance of Hhat and the potential impact that Hhat inhibition might have in pancreatic cancer and other hedgehog-driven cancers, a major goal will be to generate Hhat inhibitors with improved potency and bioavailability, to be eventually used as novel routes of therapeutic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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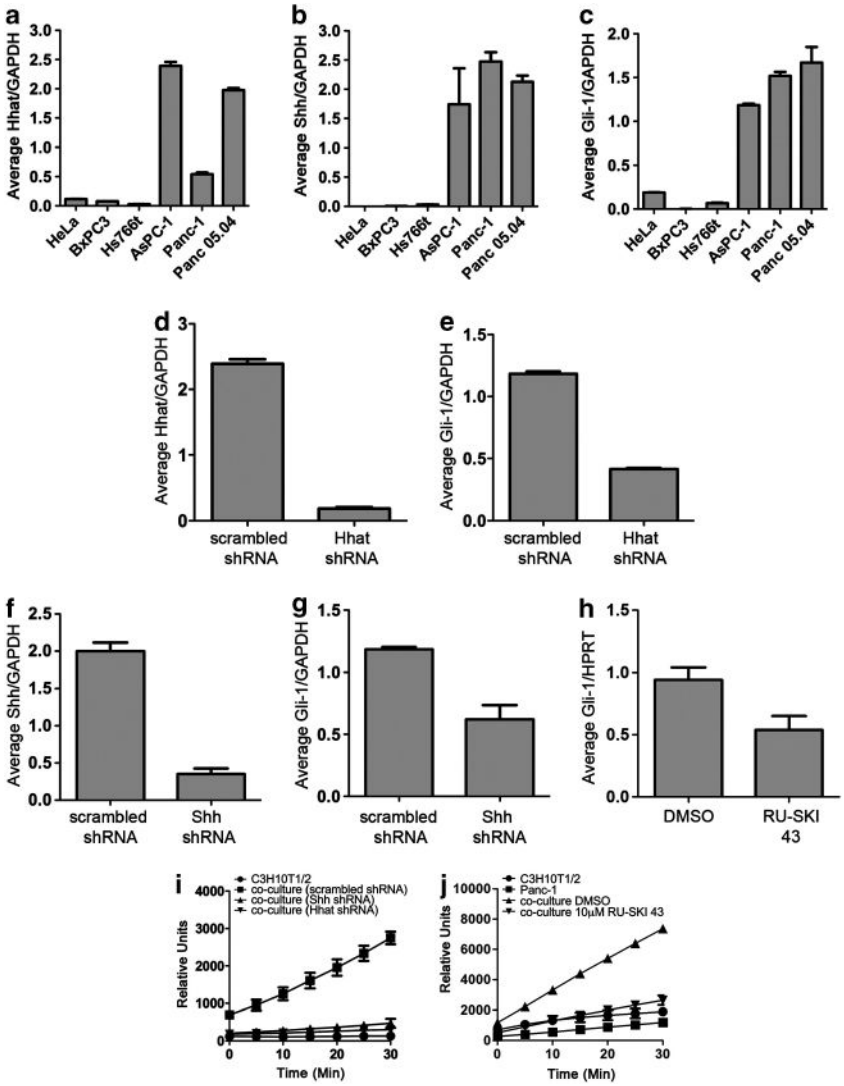

Figure 1.

Hhat inhibition blocks Shh signaling in pancreatic cancer cells. Expression of Hhat (**a**), Shh (**b**) and Gli-1 (**c**) mRNAs in different pancreatic cancer cell lines, and a control cervical cancer (HeLa) cell line, was measured by quantitative reverse transcriptase–PCR. Bars represent mean±s.d. (*n* = 2). (**d** and **e**) qRT–PCR analysis of Hhat (**d**) and Gli-1 (**e**) expression after Hhat was depleted in AsPC-1 cells using lentivirally delivered shRNA (TRCN0000035601, OpenBiosystems, Huntsville, AL, USA). A shRNA construct, carrying a scrambled sequence, was used as a control. shRNA-expressing lentiviruses were produced by co-transfecting confluent 293T cells in 15 cm plates with the shRNA plasmid, the HIV packaging vector pHRD8.2, and pcDNA3.1 VSV-G. Virus was collected 48 and 72 h later. The medium was cleared from debris by centrifugation at 500 × g for 5 min; the supernatant was filtered through a 0.45 mm filter and centrifuged at 38720 × g for 2 h at 4 °C. The pelleted virus was resuspended in medium and added to cells, and cells were selected in puromycin. Bars represent mean±s.d. (*n* = 2). The experiment was performed twice. (**f** and **g**) qRT–PCR analysis of Shh (**f**) and Gli-1 (**g**) expression after Shh was depleted in AsPC-1

cells using lentivirally delivered shRNA (TRCN0000033304, OpenBiosystems) as in (**d**) and (**e**). Bars represent mean±s.d. (*n* = 2). The experiment was performed twice. (**h**) AsPC-1 cells were treated with DMSO or 10 μM RU-SKI 43 for 72 h. Gli-1 mRNA levels were measured by qRT–PCR. Data were normalized to Gli-1 levels in DMSO-treated samples. Each bar represents mean±s.d. (*n* = 3). The experiment was performed 2–5 times. (**i**) Stable lines of Panc-1 cells expressing scrambled, Shh or Hhat shRNA were generated. The cells were co-cultured with C3H10T1/2 cells (1:4 ratio) for 4 days. The SensoLyte FDP Alkaline Phosphatase Assay Kit (AnaSpec, Fremont, CA, USA) was used to measure alkaline phosphatase levels in the cell lysates by monitoring fluorescence for 30 min at 5-min intervals on a Tecan Infinite F500 plate reader (Männdorf, Switzerland). Each point represents mean±s.d. (*n* = 3). The experiment was performed three times. (**j**) Panc-1 and C3H10T1/2 cells were co-cultured and incubated with medium containing DMSO or RU-SKI 43 for 72 h (DMSO or drug was replenished 48 h after the initial addition). Alkaline phosphatase activity in cell lysates was measured as in (**i**). Each point represents mean±s.d. (*n* = 3). The experiment was performed 2–5 times.

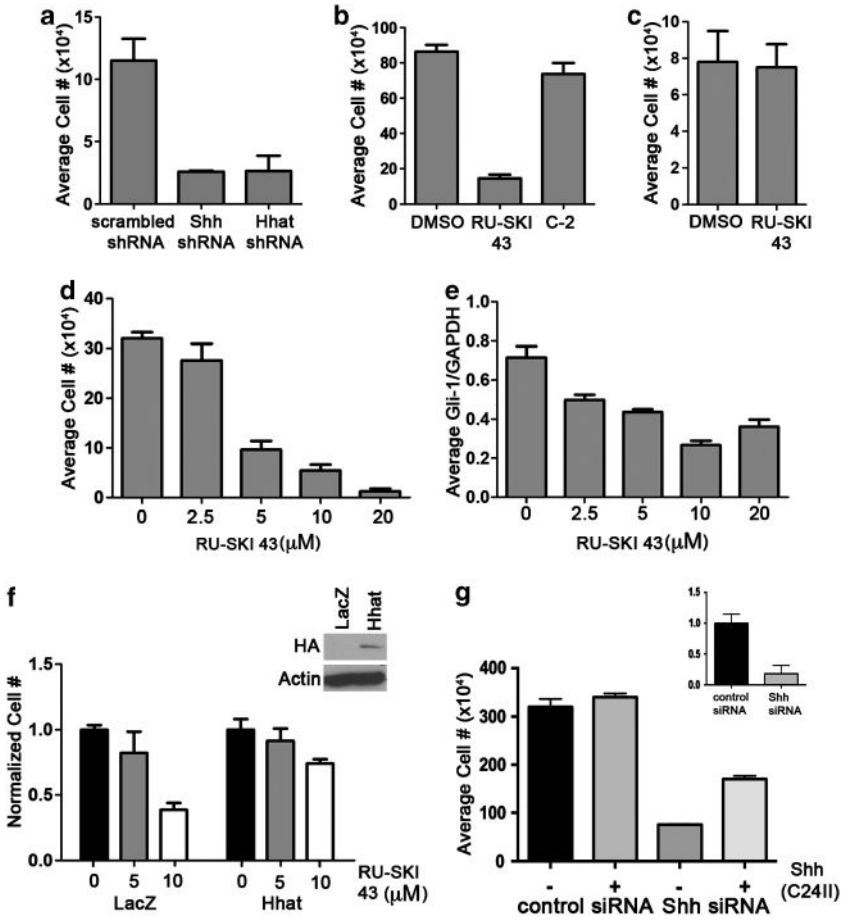

Figure 2.

Hhat inhibition blocks pancreatic cancer cell growth. (**a**) AsPC-1 cells stably expressing scrambled, Shh, or Hhat shRNAs were seeded at 2×10^4 cells/well in six-well plates and grown for 6 days; then cell numbers were quantified. Bars represent mean±s.d. ($n = 2$). The experiment was performed three times. (**b** and **c**) AsPC-1 cells (**b**) or Hs766t cells (**c**) were seeded at 5×10^4 cells/well in six-well plates, and 24 h later DMSO, 10 μM RU-SKI 43, or 10 μM C-2 was added to the medium. Cells were grown in the presence of drugs for 6 days (drugs were replenished every 48 h) and cell numbers were quantified. Bars represent mean ±s.d. ($n = 2$). The experiment was performed 2–5 times. (**d** and **e**) AsPC-1 cells were seeded at 5×10^4 cells/well in six-well plates and 24 h later were treated with varying concentrations of RU-SKI 43. Cells were grown in the presence of the drug for 6 days (drugs were replenished every 48 h), at which time cell number was counted (**d**) and Gli-1 mRNA levels were measured by qRT–PCR (**e**). Each bar represents mean±s.d. ($n = 3$). The experiment was performed twice. (**f**) Panc-1 cells were transduced with lentivirus encoding Hhat-HA or LacZ, and selected in Blasticidin S. Expression of Hhat-HA was verified by western blot (inset). A total of 5×10^4 cells/well were seeded in six-well plates and 24 h later medium with DMSO or 10 μM RU-SKI 43 was added to the cells. After 6 days of treatment (drugs were replenished every 48 h) cell number was determined. Cell number was normalized to that of the DMSO-treated samples. Each bar represents mean±s.d. ($n = 3$). (**g**) AsPC-1 cells were seeded at 5×10^5 cells/well in six-well plates and transfected on

days 0 and 3 with control siRNA or Shh siRNA. Cells were treated with buffer or 1 μM Shh (C24II) once a day, starting on day 1, and cell numbers were quantified on day 6. Bars represent mean±s.d. (n = 2). The experiment was performed twice. Relative Shh expression in control siRNA and Shh siRNA-transfected cells was quantified by qRT-PCR (inset).

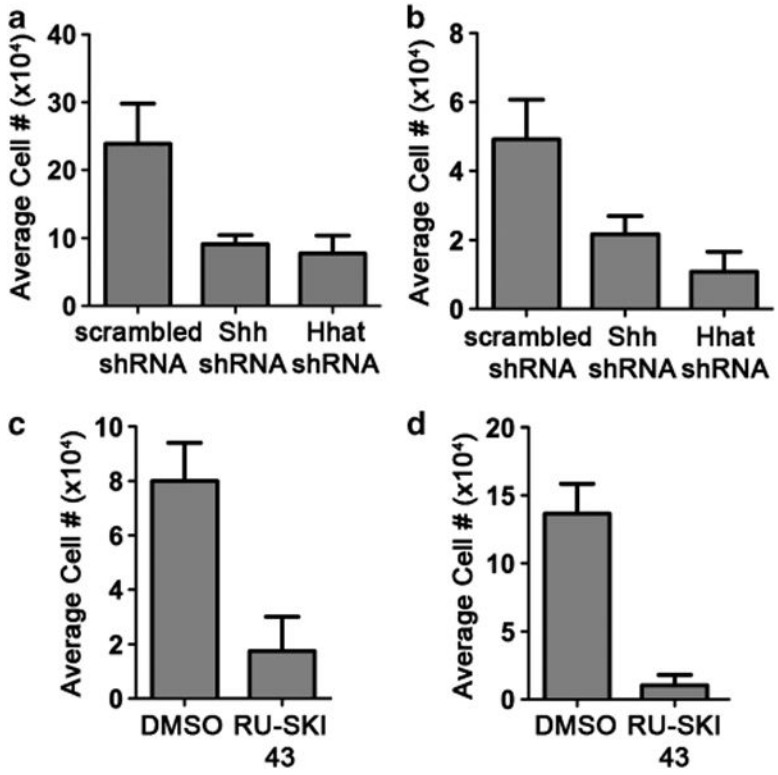

Figure 3.

Hhat inhibition blocks anchorage-independent pancreatic cancer cell growth. (**a** and b) AsPC-1 (**a**) and Panc-1 (**b**) cells stably expressing scrambled, Shh or Hhat shRNAs were seeded at 2×10^4 cells/well in 24-well low-adherence plates, and cell numbers were quantified 14 days later. Bars represent mean±s.d. ($n = 2$). Each experiment was performed twice. (**c** and **d**) AsPC-1 (**c**) and Panc-1 (**d**) cells were seeded in 24-well low-adherence plates (2×10^4 cells/well), and after 24 h DMSO or 10 μM RU-SKI 43 was added to the medium. Drugs were replenished every 48 h. Cell numbers were quantified 14 days later. Bars represent mean±s.d. ($n = 2$). Each experiment was performed twice.

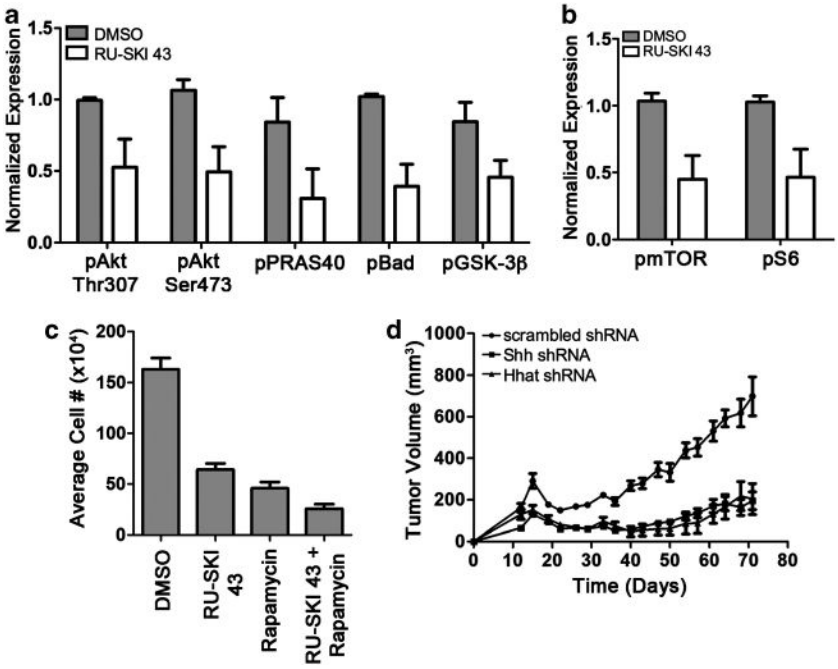

Figure 4.

Hhat inhibition affects Akt and mTOR pathways *in vitro* and reduces tumor growth *in vivo*. (**a** and **b**) Panc-1 cells were seeded in 60 mm plates (0.5×10^6 cells/plate), and 24 h later cells were treated with DMSO or 10 μM RU-SKI 43 for 48 h. Cell lysates were analyzed for protein phosphorylation or protein cleavage using the PathScan Intracellular Signaling Array kit (Cell Signaling, Danvers, MA, USA). Each signal was quantified and normalized to DMSO-treated samples. Graphs depict changes in the phosphorylation of proteins from two pathways: Akt signaling (**a**) and mTOR signaling (**b**). The experiment was performed in duplicate, and each sample was tested twice. Bars represent mean±s.d. ($n = 2$). The experiment was performed twice. (**c**) 5×10^4 Panc-1 cells/well were seeded in six-well plates and 24 h later were treated with DMSO, 10 μM RU-SKI 43, 50 nM Rapamycin (Selleckchem, Houston, TX, USA) or RU-SKI +Rapamycin. Cell number was determined after 6 days of treatment (drugs were replenished every 48 h). Cell number was normalized to that of the DMSO-treated samples. Each bar represents mean±s.d. ($n = 3$). The experiment was repeated twice. (**d**) Panc-1 cells were stably transfected with vectors carrying Shh, Hhat or a scrambled shRNA sequence. A total of 15×10^6 cells were injected into each flank of athymic female mice (2 mice per shRNA construct). Growth of the tumor xenografts was measured for 72 days (2 measurements per week). Each point represents mean±s.d. ($n = 4$). All experiments were performed in accordance with MSKCC IACUC-approved protocols.