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Authors
Hao, Fang
Xu, Qinhong
Wang, Jing
et al.

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Lipophilic statins inhibit YAP nuclear localization, co-activator activity and colony formation in pancreatic cancer cells and prevent the initial stages of pancreatic ductal adenocarcinoma in \textit{Kras}^{G12D} mice

Fang Hao\textsuperscript{1,2\*}, Qinhong Xu\textsuperscript{1,3\*}, Jing Wang\textsuperscript{1,3}, Shuo Yu\textsuperscript{1,3}, Hui-Hua Chang\textsuperscript{4,5}, James Sinnett-Smith\textsuperscript{1,5,6}, Guido Eibl\textsuperscript{4,5}, Enrique Rozengurt\textsuperscript{1,5,6\*}

\textsuperscript{1} Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California, United States of America, \textsuperscript{2} Tianjin Medical University, Tianjin, China, \textsuperscript{3} Xi’an Jiaotong University, Xi’an, China, \textsuperscript{4} Department of Surgery, David Geffen School of Medicine at UCLA, Los Angeles, California, United States of America, \textsuperscript{5} CURE: Digestive Diseases Research Center, Los Angeles, California, United States of America, \textsuperscript{6} VA Greater Los Angeles Health Care System, Los Angeles, California, United States of America

\*erozengurt@mednet.ucla.edu

Abstract

We examined the impact of statins on Yes-associated Protein (YAP) localization, phosphorylation and transcriptional activity in human and mouse pancreatic ductal adenocarcinoma (PDAC) cells. Exposure of sparse cultures of PANC-1 and MiaPaCa-2 cells to cerivastatin or simvastatin induced a striking re-localization of YAP from the nucleus to the cytoplasm and inhibited the expression of the YAP/TEAD-regulated genes Connective Tissue Growth Factor (CTGF) and Cysteine-rich angiogenic inducer 61 (CYR61). Statins also prevented YAP nuclear import and expression of CTGF and CYR61 stimulated by the mitogenic combination of insulin and neurotensin in dense culture of these PDAC cells. Cerivastatin, simvastatin, atorvastatin and fluvastatin also inhibited colony formation by PANC-1 and MiaPaCa-2 cells in a dose-dependent manner. In contrast, the hydrophilic statin pravastatin did not exert any inhibitory effect even at a high concentration (10 μM). Mechanistically, cerivastatin did not alter the phosphorylation of YAP at Ser\textsuperscript{127} in either PANC-1 or MiaPaCa-2 cells incubated without or with neurotensin and insulin but blunted the assembly of actin stress fiber in these cells. We extended these findings with human PDAC cells using primary KC and KPC cells, (expressing KrasG12D or both KrasG12D and mutant p53, respectively) isolated from KC or KPC mice. Using cultures of these murine cells, we show that lipophilic statins induced striking YAP translocation from the nucleus to the cytoplasm, inhibited the expression of Ctgf, Cyr61 and Birc5 and profoundly inhibited colony formation of these cells. Administration of simvastatin to KC mice subjected to diet-induced obesity prevented early pancreatic acini depletion and PanIN formation. Collectively, our results show that lipophilic statins restrain YAP activity and proliferation in pancreatic cancer cell models in vitro and attenuates early lesions leading to PDAC in vivo.
Introduction

Pancreatic ductal adenocarcinoma (PDAC), the most common form of pancreatic cancer, is one of the most fatal human diseases, with an overall 5-year survival rate of only 7%. It has been anticipated that PDAC will be the 3rd leading cause of cancer-related mortalities in the USA by the end of 2018 [1] and projected to become the 2nd leading cause of cancer-related mortality in the USA before 2030 [2]. Consequently, new approaches for the treatment and prevention of PDAC are urgently needed.

PDAC arises from precursor lesions, the most common of which are pancreatic intraepithelial neoplasia (PanIN) [3, 4]. Virtually all PanIN lesions and invasive PDAC entail activating mutations in the KRAS oncogene, which represent an initiating event in the development of the disease [5, 6]. In line with this concept, the model that best recapitulates the progression of human PDAC in mice involves expression of a mutant Kras (Kras<sup>G12D</sup>) from the endogenous Kras locus [7]. Administration of an obesogenic diet markedly accelerates PanIN formation and PDAC development in this model [8, 9]. The identification of novel targets and agents for prevention and interception [10] requires a detailed understanding of the signaling mechanisms and gene regulatory programs that stimulate the proliferation of PDAC cells [7].

Recent evidence indicates that the transcriptional co-activators Yes-Associated Protein (YAP) and WW-domain-containing Transcriptional co-Activator with PDZ-binding motif (TAZ), two central effectors of the highly conserved Hippo pathway [11–13], act as potent oncogenes in PDAC [14–17] and in the control of differentiation of pancreatic cells to different lineages [18]. The Hippo pathway consists of a serine/threonine kinase cascade in which Mst1/2 kinases phosphorylate and activate Lats1/2, which phosphorylate YAP and TAZ at specific residues that regulate their localization and protein stability [11, 12]. In its unphosphorylated state, YAP localizes to the nucleus where it binds and activates predominantly the TEA-domain DNA-binding transcription factors (TEAD 1–4) thereby stimulating the expression of multiple genes, including Connective Tissue Growth Factor (CTGF) and Cysteine-rich angiogenic inducer 61 (Cyr61). Independently of the Hippo pathway, YAP/TAZ localization and activity is highly responsive to the organization of the actin filament [13]. Consequently, YAP/TAZ activity represents a key point of convergence in the signaling by Ras, Rho, tyrosine kinase receptors, G protein-coupled receptors (GPCR), integrins, mechanical cues and cell density, all of which regulate the organization of the actin cytoskeleton and play a critical role in PDAC development [13, 19–23]. Several studies indicate that YAP/TAZ is up-regulated in PDAC tumor samples [24–26] and higher expression of YAP is correlated with poorer survival in PDAC patients [17, 27]. Furthermore, amplification and overexpression of Yap can substitute for mutant Kras expression in preclinical models of PDAC [24]. In view of all these findings, there is intense interest in targeting YAP/TAZ to prevent or intercept PDAC development.

Although inhibition of the activity of transcription factors or their co-activators has proven a difficult strategy, recent evidence suggests a new avenue to target YAP/TAZ activity via the use of statins in PDAC and other malignancies. Many studies showed that the mevalonate pathway is markedly upregulated in several epithelial cancers, including PDAC [28–31]. Statins are specific inhibitors of the 3-hydroxy-methylglutaryl (HMG) CoA reductase [32, 33], the rate-limiting enzyme in the generation of mevalonate, the first step in the biosynthesis of isoprenoids, leading to farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GG-PP) and cholesterol [34]. The transfer of the geranylgeranyl moiety of GG-PP to a COOH-terminal cysteine of Rho GTPases is critical for their membrane localization and function in signal transduction through actin remodeling [12]. Accordingly, YAP and TAZ act as novel sensors of mevalonate metabolism and statins as inhibitors of their nuclear localization and transcriptional
activity, at least in some cell types [35–37]. Statins, which are usually well tolerated and generally safe, have long been used to treat hypercholesterolemia and prevent cardiovascular diseases. Recent epidemiological studies imply that use of statins is associated with beneficial effects in PDAC [38–47], and other malignancies [48]. In a previous preclinical study, statin administration delayed PDAC in mice harboring Kras\(^{G12D}\) but the incidence of PDAC in this report was unusually high and the connection between statin and YAP activity was not examined [49]. Furthermore, the impact of statins on the initial stages of PDAC, namely depletion of pancreatic acini (acinar- ductal metaplasia) and formation of PanIN lesions induced by an obesogenic diet in conditional Kras\(^{G12D}\) (KC) mice remains unknown.

In the present study, we determined the impact of different statins on YAP localization, and transcriptional activity in human and murine PDAC cells. In order to determine whether statins act via stimulation of the Hippo pathway, we also examined the influence of statins on YAP phosphorylation at Ser\(^{127}\), a residue targeted by LATS1/2. Subsequently, we assessed the impact of simvastatin administration on pancreatic acini depletion and PanIN formation using KC mice subjected to diet-induced obesity (DIO), which accelerates PanIN formation and PDAC development [8, 9, 50]. Our results show that exposure of PDAC cells to lipophilic statins, including cerivastatin and simvastatin, inhibits YAP nuclear localization, transcriptional activity and colony formation via a phosphorylation-independent mechanism. Administration of simvastatin to KC mice subjected to DIO attenuated early pancreatic acini depletion and PanIN formation. Collectively, our results provide preclinical evidence that statins restrain YAP activity and proliferation in pancreatic cancer cell models in vitro and attenuates early lesions leading to PDAC in vivo.

**Material and methods**

**Cell culture**

The human pancreatic cancer cell lines PANC-1 and MiaPaCa-2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cell lines were chosen because they harbor mutations typical of human pancreatic cancer, including mutations in KRAS and TP53 (encoding the p53 protein) and deletion of CDKN2A (also known as p16 or p16\(^{INK4a}\)). These cell lines, authenticated by ATCC by short-tandem repeat analysis, were used within 15 passages and cultured for less than 6 months after recovery from frozen stocks (no authentication was done by the authors). PANC-1 and MiaPaCa-2 cells were grown in Dulbecco’s modified Eagle Medium (DMEM) with 2 mM glutamine, 100 units/mL penicillin, and 100 \(\mu\)g/mL streptomycin and 10% fetal bovine serum (FBS) at 37˚C in a humidified atmosphere containing 10% CO\(_2\).

Primary KC and KPC cells, (expressing KrasG12D or both KrasG12D and mutant p53, respectively) isolated from KC or KPC mice, were a kind gift from Dr. Ashok Saluja, (Professor and Vice-Chair, Department of Surgery, Director, Sylvester Pancreatic Cancer Research Institute, University of Miami). KC and KPC cells were validated by PCR analysis of genomic DNA. These cells were cultured in RPMI 1640 medium with 2 mM glutamine, 100 units/mL penicillin, and 100 \(\mu\)g/mL streptomycin and 5% fetal bovine serum (FBS) at 37˚C in a humidified atmosphere containing 5% CO\(_2\).

**Experimental animals**

After weaning, offspring of LSL-KrasG12D/+ and p48-Cre/+– mice were randomly allocated to a control diet (CD), a high fat, high calorie diet (HFCD), or a HFCD plus simvastatin (40mg/kg). All mice had free access to the diet, body weights were measured weekly and the general health and behavior of animals were assessed daily. After 14 weeks, mice were
euthanized and the entire pancreas was harvested for histological analysis. All studies involving animals were reviewed and approved by the Chancellor’s Animal Research Committee of the University of California, Los Angeles in accordance with the NIH Guide for the Care and Use of Laboratory Animals (protocol number: 2011–118)

**Genotyping analysis**

*LSL-KRASG12D* and *Cre* alleles were detected prior to randomizing to the experimental diets by PCR analysis of genomic DNA, as described [51]. Mutant (KC) mice expressed both *LSL-KRASG12D* and *Cre* alleles, and animals carrying neither allele were labeled wild type (WT).

**Experimental diets**

Diets were prepared by Dyets, Inc. (Bethlehem, PA). At one month of age, mice were randomized to receive either the CD, HFCD, or HFCD with simvastatin (40mg/kg) from Sigma-Aldrich, (St. Louis, MO) in the food. A detailed composition of the diets was described previously [9]. Briefly, while the CD contained 12% calories from fat, 40% of calories in the HFCD stem from fat (corn oil-based). All diets were stored at −20˚C (long-term) or 4˚C (short-term) in sealed containers and prepared under low light conditions. The diets were replaced on a weekly basis.

**Pancreas and liver histology**

Hematoxylin and eosin (H&E) stained tissue sections of the pancreas, fixed in formalin and embedded in paraffin, were assessed by gastrointestinal pathologists blinded to the experimental groups. The presence and stage of murine PanINs were analyzed as described previously [9]. For each animal approximately 100 pancreatic ducts (tail of the pancreas) were quantified and the proportion of murine PanIN-3 to the overall number of pancreatic ducts was recorded.

**Western blot analysis**

Confluent cultures of PANC-1 or MiaPaCa-2 cells, grown on 35 mm tissue culture dishes, were washed twice with DMEM and incubated in serum-free medium for 4 h and then treated as described in individual experiments. The cultures were then directly lysed in 2 × SDS-PAGE sample buffer [200 mM Tris-HCl (pH 6.8), 2 mM EDTA, 0.1 M Na$_3$VO$_4$, 6% SDS, 10% glycerol, and 4% 2-mercaptoethanol], followed by SDS-PAGE on 4–15% gels and transfer to Immobilon-P membranes (Millipore, Billerica, MA). For detection of proteins, membranes were blocked using 5% nonfat dried milk in PBS, pH 7.2, and then incubated overnight with the desired antibodies diluted in PBS containing 0.1% Tween. Primary antibodies bound to immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection with horseradish peroxidase-conjugated anti-mouse, anti-rabbit antibody and a FUJI LAS-4000 mini luminescent image analyzer. Quantification of non saturated bands was performed using the FUJI Multi Gauge V3.0 analysis program.

**Immunofluorescence**

Immunofluorescence of PANC-1 and MiaPaCa-2 cells was performed by fixing the cultures with 4% paraformaldehyde followed by permeabilization with 0.4% Triton X-100. After extensive PBS washing, fixed cells were incubated for 2h at 25˚C in blocking buffer (BB), consisting of PBS supplemented with 5% bovine serum albumin and then stained at 4˚C overnight with
YAP mouse mAb (1:200) diluted in BB. Subsequently, the cells were washed with PBS at 25˚C and stained at 25˚C for 60 min with Alexafluor 488—conjugated goat-anti mouse diluted in BB (1:100) and washed again with PBS. Nuclei were stained using a Hoechst 33342 stain (1:10,000). For staining of F-actin, fixed cells were blocked with 5% bovine serum albumin in PBS. The cells were then incubated with TRITC-conjugated phalloidin (0.25 μg/ml) in PBS for 10 min at room temperature and washed five times with PBS. Images were captured as uncompressed 24-bit TIFF files captured with an epifluorescence Zeiss Axioskop and a Zeiss (Achroplan 40/0.75W objective) and a cooled (−12˚C) single CCD color digital camera (Pursuit, Diagnostic Instruments) driven by SPOT version 4.7 software. AlexaFluor 488 signals were observed with a HI Q filter set 41001 and TRITC images with a HI Q filter set 41002c (Chroma Technology).

Image analysis
For YAP localization the average fluorescence intensity in the nucleus and just outside the nucleus (cytoplasm) was measured to determine the nuclear/cytoplasmic ratios. All Image analysis was performed using Zeiss analysis imaging software. The selected cells displayed in the appropriate figures were representative of 80% of the population.

Quantitative reverse transcription PCR (qRT-PCR)
Relative transcript expression levels of CTGF and CYR61 were determined by qRT-PCR using a TaqMan Gene Expression Assay. Briefly, total RNA was extracted from cells by using a Pure-Link RNA Mini Kit. Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit using 1 μg of total input RNA. The synthesized cDNA samples were used as templates for the real-time (RT) PCR analysis. All reactions were performed using the Applied Biosystems StepOne system and the amplifications were done using the TaqMan Fast Advanced Master Mix. The following primers were used; gene-specific Homo sapiens oligonucleotides for CTGF (Assay ID: Hs01026927_g1), CYR61 (Assay ID: Hs99999901_s1) and gene-specific Mouse oligonucleotide primers for Ctgf (Assay ID: Mm00438890_m1), Cyr61 (Assay ID: Mm00487498_m1), Birc5 (Mn00599749_m1) the internal control was 18s (Assay ID: Hs99999901_s1) all were from Life Technologies, Carlsbad, CA.

Colony formation
For cell colony formation, 500 PANC-1, MiaPaCa-2, KC or KPC cells were plated into 60-mm tissue culture dishes either in DMEM containing 10% FBS or RPMI 1640 containing 5% FBS. After 24 h of incubation at 37˚C, cultures were transferred to DMEM containing 3% FBS (PANC-1, MiaPaCa-2) or RPMI 1640 containing 1% FBS (KC, KPC) either in the absence or presence of statins, as indicated in the individual experiments. A colony consisted of at least 50 cells. Cell colony numbers from at least three dishes per condition were determined after 6 to 10 days of incubation.

Transfection of GFP-AKT-PH
PANC-1 cells were transfected with the plasmid containing a cDNA encoding a green fluorescent protein (GFP) tagged-AKT pleckstrin homology domain (pcDNA3-AKT-PH-GFP was a gift from Craig Montell, Addgene plasmid # 18836) by using Lipofectamine 3000 as suggested by the manufacturer. Analysis of the cells were performed 24 h after transfection.
Measurement of intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\))

PANC-1 cells grown on glass coverslips for 4–5 d were washed in Hanks’ balanced salt solution supplemented with 0.03% NaHCO\(_3\), 1.3 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 0.4 mM MgSO\(_4\), and 0.1% BSA (pH 7.4) (Hanks' buffer). Cells were then incubated with 5 μm fura 2-tetra-acetoxy methyl ester (fura 2-AME) for 10 min in a 37 C incubator. Coverslips were then washed with Hanks' buffer and then and mounted in a standard 1-cm path length cuvette containing Hanks' buffer, and the cuvette was placed into a Hitachi (Tokyo, Japan) F-2000 fluorospectrophotometer. The incubation medium in the cuvette was continuously stirred at 37 C. The size of the detection window allowed measurement on the order of 10\(^5\) cells. Excitation was set to 340 and 380 nm, and emission signal was collected at 510 nm, all with a 10-nm bandwidth. Samples were taken every 0.5 s using associated software (F-2000 Intracellular Cation Measurement System; Hitachi Instruments). The software created the 340/380 nm ratios, which are proportional to [Ca\(^{2+}\)]\(_i\).

Materials

DMEM, FBS, goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488, Fura-2 AM and all RT-qPCR reagents were obtained from Invitrogen (Carlsbad, CA). Neurotensin, insulin and phalloidin-TRITC were obtained from Sigma Chemical (St. Louis, MO). The geranylgeranyl transferase I (GGTase I) inhibitor GGTI 298, PI 3-Kinase inhibitor A66 and the MEK inhibitor PD0325901 were from R&D Systems (Minneapolis, MN). The dual PI3K/mTOR inhibitor NPV-BEZ235 was purchased from Selleck Chemicals (Houston, TX). Primary antibodies used were as follows: YAP (H-9, sc-271134 and 63.1, sc-101199, final dilution 1:200 for immunofluorescence), tubulin (sc-5274; final dilution 1:400), actin (sc-47778; final dilution 1:400) and GAPDH (sc-365062; final dilution 1:400) (Santa Cruz Biotechnology); phospho-YAP Ser127 (D9W2I, 13008; final dilution 1:1000), YAP (15028; final dilution 1:1000 for western blots), phospho-p70 S6 KinaseThr389 (9205; final dilution 1:1000) and phospho-S6 Ribosomal Protein Ser240/244 (5364; final dilution 1:1000) and LATS2 (5888; final dilution 1:1000) were from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase–conjugated anti-rabbit IgG and anti-mouse IgG were from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). All other reagents were of the highest grade available.

Results

Statins inhibit YAP nuclear localization and activity in sparse cultures of PDAC cells

Initially, we examined whether YAP localization depends on cell density in human PDAC cells. Specifically, PANC-1 and MiaPaCa-2 cells, plated at low densities, were fixed after 1, 3 or 7 days of culture. YAP was visualized by immunofluorescent staining. At lower cell densities (1 or 3 days in culture), YAP was localized prominently in the nucleus of PANC-1 and MiaPaCa-2 cells (Fig 1A, Left). In contrast, YAP was primarily in the cytoplasm of PANC-1 and MiaPaCa-2 cells maintained in culture for 7 days. The quantification of YAP nuclear/cytoplasmic ratio in multiple cells corroborated this conclusion (Fig 1A, Bars).

Next, we examined whether inhibition of mevalonate synthesis by statins regulates the localization of YAP in PDAC cells. Cultures of PANC-1 or MiaPaCa-2 cells were plated at low density, grown for 24 h and treated without or with cerivastatin at 0.3 μM for an additional 24 h. Then, the cultures were fixed and YAP localization was determined. As shown in Fig 1B, exposure to cerivastatin induced a striking re-localization of YAP from the nucleus to the cytoplasm.
cytoplasm, as verified by quantification of YAP nuclear/cytoplasmic ratio by image analysis (Fig 1B, Bars). Exogenously added mevalonic acid prevented the nuclear extrusion of YAP induced by exposure to cerivastatin in either PANC-1 or MiaPaCa-2 cells (Fig 1B). The results imply that statins induce YAP cytoplasmic localization in PDAC cells through inhibition of 3-hydroxy-methylglutaryl CoA reductase, the rate-limiting enzyme in mevalonic acid synthesis.

In line with the cytoplasmic localization induced by exposure to cerivastatin, treatment of PANC-1 or MiaPaCa-2 cells with this statin markedly reduced the mRNA levels of the YAP/TEAD-regulated gene CTGF (Fig 1C). CTGF is one of the best-characterized direct target gene of YAP that contains three putative YAP-TEAD binding sites (GGAATG) in its promoter region. In addition, simvastatin also inhibited CTGF expression in PANC-1 and MiaPaCa-2 (Fig 1C). Exposure to cerivastatin or simvastatin also inhibited the expression of Cyr61, another YAP/TEAD-regulated gene (Fig 1D). These results indicate that lipophilic statins regulate YAP localization and co-activator transcriptional activity in PANC-1 and MiaPaCa-2 cells.

**Statins inhibit the stimulation of YAP activity induced by neurotensin and insulin in dense cultures of PDAC cells**

In previous studies, we identified potent positive crosstalk between insulin/IGF-1 receptors and G protein-coupled (GPCR) signaling systems in PDAC cells leading to early signaling and subsequent proliferation [52–57]. Recently, we reported that stimulation of dense cultures of PANC-1 or MiaPaCa-2 cells with a combination of insulin and the GPCR agonist neurotensin promoted YAP nuclear localization and stimulated the expression of YAP/TEAD-regulated genes [58]. Here, we determined whether prior exposure to statins prevents YAP nuclear localization and transcriptional activity in confluent cultures of PDAC cells challenged with these growth-promoting agonists. PDAC cells maintained in culture for 5 days were transferred to medium containing low serum and treated with or without cerivastatin at 0.3 μM for 24 h and then stimulated with a combination of 10 ng/ml insulin and 5 nM neurotensin. In line with recent results [58], stimulation of PDAC cells with insulin and neurotensin induced robust translocation of YAP to the nucleus in either PANC-1 (Fig 2A) or MiaPaCa-2 cells (Fig 2B). Prior exposure of these cells to 0.3 μM cerivastatin prevented YAP translocation to the nucleus induced by insulin and neurotensin (Fig 2), as verified by quantification of YAP nuclear/cytoplasmic ratio by image analysis (Fig 2, Bars).

Nuclear extrusion of YAP induced by statin is expected to reduce the transcriptional activity of TEAD. Consequently, we determined the effect of statins on YAP/TEAD-regulated gene expression induced by crosstalk between insulin and neurotensin. As shown in Fig 3, stimulation of confluent PANC-1 or MiaPaCa-2 cells with neurotensin and insulin induced a marked increase in the level of CTGF and CYR61 transcripts, as determined by RT-qPCR. Treatment with cerivastatin completely blocked the increase in the expression of these genes at a
concentration as low as 0.1 μM. Similarly, treatment with simvastatin (3 μM) prevented the increase in the mRNA levels of CTGF and CYR61 induced by insulin and neurotensin. Thus, treatment with lipophilic statins induced YAP cytoplasmic localization and inhibited YAP/TEAD-regulated gene expression either in rapidly growing sparse cultures of PDAC cells (Fig 1) or in dense cultures of these cells re-stimulated via crosstalk between the GPCR agonist neurotensin and insulin (Figs 2 and 3).

Statins inhibit colony formation by PDAC cells

Having established that statins promote cytoplasmic localization and inhibit YAP/TEAD-regulated gene expression in PDAC cells, we next determined whether statins also inhibit the
proliferation of PANC-1 and MiaPaCa-2 cells by assessing the inhibitory effects of these drugs on the colony-forming ability of these PDAC cells. To this end, we plated single cell suspensions of PANC-1 or MiaPaCa-2 cells onto 60 mm dishes (500 cells per dish) in the absence or presence of increasing concentrations of different statins and determined the number of colonies formed after 8–10 days of incubation.

Addition of cerivastatin to the medium potently inhibited colony formation by PANC-1 or MiaPaCa-2 cells in a dose-dependent manner (Fig 4). Half-maximal inhibitory effect (IC$_{50}$)
Statins inhibit YAP in pancreatic cancer cells
was achieved at a concentration of cerivastatin as low as 0.03 μM. Maximal inhibition of colony formation was obtained at 0.3 μM. Addition of mevalonic acid (250 μM) reversed completely (PANC-1) or partially (MiaPaCa-2) the inhibitory effect of low concentrations (0.1–0.3 μM cerivastatin) on colony formation. These results are consistent with the notion that the statins, at low concentrations, block colony formation by PDAC cells through inhibition of 3-hydroxy-methylglutaryl CoA reductase, the rate-limiting enzyme in the generation of mevalonic acid, and with our previous results showing that knockdown of YAP/TAZ prevents colony formation by either PANC-1 or MiaPaCa-2 cells [58].

We also determined whether other lipophilic statins inhibit colony formation by PDAC cells. As shown in Fig 4, simvastatin inhibited colony formation by PANC-1 and MiaPaCa-2 cells in a dose-dependent manner with half-maximal effect achieved at 0.3 μM. In addition, atorvastatin and fluvastatin also reduced colony formation by PANC-1 cells in a dose-dependent manner. In sharp contrast, the hydrophilic statin pravastatin did not exert any inhibitory effect even at a concentration as high as 10 μM. This is likely due to the low uptake of pravastatin in pancreatic cancer cells [59]. These results indicate that cell-permeable lipophilic statins, including cerivastatin, simvastatin, atorvastatin and fluvastatin potently inhibit colony formation by PDAC cells.

Mechanism by which statins induce cytoplasmic localization and inhibit transcriptional activity of YAP: role of YAP phosphorylation and actin organization

It is widely recognized that statins deplete several important lipid intermediates, including geranylgeranyl pyrophosphate (GGPP), a critical isoprenoid in Rho prenylation. A key enzyme in this process is geranylgeranyl transferase (GGTase) that catalyzes the transfer of GG to Rho. In line with this notion, the selective inhibitor of GGTase, GGTI 298, mimicked the inhibitory effect of statins on colony formation by PDAC cells (Fig 5A).

The precise mechanism linking Rho to YAP activity in PDAC cells remains incompletely understood. In other cell types, statin treatment appears to increase YAP phosphorylation at Ser127 through a Hippo-independent but Rho-inhibited kinase pathway [35, 37]. So far, the putative Rho-sensitive kinase remains unidentified. In contrast, we found that exposure to cerivastatin did not alter the phosphorylation of YAP at Ser127 in either PANC-1 (Fig 5B) or MiaPaCa-2 (Fig 5C) cells incubated either without or with neurotensin and insulin. These results were reproduced in 9 separate experiments. In addition, we did not detect any consistent increase in the phosphorylation of YAP at Ser127 or change in total YAP in PANC-1 (Fig 5D) or MiaPaCa-2 (Fig 5E) cells treated with different concentrations of cerivastatin or simvastatin without or with stimulation by insulin and neurotensin. Accordingly, we did not detect any change in LATS phosphorylation at Thr1079 or in total LATS in response to these treatments in PANC-1 (Fig 5D) or MiaPaCa-2 (Fig 5E) cells These results indicate that statins inhibit YAP nuclear localization and co-activator activity independently of its phosphorylation at Ser127 in PDAC cells.
The organization of the actin cytoskeleton plays a central role in the regulation of YAP localization and transcriptional activity independently of YAP phosphorylation [13]. Statins disrupt Rho-dependent actin remodeling in at least some cell types [37] but their effect on the actin organization of PDAC cells has not been determined. Consequently, we examined the effect of statin exposure on actin organization in PDAC cells challenged with insulin and neurotensin, as visualized by phalloidin staining. Stimulation of PDAC cells with the combination of insulin and neurotensin induced a marked increase in the assembly of actin stress fibers, a surrogate marker of Rho signaling. Prior exposure to cerivastatin (0.1–0.3 \( \mu \)M) blunted stress fiber formation in PANC-1 (Fig 5F) and MiaPaCa-2 (Fig 5G) cells. These results imply that statins inhibit YAP via a Hippo pathway-independent disruption of the actin cytoskeleton organization.

In order to test further the specificity of the statins in PDAC cells, we examined whether treatment with cerivastatin inhibits other cellular responses induced by crosstalk between insulin and neurotensin. Because statins inhibited PI3K activity in other cell types [60], and this enzyme contributes to YAP activation in PDAC cells [58], we determined the effect of cerivastatin on the activity of PI3K in intact PANC-1 cells. To ascertain whether treatment with cerivastatin diminishes the accumulation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) in the plasma membrane, we monitored the redistribution of Akt-pleckstrin homology domain-green fluorescent protein (Akt-PH-GFP) in single PANC-1 cells. In line with our previous results, stimulation with insulin and neurotensin markedly increased membrane accumulation of Akt-PH-GFP [58]. The translocation of the PIP3 sensor to the plasma membrane was not prevented by prior exposure to cerivastatin at a concentration as high as 1 \( \mu \)M (S1 Fig). In contrast, the class I p110\( \alpha \) specific inhibitor A66, tested as a positive control, abolished the movement of Akt-PH-GFP to the plasma membrane (S1 Fig). In line with these results, treatment with cerivastatin did not prevent the stimulation of p70S6K phosphorylated on Thr\( ^{389} \), a direct marker of mTORC1 activity, and the phosphorylation of p70S6K substrate, S6 on Ser\( ^{240} \) induced by stimulation with neurotensin and insulin (S1 Fig). As a control, we verified that treatment with BEZ-235, a dual PI3K/mTOR inhibitor, completely blocked the phosphorylation of these proteins, as expected [61].
Given that an increase in the intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is one of the earliest events induced by GPCR agonists via heterotrimeric G proteins rather than monomeric GTPases (such as Rho), we also determined the effect of statins on the increase in [Ca\(^{2+}\)]. Prior exposure to cerivastatin did not prevent the increase in [Ca\(^{2+}\)] induced by subsequent stimulation with neurotensin in PANC-1 cells (S1 Fig). Collectively, these results indicate that statins inhibit YAP activation through disruption of actin organization without increasing YAP phosphorylation at Ser\(^{127}\) or interfering with other signaling events in PDAC cells, including PI3K/mTORC1 activation, and Ca\(^{2+}\) mobilization.

To further define the role of Rho, YAP and LATS in human PDAC, we analyzed the association of their expression to PDAC survival in human PDAC. We used the Pathology Atlas [62] based on the integration of publicly available data from The Cancer Genome Atlas (TCGA) and data generated within the framework of the Human Protein Atlas (HPA) and analyzed transcriptomics and survival in 176 PDAC patients [62]. In agreement with previous findings, increased YAP expression is strongly associated (p < 0.001) with unfavorable prognosis [17]. Similarly, expression of RhoA and RhoC mRNA levels is associated with poor survival of PDAC (S2 Fig). In contrast, increased mRNA expression of LATS1/2, which phosphorylate YAP at Ser\(^{127}\), is not associated with PDAC survival (S2 Fig). These findings support the notion that the Rho GTPases promote YAP activity and poor PDAC survival and thus, are in line with the conclusions drawn from the mechanistic studies presented in Fig 5.

**Effect of statins on YAP localization and activity in murine KC and KPC cells**

Next, we examined the effects of cerivastatin and simvastatin on YAP localization, YAP/TEAD-regulated gene expression and colony formation in KC cells. Treatment of KC cells with either cerivastatin or simvastatin induced striking YAP translocation from the nucleus to the cytoplasm, as verified by quantification of YAP nuclear/cytoplasmic (Fig 6A). Exposure to cerivastatin or simvastatin also inhibited the expression of the YAP/TEAD-regulated genes Ctgf, Cyr61 and Birc5 (Fig 6B). Furthermore, addition of either cerivastatin or simvastatin strikingly inhibited colony formation by KC cells in a dose-dependent manner (Fig 6C).

A recent study concluded that statin-induced depletion of mevalonic acid destabilizes mutant p53 protein and thereby exerts a more potent growth-inhibitory effects in cancer cells harboring p53 mutant [63]. Although this mechanism could explain, at least partly, the statin sensitivity of the human pancreatic cancer cells used in this study (PANC-1 and MiaPaCa-2) which contain mutated TP53, the KC cells express wild type p53. In order to determine the contribution of p53 mutation to statin sensitivity in murine PDAC cells, we also determined the effects of statins on colony formation and YAP/TEAD-regulated genes in cells isolated from the pancreas of mice expressing both KrasG12D and mutant p53 (KPC mice). We found that cerivastatin and simvastatin inhibited colony formation and expression of Ctgf and Cyr61 in KPC cells at concentrations similar to those that produced inhibitory effects in KC cells (S3 Fig). The results indicate that the inhibitory effects of statins do not depend on the expression of mutant p53 in murine PDAC cells.

**Effect of simvastatin on initial stages of PDAC development in vivo**

Recent studies demonstrated that the YAP/TAZ pathway plays a critical role in promoting the initial stages of PDAC development, namely depletion of intact acini (acinar-ductal metaplasia) and formation of PanIN lesions [14, 15]. Consequently, we determined the effect of statin on pancreatic pre-neoplastic lesions in KC mice subjected to diet-induced obesity that accelerates the appearance and stage of these lesions [16]. Specifically, cohorts of KC mice were fed...
CD, HFCD, or HFCD plus simvastatin (40mg/kg orally) for 3 months. In line with our previous results [8, 9], administration of the obesogenic diet induced a marked increase in acini depletion, inflammation and in PanIN-3 formation. Treatment with simvastatin significantly attenuated the loss of intact acini (histological images in Fig 7A and 7B; quantification in Fig 7C), markedly reduced the pancreatitis score (calculated as in [8, 9]) and the formation of PanIN-3 lesions (quantification in Fig 7E) induced by DIO. At the dose used, simvastatin did not produce any adverse clinical effects, and had no effect on weight gain (Fig 7E).

Discussion

PDAC is a clinically aggressive disease that is anticipated to become the 2nd cause of cancer fatalities during the next decade [2]. As current therapeutic approaches are limited, novel targets and agents for chemoprevention are urgently needed and will most likely arise from a more detailed understanding of the signaling mechanisms that stimulate the promotion and progression of pre-malignant (initiated) cells into invasive pancreatic cancer cells.

The highly conserved transcriptional co-activator YAP and its paralog TAZ, originally identified in *Drosophila*, are emerging as potent oncogenes for a variety of cell types [20, 64]. In this context, a number of studies indicate that YAP and TAZ are overactive in PDAC patient tumor samples [24, 65, 66] and associated with poor survival [17]. The YAP/TAZ pathway assumes an added importance in PDAC because YAP is a key downstream target of Ras signaling required for PanIN progression into invasive PDAC [14, 15]. Furthermore, YAP not only

Fig 6. Statins inhibit YAP nuclear localization, colony formation and the expression of *Ctgf, Cyr61* and *Birc5* in KC cells. A, KC cells 1 day after plating were incubated either in absence or presence of 0.3 μM cerivastatin (Cer) or 3 μM simvastatin (Ser) for 24 h. Then, the cells were then fixed with 4% paraformaldehyde and stained with an antibody that detects total YAP and with Hoechst 33342 to visualize the cell nuclei. Bars represent the ratio of nuclear/cytoplasm (50 to 75 cells). **P 0.01 as shown by paired t-test. B, KC cells were incubated either in absence or presence of cerivastatin (Cer) or simvastatin (Sim) at the indicated concentrations. Statins were added 1 day after plating and the incubation continued for 24 h. RNA was then isolated and the relative levels (n = 3) of *Ctgf, Cyr61* and *Birc5* mRNA compared with 18s mRNA were measured by RT-qPCR. Data are presented as mean ± SEM. Similar results were obtained in 3 independent experiments. C, KC cells were incubated for 6 days with various concentrations of cerivastatin or simvastatin, as indicated. The bars represent the number of colonies (mean ± SEM; n = 4 cultures per condition).

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acts downstream of K-ras but its hyper-activation can bypass the need of oncogenic K-ras in PDAC development [7, 24] and plays a critical role in the crosstalk between GPCR agonists and insulin [58]. In the light of these new developments, there is intense interest in targeting YAP/TAZ in PDAC, given the urgent need of novel strategies for combating this lethal disease.

Given the critical role of gene-regulatory programs in cancer development, inhibition of transcription factors or their co-activators in cancer cells is a logical but challenging strategy. Using sparse cultures of these PDAC cells, we show here that exposure to lipophilic statins

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Fig 7. Simvastatin prevents the disruption in histologic pancreatic architecture induced by DIO in KC mice. A and B, Representative H&E staining of the pancreas of a KC mouse fed the HFCD (A) or HFCD plus simvastatin (B). C, Percentage (%) of intact acini in KC mice fed the CD, HFCD or HFCD plus simvastatin at 3 months. D, Pancreatitis score in KC mice fed CD, HFCD or HFCD plus simvastatin at 3 months. E, Percentage (%) of PanIN-3 lesions in KC mice fed CD, HFCD or HFCD plus simvastatin at 3 months. F, Simvastatin has no effect on weight gain in KC mice fed with either CD, HFCD, or HFCD plus simvastatin at 3 months. The number of mice used were: CD = 10; HFCD = 14; HFCD+simvastatin = 14. Values are means ± s.d. *p < 0.05 vs. CD, #p < 0.05 vs. HFCD.

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induces robust translocation of YAP from the nucleus to the cytoplasm. Using dense cultures of PDAC cells, we demonstrate that statins block the translocation of YAP to the nucleus induced by crosstalk between insulin and neurotensin signaling systems, a potent mitogenic combination for these cells. Collectively, these results demonstrate that treatment with statins induces YAP cytoplasmic sequestration in PDAC cells, in agreement with recent findings in other cancer cell types [35–37].

When YAP/TAZ localize to the nucleus, they bind and activate primarily the TEA-domain DNA-binding transcription factors (TEAD 1–4) thereby stimulating the expression of a variety of genes [67], including CTGF and CYR61, well established YAP/TEAD-regulated genes. Here, we found that lipophilic statins, including cerivastatin and simvastatin, markedly reduced the mRNA levels of CTGF and CYR61 in sparse cultures of PANC-1 and MiaPaCa-2 cells and blocked the increase in the expression of these genes induced by stimulation with neurotensin and insulin in dense and serum-deprived cultures. Taken together, the results of YAP localization and expression of YAP/TEAD-regulated genes indicate that statins block YAP activity by restricting its localization to the cytoplasm. In agreement with the notion that YAP nuclear activity stimulates the expression of genes, including CTGF and CYR61, implicated in PDAC cell proliferation, we found that cell-permeable lipophilic statins, including cerivastatin, simvastatin, atorvastatin and fluvastatin potently inhibit colony formation by PANC-1 and MiaPaCa-2 cells whereas the hydrophilic statin pravastatin did not have any detectable inhibitory effect even at high concentrations.

We next examined the mechanism by which statins regulate YAP localization in PDAC cells. Canonical Hippo signals are transduced through a serine/threonine kinase cascade wherein Mst1/2 kinases, in complex with Sav1, phosphorylate and activate LATS1/2, in complex with its regulatory protein MOB1/2 [12]. The activated LATS1/2 catalyzes the phosphorylation of YAP at five different sites, including Ser127, a residue proposed to mediate cytoplasmic retention in its phosphorylated state, though the phosphorylation of this residue did not impair nuclear import of YAP in a variety of cell types [13]. Previous studies resulted in differing conclusions concerning the role of the Hippo pathway and YAP phosphorylation in the mechanism by which statins promote YAP localization to the cytoplasm [35, 37] but none of these studies were performed in PDAC cells. Our results demonstrate that exposure to lipophilic statins, at concentrations that markedly inhibit YAP nuclear localization, YAP/TEAD-regulated gene expression and colony formation, did not induce any significant change in the phosphorylation of YAP at Ser127 or LATS at Thr1079 in PDAC cells. Accordingly, we conclude that statins inhibit YAP activity through a Hippo pathway-independent mechanism.

A considerable body of evidence indicates that multiple signaling inputs regulate the activity of YAP through the organization of filamentous (F) actin [68, 69]. Rho plays a critical role in F-actin organization and in the assembly of actin stress fibers in a variety of cell types [70]. Interestingly, Rho-induced actomyosin-based tension causes YAP/TAZ activation, as well as proposed to “open” nuclear pores for YAP/TAZ nuclear import [71, 72]. Statins are specific inhibitors of the 3-hydroxy-methylglutaryl (HMG) CoA reductase [32], the rate-limiting enzyme in the generation of mevalonate, the first step in the biosynthesis of isoprenoids, leading to farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GG-PP) and cholesterol. The transfer of the geranylgeranyl moiety to a COOH-terminal cysteine of Rho GTPases is critical for their function. Here, we produced several lines of evidence indicating that statins inhibit YAP through a Rho-mediated mechanism: 1) exogenously added mevalonate reversed the inhibitory effect of low concentrations of statins on nuclear localization and at least in part, on colony formation of PDAC cells; 2) a selective inhibitor of GGTase, GGTI 298, mimicked the inhibitory effect of statins on colony formation by PDAC cells; 3) treatment of PDAC cells with cerivastatin drastically reduced stress fiber formation. These results, together with the
dissociation from YAP phosphorylation discussed above, imply that statins inhibit YAP activity by interfering with the polymerization of the actin cytoskeleton. In line with these mechanistic studies, we found that the increased expression of Rho GTPases (RhoA, RhoC) is associated with poor survival in PDAC patients.

We extended these findings to murine PDAC and to a mouse model of acinar-ductal metaplasia, PanIN formation and PDAC development. Initially, we demonstrated that treatment of cells isolated from the pancreas of KC or KPC mice with lipophilic statins induced striking YAP translocation from the nucleus to the cytoplasm, inhibited the expression of the YAP/TEAD-regulated genes Ctgf, Cyr61 and Birc5 and profoundly inhibited colony formation by these cells. The results with murine cells are in agreement with the findings in human PDAC cells and additionally imply that mutation of p53 is not necessary for high statin sensitivity, at least in murine cells.

Subsequently, we determined the impact of a lipophilic statin on early neoplastic changes in the pancreas of KC mice subjected to DIO, a condition that markedly accelerates the development of pancreatic neoplastic lesions. We found that treatment with simvastatin significantly reversed the loss of intact acini induced by DIO and markedly reduced the formation of PanIN-3 lesions. Collectively, our results provide robust preclinical evidence that statin administration provides a plausible strategy to be considered in the prevention and treatment of PDAC.

In line with this conclusion, recent epidemiological studies imply that the use of statins is associated with beneficial effects in PDAC [38–47], and other malignancies [48]. Although a recent study failed to detect an effect of statins in lowering PDAC risk [73], a follow up study of the same data reported an increased survival in PDAC patients with regular pre-diagnosis use of statins [74]. Taken together with the findings presented here and the lack of efficient strategies to oppose PDAC development, these studies warrant a comprehensive evaluation of the lipophilic statins in the primary or secondary chemoprevention of this devastating disease.

Supporting information

S1 Fig. Treatment with cerivastatin does not prevent PI3K activation or increase in \([\text{Ca}^{2+}]_{i}\) in PANC-1 cells. A: PANC-1 cells were transiently transfected with a plasmid encoding a fusion protein between GFP and the PH domain of AKT (AKT-PH-GFP). After 24h, the cultures were incubated in DMEM without or with cerivastatin at the indicated concentrations for 18h prior to stimulation with 5 nM neurotensin and 10 ng/ml insulin. Other cultures were treated with the class I p110α specific inhibitor A66 at 10 μM, tested as a positive control The intracellular distribution of AKT-PH-GFP was monitored under a fluorescence microscope. The selected cells displayed in the figures were representative of 90% of the population of GFP-positive cells. B: PANC-1 cells were treated for 24 h either in the absence or presence of cerivastatin (Cer) at the indicated concentrations for 24h. Other cultures were incubated for 2h with either the MEK inhibitor PD0325901 (1μM, PD) or the dual PI3K/mTOR inhibitor NPV-BEZ235 (1μM, BEZ). All cultures were then stimulated with 5 nM neurotensin and 10 ng/ml insulin (NT+Ins) for 30 min as indicated, and lysed with SDS–PAGE sample buffer. The samples were analyzed by SDS–PAGE and immunoblotting with phospho-p70 S6 KinaseThr-389 and phospho-S6 Ribosomal Protein Ser-240/244. Equal loading was verified by immunoblotting with GAPDH antibody.Similar results were obtained in 2 independent experiments. C: PANC-1 cells were incubated without or with cerivastatin at the indicated concentrations for 18h prior to stimulation with 5 nM neurotensin. Intracellular \([\text{Ca}^{2+}]_{i}\) was monitored as described in Materials and Methods. (TIF)
S2 Fig. Kaplan-Meier plots for RHO and LATS expression in PDAC. Images were reproduced from the Human Protein Atlas (version 17) available from www.proteinatlas.org The link is: http://www.proteinatlas.org/ENSG00000137693YAP1/pathology/tissue/pancreatic+cancerS1 (TIF)

S3 Fig. Statins inhibit colony formation and the expression of CTGF, CYR61 and BIRC5 in KPC cells. A, KPC cells were incubated for 6 days with various concentrations of cerivastatin or simvastatin, as indicated. The bars represent the number of colonies (mean ± SEM; n = 4 dishes per condition). B, KPC cells were incubated either in absence or presence of cerivastatin (Cer) or simvastatin (Sim) at the indicated concentrations. Statins were added 1 day after plating and the incubation continued for 24 h. RNA was then isolated and the relative levels (n = 3) of CTGF, CYR61 and BIRC5 mRNA compared with 18s mRNA were measured by RT-qPCR. Data are presented as mean ± SEM. Similar results were obtained in 3 independent experiments. (TIF)

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Author Contributions
Conceptualization: Guido Eibl, Enrique Rozengurt.
Data curation: James Sinnett-Smith, Guido Eibl, Enrique Rozengurt.
Formal analysis: Guido Eibl, Enrique Rozengurt.
Funding acquisition: Guido Eibl, Enrique Rozengurt.
Investigation: Fang Hao, Qinhong Xu, Jing Wang, Shuo Yu, Hui-Hua Chang, James Sinnett-Smith, Enrique Rozengurt.
Methodology: Fang Hao, Jing Wang, Shuo Yu, James Sinnett-Smith, Enrique Rozengurt.
Project administration: Enrique Rozengurt.
Resources: Enrique Rozengurt.
Supervision: James Sinnett-Smith, Guido Eibl, Enrique Rozengurt.
Validation: Guido Eibl, Enrique Rozengurt.
Writing – original draft: Enrique Rozengurt.
Writing – review & editing: James Sinnett-Smith, Guido Eibl, Enrique Rozengurt.

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