GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Role of A-Kinase Anchoring Protein Phosphorylation in Alcohol-Induced Liver Injury and Hepatic Stellate Cell Activation

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Accepted for publication
November 21, 2017.

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Alcoholic liver injury is associated with hepatic stellate cell (HSC) activation. A-kinase anchoring protein 12 (AKAP12) scaffolds protein kinase C and cyclin-D1, which is regulated by its phosphorylation, and spatiotemporally controls cell proliferation, invasiveness, and chemotaxis. HSC activation induces AKAP12 expression, but the role of AKAP12's scaffolding activity in liver function is unknown. Because AKAP12 phosphorylation is enhanced in ethanol-treated HSCs, we examined AKAP12's scaffolding functions in alcohol-mediated HSC activation and liver injury. AKAP12 expression, interaction, and phosphorylation were assayed in *in vitro* and *in vivo* ethanol models and human subjects by real-time PCR, coimmunoprecipitation, immunoblotting, and phosphorylated proteomics/Phos-tag. Ethanol induced AKAP12 phosphorylation in the liver and in primary HSCs, but not in hepatocytes. AKAP12's scaffolding activity for protein kinase C/cyclin-D1 decreased in ethanol-treated HSCs but not hepatocytes. AKAP12 negatively regulated HSC activation, which was reversed by ethanol-mediated AKAP12 phosphorylation. AKAP12 interacted with heat shock protein 47 (HSP47), which chaperones collagen and induces its secretion. Ethanol inhibited AKAP12-HSP47 and induced HSP47-collagen interaction. Ethanol-induced phosphorylated AKAP12 was unable to bind to HSP47 compared with its unphosphorylated counterpart, thereby proving that ethanol-mediated phosphorylation of AKAP12 inhibited the HSP47-AKAP12 scaffold. Silencing AKAP12 facilitated the chaperoning of collagen by HSP47. Hence, AKAP12 scaffolds HSP47 and regulates collagen-HSP47 interaction. Ethanol quenches AKAP12's scaffolding activity through phosphorylation and facilitates HSC activation. (*Am J Pathol* 2018, 188: 640–655; <https://doi.org/10.1016/j.ajpath.2017.11.017>)

Alcoholic liver disease (ALD) encompasses a wide range of pathologic conditions, ranging from steatosis to advanced conditions, such as alcoholic hepatitis, fibrosis, and cirrhosis, depending on the dose and duration of alcohol intake.¹ There are no approved antifibrotic drugs to prevent disease progression in patients with moderate ALD.²

Hepatic stellate cells (HSCs) constitute approximately 5% to 8% of the normal liver and store vitamin A in the form of retinyl esters.³ They are highly responsive to fibrotic stimuli.³ Acute and chronic liver damage by alcohol intake causes HSCs to undergo the process of activation.⁴ Activated HSCs lose their vitamin A, become highly proliferative and fibrogenic, and exhibit enhanced expression of extracellular matrix components. HSC activation associated

with increased collagen production is an early event in alcoholic liver injury before the onset of fibrosis.⁵ Collagen chaperoning and maturation, promoted by the heat shock

Supported by NIH grant 5R00AA017774-05, Applied Cell Biology and Physiology Division seed funding, Biomedical Sciences Department, Cedars-Sinai Medical Center (K.R.); NIH grant K01AA022372 (M.L.T.); liver research group core support, Division of Digestive and Liver Diseases, Department of Medicine, Cedars-Sinai Medical Center (K.R. and J.B.); Applied Cell Biology and Physiology division seed funding, Biomedical Sciences Department, Cedars-Sinai Medical Center (N.M.); and grant R24AA025017, National Institute on Alcohol Abuse and Alcoholism—supported Clinical Resource for Alcoholic Hepatitis Investigations, Johns Hopkins University (Z.S.).

K.R. and M.L.T. contributed equally to this work as senior authors.

Disclosures: None declared.

protein 47 (HSP47), is an essential event during HSC activation.^{6,7} HSP47 is known to inhibit collagen aggregation by binding procollagen in the endoplasmic reticulum and facilitating triple helix formation.⁷ Silencing HSP47 in HSCs decreases extracellular collagen,⁷ reversing liver fibrosis.⁸

A-kinase anchoring proteins (AKAPs) are involved in the spatiotemporal control of cellular signaling.⁹ They regulate intracellular signaling by guiding the anchored enzyme pools to their physiological substrates at specific cellular locations.^{9,10} AKAP12/gravin (in humans) or SSECKS (in mouse) is a member of the AKAP family that acts as a scaffold protein for protein kinases A and C (PKC) and cyclin-D1 (CCND1).¹¹ AKAP12-mediated scaffolding of PKC is known to attenuate PKC activation and suppress oncogenic proliferation, invasiveness, chemotaxis, and senescence.^{12,13} Of the known isoforms of PKC, PKCs α , δ , and ϵ interact with AKAP12; however, only PKC α and δ activity is induced in the absence of AKAP12.^{13,14}

The scaffolding activity of AKAP12 is altered by phosphorylation. Prephosphorylation of AKAP12 by PKC suppresses its interaction with PKC itself and increases PKC activity.¹⁵ Platelet-derived growth factor (PDGF)—induced tyrosine phosphorylation of AKAP12 inhibits its binding to F-actin, thereby modulating cytoskeletal reorganization and enhancing cellular motility.^{15,16} AKAP12 phosphorylation also enhances growth-associated functions. Phosphorylation of AKAP12 at a PKC phosphorylation site (S507/515) prevents the sequestration of CCND1 by AKAP12, leading to nuclear translocation of CCND1, allowing cell cycle progression.^{11,13} AKAP12 phosphorylation by cyclin-dependent kinase 1 at a threonine residue (T766) enhances the recruitment of the polo-like kinase in human glioblastomas to ensure efficient mitotic progression.¹⁷ The first 1000 amino acids of human and rodent AKAP12 share 83% identity, followed by approximately 600 amino acids with <20% identity (<http://atlasgeneticsoncology.org/Genes/AKAP12ID607ch6q25.html>, last accessed October 2014). The AKAP12 region containing all of the phosphorylation sites for PKC, CCND1, and polo-like kinase interaction^{11,13,15–17} is highly conserved between humans and rodents.

We and others have demonstrated that site-specific protein phosphorylation plays an important role during HSC activation^{18,19} and during early inflammatory responses in Kupffer cells of the liver.²⁰ The role of AKAP12 scaffolding activity or post-translational modifications in liver function are not yet reported. It was previously shown that AKAP12 expression is induced during HSC activation and is required for it.²¹ However, mechanistic insight into the function of AKAP12 in normal HSCs and fibrotic HSCs was not deciphered. Using mass spectrometry, enhanced levels of AKAP12 phosphorylated (phospho)-peptides were identified in culture-activated and ethanol-treated HSCs compared with controls. Liver injury caused by ethanol exposure or bile duct ligation (BDL) in mice increased AKAP12 phosphorylation. Livers of human patients with ALD/alcoholic hepatitis exhibited increased AKAP12 phosphorylation

compared with normal subjects. On the basis of the human disease data, the effect of ethanol on AKAP12 phosphorylation in liver cells was further investigated. AKAP12 phosphorylation was found to be induced in both primary human and mouse HSCs but not in primary hepatocytes on ethanol exposure. In HSCs, phosphorylation was associated with inhibition of AKAP12's scaffolding activity toward known players, such as cyclin-D1 and PKC α , and toward the collagen chaperone, HSP47. This scaffolding function of AKAP12 may have important implications in collagen maturation associated with ethanol-induced HSC activation and liver injury.

Materials and Methods

Materials

Ethanol (molecular biology grade, 200 proof) and acetaldehyde were purchased from Sigma (St. Louis, MO). All other reagents used were of molecular biology grade and are described under specific methods.

Cell Culture and Treatments

Primary quiescent human HSCs from single donor livers were purchased from ScienCell Inc. (San Diego, CA) and maintained in stellate cell medium+2% serum+1% growth supplements+1% penicillin/streptomycin (ScienCell Inc.). Primary human hepatocytes obtained from the cell culture core at the University of Southern California research center for liver diseases were maintained in Dulbecco's modified Eagle's medium (high glucose) containing 10% serum and 1% penicillin/streptomycin. Primary mouse HSCs and mouse hepatocytes were isolated from 3-month-old male C57BL/6 mice using the core services provided by the Liver Center at Cedars-Sinai Medical Center (Los Angeles, CA), according to previously published protocols.^{22,23} Mouse HSCs were cultured in Dulbecco's modified Eagle's medium (low glucose) containing 10% serum and 1% penicillin/streptomycin. Mouse hepatocytes were cultured like human hepatocytes, as described earlier in this paragraph. Primary HSC quiescence and hepatocyte differentiation was maintained by attachment on Matrigel matrix (Corning Life Sciences, Corning, NY), according to published literature.^{24,25} HSCs were treated with 100 mmol/L ethanol for 48 hours, and hepatocytes were treated with 100 mmol/L ethanol for 24 hours. HSCs or hepatocytes were treated with 150 μ mol/L of acetaldehyde for 24 hours, as reported previously.¹⁸ Quiescent HSCs were culture activated on plastic, as described previously.¹⁹ Quiescent human HSCs were treated with 40 ng/mL of active PDGF-BB (Abcam, Cambridge, MA) for 6 or 18 hours.

Mouse Tissues/Ethanol Feeding

Liver tissues from 10-day ethanol+binge feeding mouse model and pair-fed controls were kindly provided by Dr. Bin

Gao's laboratory (National Institute on Alcohol Abuse and Alcoholism, NIH, Bethesda, MD). The method of ethanol feeding was according to their published work²⁶ and briefly involved feeding 8- to 10-week-old C57BL/6 male mice with an ethanol Lieber-DeCarli diet containing 5% (v/v) ethanol for 10 days, followed by a binge dose of ethanol (single binge oral gavage of 5 grams/kilogram body weight ethanol) on the 11th day. Control groups were pair fed with the isocaloric control diet.

Mouse HSCs and hepatocytes isolated from livers of acute ethanol feeding in 12-week-old female mice (single binge oral gavage of 5 g/kg body weight ethanol) were provided by M.L.T. The protocols for ethanol feeding were approved by the Institutional Animal Care and Use Committee of the Cedars-Sinai Medical Center. Animals were treated humanely, and all procedures were compliant with the institution's guidelines for the use of laboratory animals. Protein extracts and paraffin-embedded slides from 10-day-old BDL mice livers and the sham controls were a kind gift from Dr. Heping Yang (Cedars-Sinai Medical Center).

Human Liver Specimens

Three normal human and three alcoholic hepatitis liver samples were processed and provided by Dr. Zhaoli Sun through the National Institute on Alcohol Abuse and Alcoholism—supported Clinical Resource for Alcoholic Hepatitis Investigations at Johns Hopkins University (Baltimore, MD). The procedure for collecting tissues from explanted livers or biopsy specimens from donor livers was approved by Institutional Review Boards at Johns Hopkins Medical Institutions (IRB00107893 and IRB00021325). All samples were coded, and private information of patients or specimens was not individually identifiable. Briefly, cut tissue samples (<0.5 cm in at least one dimension and submerged) from explanted livers in patients with severe alcoholic hepatitis during liver transplantation or wedge biopsy specimens from the donor livers (normal control) were snap frozen in liquid nitrogen and stored at -80°C . Three deidentified normal and three ALD liver tissues (pathologically alcoholic cirrhosis) were provided by the Kansas University Medical Center Liver Center tissue bank.

Plasmid Vectors and siRNA

AKAP12 overexpression vector (pReceiver-M02-CMV-*AKAP12*) and empty vector (pReceiver-M02) were purchased from Genecopoeia (Rockville, MD). Silencer Select siRNAs for *AKAP12* and the negative control were purchased from ThermoFisher Scientific (Rockford, IL).

Transient Transfection Assays

Expression vectors (1 μg) were transfected into quiescent HSCs on Matrigel (0.3×10^6 cells) in a 6-well plate format for 65 hours using the JetPrime reagent, according to the

manufacturer's protocol (Polyplus Transfection, New York, NY). Reverse transfection of siRNAs was performed in HSCs (0.3×10^6 cells) plated on 6-well plates for 65 hours using the RNAiMAX (Invitrogen, Carlsbad, CA), as described.¹⁹ The siRNA transfection efficiency of Lipofectamine RNAiMax in cells was determined by the BLOCK-iT Alexa Fluor Red Fluorescent Oligo protocol (Invitrogen), as previously described.²⁷ Ethanol cotreatment was performed during the last 48 hours of transfection.

Phospho-Proteomics Analysis

Phosphorylated protein from control/ethanol-treated human HSCs or quiescent (day 0)/culture-activated (day 7) human HSCs was purified from the unphosphorylated fraction using the phospho-protein isolation kit (Qiagen, Valencia, CA), as described.¹⁹ Enriched phospho-peptides were subjected to mass spectrometry using the services of the proteomics core at University of Southern California.

Quantitative PCR

Total RNA from cells was isolated using the RNA isolation kit (Zymo Research, Irvine, CA), and total RNA from tissue was isolated using the Trizol reagent, according to the manufacturer's protocol (ThermoFisher Scientific). RNA reverse transcribed using M-MLV RT (Invitrogen) was subjected to quantitative RT-PCR using TaqMan probes for human *AKAP12*, mouse *Akap12*, housekeeping genes, *HPRT1* (human), and *Gapdh* (mouse) (ABI, Foster City, CA).^{27,28} The thermal profile consisted of the following: initial denaturation: 95°C for 3 minutes, 45 cycles; 95°C , 3 seconds; 60°C , 30 seconds. The C_T value of the target genes was normalized to that of control gene to obtain the ΔC_T . The ΔC_T was used to find the relative expression of target genes, according to the following formula: relative expression = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ of target genes in experimental condition – ΔC_T of target gene under control condition.²⁸

Western Blotting

Total protein from cells or tissues was prepared using EDTA-free radioimmunoprecipitation assay buffer containing protease inhibitor cocktail and phosphatase inhibitors (Sigma). It was separated on SDS-PAGE following standard protocols (Amersham BioSciences, Piscataway, NJ). The antibodies used in this study are listed in Table 1. Western blots were quantified by densitometry using the ImageJ densitometry program (NIH).

Coimmunoprecipitation

Coimmunoprecipitation of proteins from cells or tissue extracts was performed, as published by us previously.¹⁹ For the coimmunoprecipitation analysis in this study, 500 μg of

Table 1 Antibodies Used in This Study

Name	Citation (PubMed ID)	Supplier (location)	Catalog number	Clone number
Anti-AKAP12 antibody	25188285	Abcam (Cambridge, MA)	ab49849	JP74
Antiphosphoserine antibody	19383294	Abcam	ab9332	Polyclonal IgG
Antiphosphotyrosine antibody	26310847	Abcam	ab9319	Polyclonal IgG
Anti- α -smooth muscle actin antibody	20043323	Abcam	ab5694	Polyclonal IgG
Anti-PKC α antibody		Genetex (Irvine, CA)	GTX130453	Polyclonal IgG
Anti-cyclin D1 antibody	25956904	Abcam	Ab10540	DCS-6
Anti-collagen I α (1) antibody	25294683	Novus Biologicals (Littleton, CO)	NB600-450	COL-1
Anti-heat shock protein 47 antibody	10811611	Novus Biologicals	MAB9166-100	950806
Anti-phospho-p44/p42 MAPK (ERK1/2) (Thr202/Tyr204) antibody	28220783	Cell Signaling Technologies (Danvers, MA)	9101	Polyclonal IgG
Anti- β -actin—peroxidase antibody (control for <i>in vitro</i> experiments)	27831566	Sigma (St. Louis, MO)	A3854	AC-15
Anti-GAPDH antibody (control for <i>in vivo</i> experiments)	28761447	Cell Signaling Technologies	2118	14C10
Anti-desmin antibody	23308088	Dako (Agilent; Santa Clara, CA)	M0760	D33

AKAP, A-kinase anchoring protein; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ID, identification; MAPK, mitogen-activated protein kinase; PKC, protein kinase C.

extract was incubated with 2 μ g of antibody (Table 1) under cold conditions and then processed for Western blotting.

Phos-tag Assays

Phos-tag acrylamide (Wako Chemicals, Richmond, VA) provides a phosphate affinity SDS-PAGE for mobility shift detection of phosphorylated proteins.²⁹ Manganese-Phos-tag gels (15 μ mol/L) were prepared for separation of high-molecular-weight AKAP12 from its phospho-protein using a 3% (w/v) polyacrylamide gel strengthened with 0.5% (w/v) agarose, following a published protocol.²⁹ Extracts treated with alkaline phosphatase (ALP; Sigma) were used as a negative control to prove that the shifted bands were phosphorylated. Extract (50 μ g) was treated with 50 U of ALP for 1 hour at 30°C. Samples were mixed with 6 $\times$ SDS-loading dye and heated at 95°C for 5 minutes before loading onto gel.

Vitamin A Autofluorescence

UV-excited autofluorescence of human HSCs at 330 nm was captured by fluorescence microscopy, as described previously.³⁰ Areas of fluorescent cells were measured by the basic intensity quantification method from the ImageJ software version 1.50i (NIH).

Duolink PLA

Phosphorylation of AKAP12 in paraffin-embedded tissue sections (5 μ m thick) from control/ethanol livers or control/BDL livers was performed by the Duolink proximity ligation assay (PLA) kit (Sigma). AKAP12 or phospho-serine (P Ser) antibody (Table 1) was conjugated to PLA minus or plus complementary oligonucleotide arms (PLA minus, catalog

number DUO92010; PLA plus, catalog number DUO92009) using the conjugation buffer provided in the kit. Deparaffinized liver tissue sections after serial hydration were antigen retrieved by heating in tris-EDTA buffer. Tissue sections were then permeabilized with tris-buffered saline containing 0.5% Triton X-100 for 30 minutes at room temperature and washed further with tris-buffered saline.³¹ Slides were blocked with the Duolink kit blocking buffer and then incubated with a mixture of AKAP12 (minus)— and P Ser (Plus)—conjugated antibodies in the PLA probe diluent solution at a 1:100 dilution containing 1.25 μ g antibody overnight at 4°C. Sections showing positive AKAP12 phosphorylation were detected when both the AKAP12 and P Ser antibody bound to phospho-AKAP12 together. Because of the close proximity of the antibodies on a single protein, the oligonucleotide arms could be ligated and amplified, following the PLA kit red detection protocol (catalog number DUO92008). Stained sections were mounted with VECTASHEILD mounting medium (Vector Laboratories Inc., Burlingame, CA) containing DAPI. Images were acquired as bright red spots of amplified signal from phospho-AKAP12 using a Keyence BZ-X710 inverted fluorescent microscope (Itasca, IL). For costaining of PLA signals with desmin-positive HSCs in the liver, antigen-retrieved sections were blocked with 5% goat serum at room temperature for 10 minutes and incubated with desmin antibody (Table 1) for 16 hours at 4°C, followed by secondary antibody, anti-mouse Alexa Fluor 488 (green fluorescence; Abcam, Cambridge MA). Then, slides were further incubated with PLA antibodies, and images were acquired as described earlier in this paragraph.

Two-Step Chemical Cross-Linking/Mass Spectrometry

Binding partners of AKAP12 in human HSCs were identified using a two-step chemical cross-linking procedure, followed

Figure 1 Phospho-proteomics analysis reveals AKAP12 phosphorylation in hepatic stellate cells (HSCs) during profibrogenic stimulation or culture activation. **A:** Phospho-protein from control or ethanol-treated HSCs was analyzed by mass spectrometry, as described in *Materials and Methods*. **B:** Phospho-protein from quiescent and activated HSCs was analyzed by mass spectrometry, as described in *Materials and Methods*. **White boxes (A and B)** indicate the protein fraction containing AKAP12 unique phospho-peptides. $n = 3$ control (**A**); $n = 9$ ethanol (**A**); $n = 7$ quiescent (**B**); $n = 45$ activated (**B**).

by mass spectrometry. AKAP12 antibody was cross-linked on protein A/G beads using the Pierce Crosslink Immunoprecipitation Kit (ThermoFisher Scientific), following a published protocol.³² Briefly, AKAP12 antibody was immobilized on protein A/G beads using disuccinimidyl suberate and allowed to bind to HSC extracts containing AKAP12 and its interacting proteins. Subsequently, dithiois[succinimidyl]propionate, a

cleavable cross-linker, was introduced to produce stable complexes between the interacting proteins. The cross-linked proteins were eluted, according to the manufacturer's instructions (ThermoFisher Scientific), and submitted to the mass spectrometry and biomarker discovery core service at Cedars-Sinai Medical Center for proteomics analysis.

Statistical Analysis

Data are expressed as means $\pm$ SEM from at least three experiments. Quantitative PCR and densitometric analysis (as shown with each Western blot) were expressed as fold over controls. Statistical analysis was performed using analysis of variance, followed by *t*-test. Significance was defined as $P < 0.05$.

Results

Phospho-Proteomics Analysis Reveals AKAP12 Phosphorylation in HSCs during Profibrogenic Stimulation or Culture Activation

The preliminary work on phospho-proteome analysis of ethanol-treated HSCs (*Figure 1A*) and culture-activated HSCs (*Figure 1B*) revealed a high number of AKAP12 phospho-peptides compared with controls. These results were later confirmed by Phos-tag—Western blotting.

Ethanol- or BDL-Mediated Liver Injury Induces AKAP12 Phosphorylation in Mice

Ethanol administration in mice inhibited the expression of both Akap12 mRNA and unphosphorylated AKAP12 protein (*Figure 2A*) by 70% compared with pair-fed controls. However, ethanol feeding was associated with an 11-fold induction in AKAP12 phospho-protein and a 32-fold increase in the AKAP12 phospho-protein/AKAP12 ratio compared with controls (*Figure 2A*). Phos-tag results were further confirmed by PLA staining, showing high levels of AKAP12 phosphorylation in ethanol livers compared with control (*Figure 2B*). Also, colocalization of desmin-positive HSCs with PLA signals of phospho-AKAP12 was observed in ethanol-treated livers (*Figure 2C*). Control livers also exhibited desmin-positive HSC staining, but colocalization with phospho-AKAP12 was not detected (*Figure 2C*). Unlike ethanol exposure, other modes of injury, such as BDL, slightly increased Akap12 mRNA (twofold) (*Supplemental Figure S1*) and unphosphorylated AKAP12 protein (1.23-fold) (*Supplemental Figure S1*) compared with sham control. However, similar to ethanol, BDL drastically induced AKAP12 phosphorylation by sevenfold compared with sham livers (*Supplemental Figure S1*).

Figure 2 Ethanol induces AKAP12 phosphorylation in mice. **A:** Control or ethanol-fed livers were subjected to Akap12 quantitative PCR (left panel) or AKAP12 Phos-tag analysis (right panel) (Materials and Methods). **B:** Tissue slides from the described experiment were processed for proximity ligation assay (PLA) staining, as described in Materials and Methods. The data are representative images from four preparations. **Arrows** indicate the PLA-amplified signal from phospho-AKAP12 (P-AKAP12). **C:** Tissue sections, as in **B**, were first stained with desmin antibody and overlaid with PLA antibodies, as described in Materials and Methods. Data from three control and three ethanol tissues are shown. **Red arrows** indicate cells showing colocalization of phospho-AKAP12 with desmin; **white arrows**, cells showing desmin staining alone. Data are expressed as means $\pm$ SEM (**A**). $n = 9$ ethanol-fed and control livers (**A**). **** $P < 0.001$ versus pair-fed control. Original magnification: $\times 20$ (**B** and **C**, main images); $\times 40$ (**B** and **C**, insets). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

AKAP12 Phosphorylation Is Induced in Human Alcoholic Livers

Human liver tissues from alcoholic hepatitis subjects or ALD subjects (alcoholic cirrhosis) exhibited a 70% to 80% reduction in unphosphorylated AKAP12 protein compared with corresponding normal, without a significant change in AKAP12 mRNA (Figure 3). This was

associated with a 10- to 22-fold induction in AKAP12 phosphorylation (AKAP12 phospho-protein) and a 25- to 100-fold increase in the AKAP12 phospho-protein/AKAP12 ratio compared with normal livers (Figure 3). Alcoholic hepatitis livers or ALD livers exhibited a five-fold to sixfold increase in α -smooth muscle actin (α -SMA) protein, a marker of HSC activation, compared with normal livers (Figure 3).

Figure 3 AKAP12 phosphorylation (P-AKAP12) is induced in human alcoholic livers. **A:** Livers from normal or alcoholic hepatitis subject were analyzed by AKAP12 quantitative PCR (**left panel**) or Phos-tag gels for AKAP12 phosphorylation (**right panel**). Western blotting of α -smooth muscle actin (α -SMA) was used as a marker for hepatic stellate cell activation. **B:** Normal or alcoholic liver disease (ALD) subject livers that were pathologically identified as alcoholic cirrhosis were processed as in **A**. Data are expressed as means $\pm$ SEM (**A** and **B**). $n = 3$ alcoholic hepatitis/normal samples in duplicate (**A**) and ALD and normal samples in duplicate (**B**). * $P < 0.05$ versus normal. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Ethanol Inhibits the Expression of AKAP12 in Both HSCs and Hepatocytes but Specifically Induces AKAP12 Phosphorylation in HSCs

Ethanol exposure inhibited the expression of AKAP12 mRNA in human HSCs and hepatocytes by 30% compared with control (Figure 4A). *Akap12* mRNA was inhibited by 30% to 60% in mouse HSCs and hepatocytes treated with ethanol compared with control (Figure 4A). Ethanol inhibited the expression of AKAP12 protein by 60% in human HSCs, human hepatocytes, mouse HSCs, and mouse hepatocytes compared with control (Figure 4B). Phos-tag analysis of total protein from human HSCs revealed that ethanol exposure strongly induced the phospho-shift of AKAP12 (Figure 4C). Ethanol-treated HSC extracts treated with ALP were used as a negative control to prove that the shifted bands were phosphorylated (Figure 4C). Compared with human HSCs, primary human hepatocytes did not exhibit an AKAP12 phospho-shift on ethanol exposure compared with control cells and ethanol-treated ALP

samples (Figure 4C). Coimmunoprecipitation with P-Ser antibody revealed that ethanol induced the phospho-serine phosphorylation of AKAP12 by 2.37-fold compared with control (Figure 4D), thereby inducing the ratio of P-Ser-AKAP12/AKAP12 by fourfold compared with control (Figure 4D). The changes in tyrosine phosphorylation appeared to be variable among different HSC preparations treated with ethanol and were not significantly different compared with control (data not shown). The described *in vitro* results were further confirmed by isolating HSCs, hepatocytes, and Kupffer cells from mice fed with a single acute binge dose of ethanol. Both HSCs and hepatocytes from ethanol livers exhibited a decrease in expression of AKAP12, but only ethanol liver HSCs showed induction of AKAP12 phosphorylation compared with control (Supplemental Figure S2). Kupffer cells did not exhibit any expression of AKAP12 (Supplemental Figure S2). Apart from ethanol, other modes of *in vitro* HSC activation and the metabolite of ethanol, acetaldehyde, induced AKAP12 phosphorylation but had differential effects on its

Figure 4 Ethanol inhibits the expression of AKAP12 in both hepatic stellate cells (HSCs) and hepatocytes but specifically induces AKAP12 phosphorylation (P-AKAP12) in HSCs. Quiescent HSCs or differentiated hepatocytes on Matrigel were treated with ethanol (*Materials and Methods*). **A:** Quantitative PCR was performed using probes for human AKAP12, human hypoxanthine phosphoribosyltransferase 1 (HPRT1), mouse Akap12, and mouse glyceraldehyde-3-phosphate dehydrogenase. **B:** Total protein was immunoblotted with AKAP12 antibody (*Materials and Methods*). **C:** Human HSC or hepatocyte protein was analyzed by Phos-tag (*Materials and Methods*). A representative Phos-tag gel from three preparations is shown. **D:** Control or ethanol-treated human HSC protein was immunoprecipitated with phosphoserine (Pser) antibody, followed by Western blotting for AKAP12. Data are expressed as means $\pm$ SEM (**A**, **B**, and **D**). $n = 3$ preparations (**A** and **B**); $n = 4$ experiments (**D**). * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.001$ versus control. ALP, alkaline phosphatase; IP, immunoprecipitation; WB, Western blotting.

expression. Culture activation of primary human HSCs at day 7 caused a moderate induction in total AKAP12 protein, as reported previously in rat HSCs,²¹ and a major induction in phospho-AKAP12 (Supplemental Figure S3A). PDGF, a potent mitogen for HSCs, induced both phospho-serine and phospho-tyrosine phosphorylation of AKAP12 in HSCs but did not affect its total expression (Supplemental Figure S3B). Like ethanol, acetaldehyde induced the phospho-form of AKAP12 in human HSCs but not in hepatocytes compared with respective controls and ALP-treated samples (Supplemental Figure S4). However, no effect of acetaldehyde was found on the expression of the unphosphorylated AKAP12 protein in human HSCs (Supplemental Figure S4). Hence, AKAP12 phosphorylation was induced during HSC activation regardless of the stimulus: ethanol or acetaldehyde exposure, culture activation, or PDGF treatment.

Scaffolding Activity of AKAP12 toward PKC α and CCND1 Is Reduced in Ethanol-Treated Mice and in Ethanol-Treated Primary Mouse HSCs *in Vitro*, but Not Hepatocytes

The activity of AKAP12 to scaffold PKC α and CCND1 has been demonstrated in recombinant pull-down assay systems, transfected 293T cells, and NIH3T3 fibroblasts.^{13,15} It was examined whether ethanol influenced AKAP12's scaffolding ability toward these known proteins in the liver. Ethanol-fed mice exhibited a 72% and 58% decrease in AKAP12-PKC α and AKAP12-CCND1 interaction, respectively, compared with controls (Figure 5A). Mouse HSCs treated with ethanol exhibited a 65% decrease in AKAP12's interaction with PKC α and CCND1, without a change in the total expression of these proteins compared with control (Figure 5B). AKAP12

Figure 5 AKAP12’s scaffolding activity toward protein kinase C (PKCα) and cyclin-D1 (CCND1) is reduced in ethanol-treated mice and in *in vitro* ethanol-treated primary mouse hepatic stellate cells (HSCs) but not in hepatocytes. HSCs, hepatocytes, or total liver was subjected to immunoprecipitation (IP) with AKAP12 antibody, followed by Western blotting (WB) for PKCα or CCND1 (*Materials and Methods*). **A:** Coimmunoprecipitation of control or ethanol-fed mouse livers. **B:** Coimmunoprecipitation of control or ethanol-treated mouse HSCs. **C:** Coimmunoprecipitation of control or ethanol-treated mouse hepatocytes. Data are expressed as means ± SEM (**A–C**). *n* = 4 experiments (**A**); *n* = 3 HSC preparations (**B**) and hepatocyte preparations (**C**). **P* < 0.05, *****P* < 0.001 versus pair-fed control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

interacted with PKCα and CCND1 in normal mouse hepatocytes but did not exhibit any change in its interaction with these proteins on ethanol exposure (Figure 5C).

AKAP12’s Scaffolding Activity toward PKCα and CCND1 Is Reduced in Alcoholic Hepatitis Livers and in *in Vitro* Ethanol-Treated Primary Human HSCs, but Not Hepatocytes

It was further examined whether AKAP12’s scaffolding ability for PKCα and CCND1 was altered in alcoholic hepatitis subject livers. The AKAP12-PKCα and AKAP12-CCND1 interaction was reduced by 43% and 53%, respectively, in alcoholic hepatitis livers compared with normal (Figure 6A). Similar to mouse HSCs (Figure 5B), primary human HSCs treated with ethanol exhibited a 65% decrease in AKAP12’s interaction with PKCα and CCND1, without a change in the total expression of these proteins compared with control (Figure 6B). Similar to mouse hepatocytes (Figure 5C), AKAP12 interacted with PKCα and CCND1 in primary

human hepatocytes but did not exhibit any change in its interaction with these proteins on ethanol exposure (Figure 6C).

AKAP12 Negatively Regulates Activation of Normal Human HSCs but Promotes Ethanol-Mediated HSC Activation in These Cells

So far, the results have suggested that ethanol deregulates AKAP12 by phosphorylation and alters its scaffolding activity in HSCs but not in hepatocytes. The role of AKAP12 in normal HSCs under basal and ethanol-treated conditions was further examined. These results demonstrated that a 77% decrease of AKAP12 protein was caused by silencing increased HSC activation, as detected by activation marker expression (3.5-fold increase in collagen and 1.5-fold increase in α-SMA compared with negative control) (Figure 7A). Ethanol exposure increased collagen and α-SMA levels in normal HSCs; however, AKAP12 silencing inhibited the ethanol-mediated induction of HSC

Figure 6 AKAP12’s scaffolding activity toward protein kinase C (PKCα) and cyclin-D1 (CCND1) is reduced in alcoholic hepatitis livers and in *in vitro* ethanol-treated primary human hepatic stellate cells (HSCs) but not in hepatocytes. HSCs, hepatocytes, or total liver was subjected to immunoprecipitation (IP) with AKAP12 antibody, followed by Western blotting (WB) for PKCα or CCND1 (*Materials and Methods*). **A:** Coimmunoprecipitation of normal or alcoholic hepatitis subject livers. **B:** Coimmunoprecipitation of control or ethanol-treated human HSCs. **C:** Coimmunoprecipitation of control or ethanol-treated human hepatocytes. Data are expressed as means ± SEM (**A–C**). *n* = 3 experiments in duplicate (**A**), HSC preparations (**B**), and hepatocyte preparations (**C**). **P* < 0.05, ****P* < 0.005 versus control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

activation markers (Figure 7A). Consistent with the silencing results, a threefold induction in AKAP12 protein overexpressing the *AKAP12* vector caused a dramatic 70% to 80% decrease in the basal level of collagen and α-SMA but sustained the expression of HSC activation markers on ethanol exposure (Figure 7B). It was also tested whether vitamin A autofluorescence, a marker of HSC quiescence, was altered by AKAP12. *AKAP12* vector induced vitamin A autofluorescence in normal HSCs by twofold, whereas ethanol inhibited it by 70%, as reported previously³³ (Figure 7C). However, *AKAP12* overexpression was unable to completely reverse the decrease of vitamin A autofluorescence caused by ethanol (Figure 7C). These results indicate that in normal HSCs, AKAP12 negatively regulates HSC activation and maintains HSC quiescence but acquires a proactivation capability on exposure to ethanol.

Ethanol Exposure Inhibits the Interaction of AKAP12 with the Collagen Chaperone, HSP47, in Human HSCs

To decipher the mechanism by which AKAP12 may regulate HSC activation, chemical cross-linking/coimmunoprecipitation and proteomics were performed to identify the interacting partners of AKAP12 in human HSCs. AKAP12 exhibited high-scoring interactions with a family of collagens and the collagen chaperone (HSP47) (Table 2) as well as known kinases, such as PKCα,^{13,14} and some novel kinases, such as death-associated protein kinase 1. The proteomics analysis was confirmed by coimmunoprecipitation of AKAP12 with HSP47 in HSCs (Figure 8A). Ethanol exposure inhibited AKAP12-HSP47 interaction by 70% and concomitantly increased collagen-HSP47 interaction by twofold (Figure 8A). Ethanol treatment increased basal collagen expression but did not alter total HSP47 level

Figure 7 AKAP12 negatively regulates activation of normal human hepatic stellate cells (HSCs) but promotes ethanol-mediated HSC activation in these cells. **A:** HSCs were transfected with AKAP12 or negative control siRNA (*Materials and Methods*). AKAP12 quantitative PCR and Western blotting for collagen, α -smooth muscle actin (α -SMA), or AKAP12 was performed. **B:** HSCs were transfected with AKAP12 vector or empty vector (EV; *Materials and Methods*). AKAP12 quantitative PCR and Western blot for collagen, α -SMA, or AKAP12 was performed. **C:** Human HSCs on Matrigel were transfected as in **B** and were visualized under bright-field or UV autofluorescence, as described in *Materials and Methods*. Representative images and ImageJ fluorescence quantification are shown. Data are expressed as means $\pm$ SEM (**A** and **B**). $n = 5$ experiments (**A** and **B**); $n = 3$ experiments (**C**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, and **** $P < 0.001$ versus negative control siRNA; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ versus control empty vector; ‡ $P < 0.05$ versus control; § $P < 0.05$ versus ethanol; §§§§ $P < 0.001$ versus AKAP12 vector alone.

(Figure 8A). Because ethanol induced AKAP12 phosphorylation and this is known to regulate its scaffolding ability in different systems,¹² it was examined whether phospho-AKAP12 exhibited altered ability to interact with HSP47. Unphosphorylated AKAP12, which normally decreases on ethanol exposure (Figure 1), exhibited a 70% decrease in binding to HSP47 compared with control HSCs that showed strong binding (Figure 8B). However, both basal phospho-AKAP12 and ethanol-induced phospho-AKAP12 exhibited a dramatic (70% to 80%) decrease in HSP47 binding compared with control (Figure 8B). This decrease in binding was evident despite a 6.9-fold induction of phospho-AKAP12 in the ethanol-treated HSCs compared with control (Figure 8B). HSP47 also immunoprecipitated less AKAP12 from total liver of ethanol-fed mice (60% decrease

compared with control) and alcoholic hepatitis subjects (95% decrease compared with normal) (Figure 8, C and D).

AKAP12 Regulates the Ability of Collagen to Interact with HSP47

AKAP12 silencing by 62% in normal HSCs increased the interaction of collagen with HSP47 by twofold compared with negative control siRNA (Figure 9A). Consistently, a twofold overexpression of AKAP12 caused an 80% decrease in collagen-HSP47 interaction compared with negative control (Figure 9B). These results show that AKAP12 negatively regulates collagen’s interaction with HSP47 in HSCs.

Table 2 Proteins Cross-Linked to AKAP12 in Normal HSCs

Accession	Description	Score	Sequence coverage, %	Unique peptides, <i>n</i>	MW, kDa
P02452	Collagen α -1(I) chain (GN= <i>COL1A1</i> human)	101.68	17.01	9	138.9
P50454	Heat shock protein 47 (GN= <i>SERPINH1</i> human)	104.51	32.78	11	46.4
P02461	Collagen α -1(III) chain (GN= <i>COL3A1</i> human)	86.31	12.07	8	138.5
P17252	Protein kinase C α type (GN= <i>PRKCA</i> human)	10.29	6.57	12	73.0
Q6PJY1	Far upstream element-binding protein (GN= <i>KHSRP</i> human)	11.57	7.24	2	32.0
B7ZLE7	Death-associated protein kinase 1 (GN= <i>DAPK1</i> human)	8.97	8.2	5	152.4

AKAP12 antibody was cross-linked to beads, and HSC extracts were immunoprecipitated to identify AKAP12 binding partners (*Materials and Methods*). Samples of cross-linked proteins were submitted for proteomics. Unique proteins with high score that interacted with AKAP12 were identified. Score indicates the sum of the ion scores of all peptides that were identified; sequence coverage, the percentage of the protein sequence covered by identified peptides; and unique peptides, the number of peptides unique to a protein group.

AKAP, A-kinase anchoring protein; GN, gene name; HSC, hepatic stellate cell; MW, molecular weight.

Discussion

AKAP12 is a scaffold protein for PKC and CCND1 that can alter the localization of these targets and, hence, control important processes, such as cytokinesis, cell proliferation, and cell migration.¹² It has been known for some time that site-specific phosphorylation of AKAP12 alters its scaffolding activity toward PKC α , CCND1, polo-like kinase, and F-actin.^{12,16,17}

The role of AKAP12 scaffolding activity and post-translational modifications in liver function is undescribed so far. In general, it is known that ethanol can alter

scaffolding-associated signaling in cells.³⁴ The goal of this work is to understand the role of AKAP12 in normal liver function and during profibrogenic stimulation caused by ethanol exposure. It was previously reported by You et al²¹ that AKAP12 expression is induced during rat HSC activation and is required for it. Their findings showed that preactivated HSCs treated with AKAP12 siRNA exhibited a decrease in HSC activation, but the molecular mechanism was not deciphered.²¹ During a phospho-proteomics analysis of primary human HSCs treated with ethanol or culture-activated human HSCs, a high number of unique AKAP12 phospho-peptides were identified compared with the

Figure 8 Ethanol exposure inhibits the interaction of AKAP12 with the collagen chaperone, heat shock protein 47 (HSP47), in hepatic stellate cells (HSCs). **A:** Control or ethanol-treated HSCs were immunoprecipitated (IP) with AKAP12 or collagen and then Western blotted (WB) for HSP47 or collagen (*Materials and Methods*). **B:** IP blot: Phosphorylated and unphosphorylated fractions of control or ethanol-treated HSCs were purified using Qiagen columns (*Materials and Methods*) and subjected to immunoprecipitation with HSP47 antibody, followed by Western blotting for AKAP12. Phos-tag blot: AKAP12 phosphorylated (P-AKAP) and unphosphorylated Qiagen fractions from control or ethanol-treated HSCs were compared on Phos-tag gels. Phosphorylated extracellular signal-regulated kinase (p-ERK) was a positive control and is found mainly in the phosphorylated fraction. **C:** Control or ethanol-fed mouse livers were immunoprecipitated with HSP47 antibody followed by Western blotting for AKAP12. Three experiments are shown. **D:** Normal or alcoholic hepatitis liver extracts were immunoprecipitated with HSP47 antibody, followed by Western blotting for AKAP12. Experiments were performed in duplicate. Data are expressed as means $\pm$ SEM (**A–D**); *n* = 5 experiments (**A** and **C**); *n* = 3 experiments (**B** and **D**). **P* < 0.05, ***P* < 0.01 versus control; †*P* < 0.05, ††*P* < 0.01, and †††*P* < 0.001 versus unphosphorylated control; ††††*P* < 0.001 versus pair-fed control; †††††*P* < 0.001 versus normal. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 9 AKAP12 regulates the ability of collagen to interact with heat shock protein 47 (HSP47). **A:** Hepatic stellate cells (HSCs) transfected with AKAP12 or negative control siRNA immunoprecipitated (IP) with collagen antibody, followed by HSP47 immunoblotting. **B:** HSCs transfected with AKAP12 vector or empty vector control were immunoprecipitated with collagen antibody, followed by HSP47 Western blotting (WB). Data are expressed as means ± SEM (**A** and **B**). $n = 5$ experiments (**A** and **B**). * $P < 0.05$, **** $P < 0.001$ versus negative control; †† $P < 0.01$, †††† $P < 0.001$ versus empty vector.

respective controls. Because AKAP12 phosphorylation has been associated with deregulation of important cellular functions,¹² we hypothesized that AKAP12 phosphorylation may also play a role in profibrogenic stimulation in the liver and HSCs may be an important site of this post-translational modification.

The expression and phosphorylation of AKAP12 was first examined in alcohol-fed mice, human alcoholic hepatitis, and ALD subject livers. The ethanol+binge-feeding mice used in this study exhibit liver injury, inflammation, and fatty liver, and mimic acute-on-chronic alcoholic liver injury in patients. However, long-term fibrosis is not observed. In this early event of injury, high AKAP12 phosphorylation, associated with an overall decrease in AKAP12 mRNA and protein, was found. AKAP12 phosphorylation is also high in alcoholic hepatitis subjects who exhibit increased HSC activation (high α -SMA levels). Liver injury caused by 10-day BDL caused a small increase in AKAP12 expression but highly induced AKAP12 phosphorylation in the liver. In the carbon tetrachloride model of liver injury, an induction in AKAP12 expression has been reported.²¹ Different

modes of injury have differential effects on AKAP12 expression: ethanol is inhibitory, whereas BDL/carbon tetrachloride causes induction.²¹ However, these results strongly suggest that regardless of the mode of liver injury and its effect on AKAP12 expression, there is a major induction in AKAP12 phosphorylation.

Given access to both ethanol-fed mouse models and human ALD tissue to examine AKAP12 phosphorylation, this work was focused mainly on ethanol as a mediator of this effect. We examined which cells of the liver may respond to ethanol-mediated AKAP12 phosphorylation. Both *in vitro* cultured primary hepatocytes and HSCs (mouse and human) expressed AKAP12, as previously reported,²¹ and ethanol exposure inhibited both the mRNA and protein of AKAP12. Hence, AKAP12 may be transcriptionally and/or post-transcriptionally regulated in liver cells. Interestingly, ethanol caused a major increase in the phosphorylation of AKAP12 in HSCs but not in hepatocytes. These *in vitro* results were further confirmed by performing *in vivo* single binge feeding of ethanol in mice. This also mildly increased AKAP12 phosphorylation in HSCs but not in hepatocytes of the liver. Ethanol's effect was compared with other modes of HSC activation, such as plastic culture and treatment with the mitogen, PDGF. Both these modes also resulted in increased AKAP12 phosphorylation. The only difference between these agents was that ethanol inhibited total AKAP12 expression, whereas culture activation slightly induced it and PDGF had no effect. Like ethanol, PDGF treatment increased P_{Ser} phosphorylation in HSCs. Tyrosine phosphorylation was also induced by PDGF (but not by ethanol) in HSCs, and this has previously been shown to decrease AKAP12's scaffolding activity toward F-actin in NIH3T3 cells.¹⁶ The described results suggest that different profibrogenic agents may differentially regulate AKAP12 expression but appear to have a common effect on AKAP12 phospho-induction.

Because AKAP12 did not show any phospho-shift in Phos-tag analysis of ethanol-treated hepatocytes, it appears that HSCs may be the main players exhibiting AKAP12 phosphorylation on ethanol exposure. Among the non-parenchymal cells, AKAP12 is expressed in von Willebrand factor-positive sinusoidal endothelial cells but not in Kupffer cells.²¹ In this work, HSCs and hepatocytes were examined; however, the possibility of sinusoidal endothelial cells exhibiting alterations in AKAP12 phosphorylation on ethanol exposure cannot be ruled out. Because expression of AKAP12 was not observed from Kupffer cells of normal or ethanol-treated livers, these cells were not examined further. Like ethanol, its toxic metabolite, acetaldehyde,¹⁸ also induced AKAP12 phosphorylation in HSCs but not in hepatocytes. Both hepatocytes and HSCs express AKAP12 as well as alcohol-metabolizing enzymes, class I alcohol dehydrogenases, and aldehyde dehydrogenases.¹⁸ The only difference between these cells is that hepatocytes also metabolize alcohol via the microsomal ethanol oxidizing system (namely, cytochrome P4502E1), whereas HSCs do

Figure 10 Summary of mechanisms of action of phospho-AKAP12 in alcoholic liver injury. In normal liver, AKAP12 acts as a scaffold protein for protein kinase C (PKC α) and cyclin-D1 (CCND1). Both hepatocytes and hepatic stellate cells (HSCs) exhibit these functions. Normal HSCs also exhibit specific interaction of AKAP12 with the collagen chaperone, heat shock protein 47 (HSP47), and through this interaction, they might regulate collagen maturation and secretion. During alcoholic liver injury, there is decreased interaction of AKAP12 with PKC α and CCND1 in HSCs because of enhanced AKAP12 phosphorylation and loss of its scaffolding activity. Loss of this scaffolding activity may result in changes in HSC proliferation, cytokinesis, and altered signaling. HSC-specific scaffolding of HSP47 is lost when AKAP12 is phosphorylated on alcohol exposure, and this may facilitate HSP47-collagen chaperoning that may further induce collagen maturation and secretion.

not express cytochrome P4502E1. The overall metabolic potential of hepatocytes is higher than that of HSCs¹⁸; still, alcohol and acetaldehyde preferentially induce AKAP12 phosphorylation in HSCs. Hence, at this point, this effect cannot entirely be linked to altered alcohol metabolism in the two cell types. However, preferential AKAP12 phosphorylation in ethanol-treated HSCs but not hepatocytes may suggest a cell type-specific profibrogenic role of this modification in alcoholic liver. The scaffolding activity data discussed later support this hypothesis.

In vivo, ethanol inhibited the scaffolding activity of AKAP12 for PKC α and CCND1 in the liver of mice and in human subjects. Correspondingly, *in vitro* analysis in primary cells (both mouse and humans) revealed that of the two liver cell types, HSCs exhibited decreased interaction of AKAP12 with PKC α and CCND1 on ethanol exposure, whereas hepatocytes did not exhibit any changes in this interaction. Hence, a positive correlation exists between increased AKAP12 phosphorylation and decreased AKAP12 scaffolding activity in HSCs. Because hepatocytes do not show phosphorylation of AKAP12 on ethanol exposure, this may be a probable reason why AKAP12's scaffolding activity remained unchanged in these cells. Despite no AKAP12 phosphorylation or scaffolding activity change in the hepatocytes, ethanol-treated liver exhibits reduced AKAP12 scaffolding activity and this might be attributed to HSCs and other cell types, such as sinusoidal endothelial cells.

Scaffolding toward PKC α and CCND1 is a general activity of AKAP12 found in HSCs, hepatocytes, and total

liver and in pull-down assays from 293 T-overexpression systems and NIH3T3 fibroblasts.^{13,15} Because AKAP12 was highly phosphorylated in ethanol-treated HSCs, it was examined whether it played any specific function in normal HSCs that were altered by ethanol. Decreasing the level of AKAP12 increased HSC activation, whereas forced expression negatively controlled it. These data suggest that AKAP12 is an antifibrogenic protein that may play a role in maintaining HSC quiescence. Vitamin A autofluorescence examination in quiescent HSCs revealed that ethanol exposure completely reversed the ability of AKAP12 to maintain quiescence and transformed it into a protein with proactivation capabilities. The effect of AKAP12 on suppression of collagen expression was particularly prominent compared with the other activation marker, α -SMA. It was suspected that the ability of ethanol to reverse AKAP12's antifibrogenic properties was linked to its increased phosphorylation, which may have resulted in lower scaffolding activity toward some key components of the HSC machinery.

To unravel the underlying mechanisms of AKAP12's ability to negatively regulate ethanol-mediated HSC activation, chemical cross-linking/proteomics analysis was performed to identify AKAP12 binding partners in HSCs. This revealed an interesting profile of several collagen-binding proteins and collagens. One of these high-scoring interactions is that of the collagen chaperone HSP47 that is expressed only in HSCs and is required for collagen maturation and secretion during HSC activation.^{6–8,35} In HSCs, ethanol exposure induces collagen-HSP47 interaction and

concomitantly inhibits the strong interaction between AKAP12 and HSP47. Moreover, the ethanol-inducible phosphorylated AKAP12 is unable to interact with HSP47 compared with its unphosphorylated form, proving that phosphorylation inhibits the scaffolding activity of AKAP12 toward HSP47. These combined results suggest that loss of AKAP12-HSP47 scaffolding is associated with increased chaperoning of collagen by HSP47. *In vivo*, ethanol fed-mice and alcoholic hepatitis patient livers also exhibited decreased AKAP12-HSP47 interaction compared with controls, even though there are reports that HSP47 expression is induced on moderate ethanol feeding in HSCs and is also high in ALD patients.^{6,36} Further evidence of the role of AKAP12 in modulating the collagen-HSP47 axis comes from the results on AKAP12 silencing/over-expression. Moderate silencing of AKAP12 in HSCs strongly induces the HSP47-collagen interaction, and a small increase in AKAP12 expression strongly inhibits the interaction.

Together, these data support the hypothesis that AKAP12 works as a quencher of HSP47-mediated collagen chaperoning by itself interacting with HSP47. The findings summarized in Figure 10 show that ethanol-mediated phosphorylation of AKAP12 in HSCs inhibits its anti-fibrogenic activity toward HSP47, and this may facilitate collagen chaperoning by HSP47. Cross-linking proteomics also identified PKC α and death-associated protein kinase 1 as interacting kinases for AKAP12. These may play a role in AKAP12 phosphorylation and may possibly regulate the AKAP12-HSP47/collagen scaffold. The overall data indicate that the effect of ethanol on AKAP12 is probably associated with profibrogenic regulation in HSCs, and hepatocytes may not be involved in this effect. However, the possibility that other unknown functions of AKAP12 in hepatocytes may exist that could have an association with ALD and may be investigated in the future cannot be ruled out. Identifying AKAP12 phosphorylation sites and kinases that are modulated by ethanol exposure and may be involved in regulating the AKAP12-HSP47 interaction and HSC activation in general would be the subject of further investigation.

Acknowledgments

We thank the liver research group (Cedars-Sinai Medical Center), headed by Dr. Shelly Lu, for core support; Dr. Bin Gao [National Institute on Alcohol Abuse and Alcoholism (NIAAA)] for scientific advice and samples from ethanol-fed mouse model; the Kansas University Liver Bank for alcoholic liver disease samples; Dr. Hidekazu Tsukamoto (Southern California Research Center for ALPD and Cirrhosis) for scientific advice; Dr. Heping Yang (Cedars-Sinai Medical Center) for providing protein extracts and paraffin-embedded slides from 10-day-old bile duct ligation mice livers and the sham controls; Dr. Zhaoli Sun [NIAAA-

supported Clinical Resource for Alcoholic Hepatitis Investigations at Johns Hopkins University (R24AA025017)] for providing and processing three normal human and three alcoholic hepatitis liver samples; and the Kansas University Medical Center Liver Center tissue bank for providing three deidentified normal and three ALD liver tissues (pathologically alcoholic cirrhosis).

K.R. conceptualized the study, performed experiments, and wrote the manuscript; M.L.T. performed experiments and reviewed the manuscript; J.B. and N.M. performed experiments; Z.S. processed and provided human liver specimens for the study.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.11.017>.

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