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Transcription Factor Forkhead Box O1 Mediates Transforming Growth Factor-β1—Induced Apoptosis in Hepatocytes

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Dysregulation of hepatocyte apoptosis is associated with several types of chronic liver diseases. Transforming growth factor-β1 (TGF-β1) is a well-known pro-apoptotic factor in the liver, which constitutes a receptor complex composed of TGF-β receptor I and II, along with transcription factor Smad proteins. As a member of the forkhead box O (Foxo) class of transcription factors, Foxo1 is a predominant regulator of hepatic glucose production and apoptosis. This study investigated the potential relationship between TGF-β1 signaling and Foxo1 in control of apoptosis in hepatocytes. TGF-β1 induced hepatocyte apoptosis in a Foxo1-dependent manner in hepatocytes isolated from both wild-type and liver-specific Foxo1 knockout mice. TGF-β1 activated protein kinase A through TGF-β receptor I—Smad3, followed by phosphorylation of Foxo1 at Ser273 in promotion of apoptosis in hepatocytes. Moreover, Smad3 overexpression in the liver of mice promoted the levels of phosphorylated Foxo1-S273, total Foxo1, and a Foxo1-target pro-apoptotic gene *Bim*, which eventually resulted in hepatocyte apoptosis. The study further demonstrated a crucial role of Foxo1-S273 phosphorylation in the pro-apoptotic effect of TGF-β1 by using hepatocytes isolated from Foxo1-S273A/A knock-in mice, in which the phosphorylation of Foxo1-S273 was disrupted. Taken together, this study established a novel role of TGF-β1 → protein kinase A → Foxo1 signaling cascades in control of hepatocyte survival. *(Am J Pathol 2023, 193: 1143—1155; https://doi.org/10.1016/j.ajpath.2023.05.007)*

Apoptosis, also known as programmed cell death, has been recognized as a process of genetically determined elimination of cells.[1] In the liver, hepatocytes are the major functional cells to execute metabolic functions.[2] Apoptosis of hepatocytes plays an essential role in removing old or damaged cells during hepatic tissue development and regeneration. Aberrant apoptosis of hepatocytes is associated with adverse effects in the liver. As documented, apoptotic activity is strongly increased in several acute and chronic liver diseases, including viral hepatitis,[3] alcoholic steatohepatitis,[4,5] nonalcoholic steatohepatitis,[6] fibrosis,[7,8] and hepatocellular carcinoma.[9] These observations highlight the mechanistic importance of hepatocyte apoptosis in the pathogenesis of various types of liver injury and diseases.[10]

Given the critical roles of hepatocyte apoptosis in liver functions, pro-apoptotic factors that trigger hepatocyte apoptosis have been characterized, of which include transforming growth factor (TGF)-β1.[11] The canonical TGF-β1 signaling is composed of a ligand-dependent assembly of a receptor complex, including TGF-β receptor I (TβRI) and TGF-β receptor II (TβRII), followed by the receptor kinase

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activation and subsequent phosphorylation of Smad2/3 proteins, which are transcriptional factors regulating the target apoptotic genes.[12] In addition, the TGF-β1 and Smad proteins also increase the production of reactive oxygen species and promote the hepatocyte apoptosis.[13]

As a member of the forkhead box O (Foxo) class of transcription factors, Foxo1 has a dominant role in hepatic glucose production enhancing the transcriptional activity of rate-limited enzymes responsible for gluconeogenesis.[14] Specifically, Foxo1 integrates both insulin and glucagon signaling via phosphorylation at different serine residues. In the feeding liver, when insulin signaling is activated, the phosphorylation/activation of Akt induces Foxo1 phosphorylation at Ser253, followed by Foxo1 ubiquitination and degradation.[15] By contrast, Foxo1 is activated by glucagon signaling via protein kinase A (PKA)−mediated phosphorylation at Ser273, which then promotes Foxo1 nuclear translocation and activates the transcription of target genes.[16]

Interestingly, a role of Foxo1 in control of apoptosis has been reported, suggesting that the transcriptional activity of Foxo1 goes beyond glucose metabolism. The acetylation of Foxo1 or Foxo3 activates the transcription of pro-apoptotic genes, such as *Bim* and *Puma*, in HepG2 cells.[17] Dephosphorylation of Foxo1 at Ser253 by protein phosphatase-1 promotes pro-apoptotic activity of Foxo1 in mouse fibroblasts.[18] Cyclin-dependent kinase 2 phosphorylates Foxo1 at Ser249 in human in response to DNA damage, followed by transcriptional activations of *Bim*, *Fas ligand*, and *TRAIL* in promotion of apoptosis.[19] Conversely, inhibition of Foxo1 protects pancreatic β cells from fatty acid−mediated apoptosis.[20] Moreover, the protein level of Foxo1 is strongly decreased in the liver of Smad3 knockout mice compared with that of wild-type (WT) mice.[21] Following reports of the phosphorylation of Foxo1 by PKA,[16] the current study further investigated whether PKA → Foxo1 signaling axis regulates hepatocyte apoptosis.

The present study examined the role of Foxo1 in TGF-β1−induced apoptosis in hepatocytes as well as the potential molecular mechanisms and tested the hypothesis that Foxo1 mediates the TGF-β1−induced apoptosis in hepatocytes.

Materials and Methods

Animals

Male C57/BL6 mice at the ages of 8 to 12 weeks were used in this study. Liver-specific Foxo1 knockout (L-FKO) mice were generated by the method described previously.[22,23] The floxed Foxo1 (Foxo1$^{L/L}$) mice were used as the WT control of the L-FKO mice. Foxo1-S273A knock-in mice were generated using the clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR/Cas9) approach, as described previously.[16] The Foxo1-S273$^{+/+}$ mice were used as the control of the Foxo1-S273A mice. The β1^{glo} mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were crossed with albumin-Cre mice to generate liver-specific TGF-β1 overexpression mice (L-TGF-β1OE). To generate liver-specific TGF-β1 overexpression in S273A/A mice (L-TGF-β1OE::S273A/A), L-TGF-β1OE mice were crossed with S273A/A mice. Control, S273A/A, L-TGF-β1OE, and L-TGF-β1OE::S273A/A mice at the age of 10 weeks were fed with nonalcoholic steatohepatitis (NASH) diet (Research Diet, New Brunswick, NJ; D17010103) for 5 months. All animal experiments were performed by animal protocols that were approved by Institutional Animal Care and Use Committee at Texas A&M University (College Station, TX).

Cell Isolation and Culture

Primary hepatocytes were isolated and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin-streptomycin, as described previously.[16] Primary hepatocytes were treated with 10 ng/mL of TGF-β1 for 24 hours, followed by subsequent assays. For the experiments using inhibitors, PKA inhibitor H89 (10 μmol/L) or TβRI inhibitor SB431542 (10 μmol/L) was given to cells 0.5 hours before the addition of TGF-β1.

Western Blot Analysis

Cells were lysed in radioimmunoprecipitation assay buffer (500 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mmol/L EDTA, phosphatase inhibitor, protease inhibitor, and 1 mmol/L phenylmethylsulfonyl fluoride). Nuclear and cytoplasmic proteins from primary hepatocytes were isolated by using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Waltham, MA), as described previously.[16] Protein concentration was quantified by bicinchoninic acid assay kit (Thermo Fisher Scientific). The protein sample was separated by SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking in tris-buffered saline with Tween 20 containing 5% bovine serum albumin (Sigma Aldrich, St. Louis, MO), membranes were incubated with primary antibodies indicated below overnight at 4°C. Then, goat anti-rabbit secondary antibody was added (1:5000) for 1-hour incubation at room temperature. The immunoblot signals were developed using Clarity western enhanced chemiluminescence (ECL) blotting substrates (Bio-Rad, Hercules, CA). Antibodies against Foxo1 (1:1000; CST 2880), Smad3 (1:1000; CST 9523), cleaved caspase-3 (1:1000; CST 9664), caspase-3 (1:1000; CST 9662), phosphorylated (p)-PKA substrates (1:1000; CST 9624), cAMP-response element binding protein (CREB) (1:1000; CST 9197), p-CREB-S133 (1:1000; CST 9198), Bim (1:1000; CST 2819), glyceraldehyde-3-phosphate dehydrogenase (1:1000; CST 5174), and histone H3 (1:1000; CST 9715)

were all purchased from Cell Signaling Technology (Danvers, MA). The antibody against p-Foxo1-S273 was generated according to the method previously described.[16]

Real-Time PCR

TRIzol reagent (Thermo Fisher Scientific) was used for total RNA isolation. cDNA was synthesized using the iScript cDNA synthesis Kit (Bio-Rad). mRNA level of each interested gene was measured using SYBR Green supermix (Bio-Rad) with primers previously described[16] by a real-time PCR system (Bio-Rad real-time PCR). Cyclophilin was used as the internal control.

Caspase 3 Activity Assay

Caspase 3 activity was determined by a caspase 3 colorimetric assay kit (Abcam, Cambridge, MA), according to the manufacturer's protocol. Briefly, the cells were lysed in chilled cell lysis buffer, followed by incubation for 10 minutes on ice. Afterward, the cell lysate was centrifuged at 10,000 × *g* for 5 minutes at 4°C. The supernatants were collected for protein quantification using Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific), and protein content of each sample was then adjusted to 100 μg protein per 50 μL cell lysis buffer for each assay. Each sample was incubated with reaction buffer containing 10 mmol/L dithiothreitol and 200 μmol/L aspartyl-glutamyl-valyl-aspartyl-p-nitroanilide (DEVD-p-NA) at 37°C for 90 minutes. Caspase 3 activity was determined by measuring the absorbance at 405 nm.

Transfection of siRNA

siRNA of Smad3 and control nontargeting siRNA were purchased from Thermo Fisher Scientific. Freshly isolated primary hepatocytes were cultured for 3 hours, then transfected with 60 pmol of siRNA for 24 hours by Lipofectamine 3000 (Thermo Fisher Scientific). Scramble siRNA was used as the negative control. Afterward, the cells were treated by 10 ng/mL of TGF-β1 for 24 hours.

Apoptosis Detection

Cell apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining using TACS TdT-Fluor *in situ* apoptosis detection kit (4812 to 30-K; R&D Systems, Minneapolis, MN), according to the manufacturer's protocol. Briefly, the cells were fixed by 70% ethanol for 10 minutes and incubated with proteinase K solution for 30 minutes at room temperature. After two times of washing by deionized water, the cells were incubated with 1× TdT labeling buffer for 5 minutes under room temperature. Then, labeling reaction mix was added to cells for incubation at 37°C for 1 hour. The reaction was ended by adding 1× TdT stop buffer. To elicit the fluorescent signal, the Strep-Fluor solution (R&D Systems) was added to the cells for incubation at room temperature for 20 minutes in the dark. At the end, the cells were viewed under a fluorescent confocal microscope (Leica Biosystems, Wetzlar, Germany).

Smad3 Overexpression

Adenovirus-expressing green fluorescent protein and adenovirus-expressing Smad3 were purchased from VectorBuilder Inc. (Chicago, IL). A total volume of 100 μL phosphate-buffered saline—diluted virus containing 10^9 viral genome copies was injected into the tail vein of the recipient mice (male; aged 15 weeks). Tissues were collected after 3 weeks of the injection. Tissues used for the apoptosis detection were fixed by formalin and embedded in paraffin for tissue sectioning. Hepatic apoptosis was detected by TUNEL the same as described previously.

Statistical Analysis

Data were analyzed by one-way analysis of variance to determine the significance of the model. Differences between groups were determined by the one-tailed unpaired *t* test or the Tukey *post hoc* test for the data set with two groups or more than two groups, respectively. Results are presented as means ± SEM. $P < 0.05$ was considered as statistically significant.

Results

TGF-β1 Regulates Foxo1 Expression and Induces Apoptosis in Hepatocytes

TGF-β1 induces apoptosis in hepatocytes within 24 hours.[24] Herein, the mRNA level of a Foxo1-regulated pro-apoptotic gene *Bim* was increased by 9.01-fold by the TGF-β1 treatment (Figure 1A), which suggested the involvement of Foxo1 in TGF-β1—induced apoptosis in primary hepatocytes.

A study into the role of Foxo1 in the TGF-β1 signaling indicated that the protein level of total Foxo1 was significantly increased by 2.25-fold on the 24-hour TGF-β1 treatment (Figure 1B). However, the mRNA level of Foxo1 was unaffected by the TGF-β1 treatment (Figure 1A), which suggested that the regulatory role of TGF-β1 in Foxo1 activation is at the protein translation level.

The stability of Foxo1 is enhanced by PKA signaling through the phosphorylation at Ser273.[16] Therefore, the level of p-Foxo-S273 was examined in the hepatocytes after TGF-β1 treatment. TGF-β1 increased the p-Foxo1-S273 level by 1.33-fold (Figure 1B), indicating that the regulation of Foxo1 by TGF-β1 occurs via the phosphorylation at Ser273.

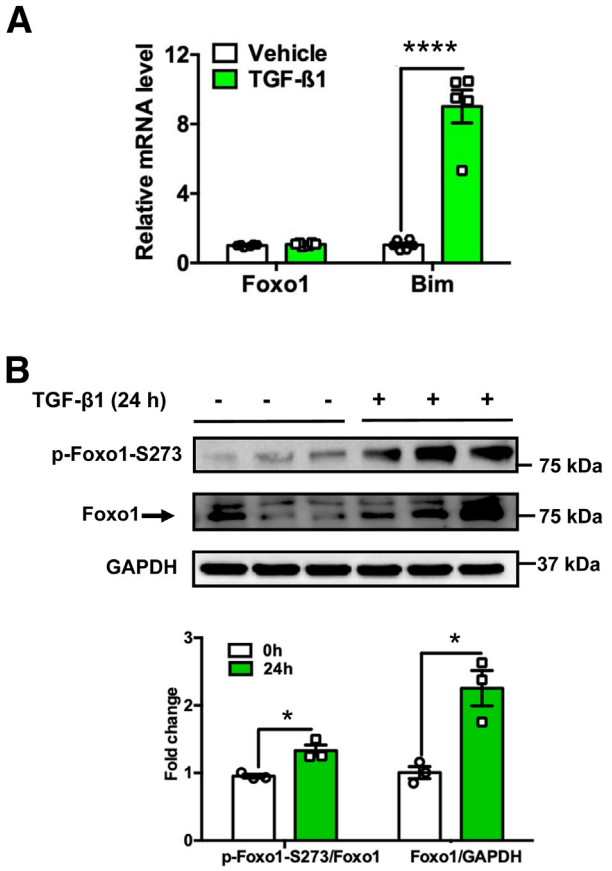

Figure 1 Transforming growth factor (TGF)-β1 activates forkhead box 01 (Foxo1) and induces apoptosis in primary hepatocytes. Primary hepatocytes isolated from the wild-type control mice were treated by 10 ng/mL of TGF-β1 for 24 hours. **A:** mRNA levels of Foxo1 and Bim were analyzed by real-time quantitative PCR. Cyclophilin was used as the loading control. **B:** Protein levels of total Foxo1 and phosphorylated (p)-Foxo1-S273 were analyzed by Western blot analysis and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are presented as the means ± SEM (**A** and **B**). $n = 5$ of each group (**A**); $n = 3$ of each group (**B**). $^{*}P < 0.05$, $^{****}P < 0.0001$ between assigned groups using *t*-test.

TGF-β1 Induces Apoptosis of Primary Hepatocytes Dependent on Foxo1

Because TGF-β1 up-regulates Foxo1 protein level and improves apoptosis in the primary hepatocytes, the contribution of Foxo1 to the TGF-β1–induced apoptosis in primary hepatocytes was examined next. Foxo1-deficient primary hepatocytes were isolated from the L-FKO mice. As shown in Figure 2A, the up-regulation of cleaved caspase 3 by TGF-β1 was completely abolished by Foxo1 deletion in primary hepatocytes. The effect of TGF-β1 on caspase 3 activity was reduced by 38% owing to Foxo1 deficiency ($P = 0.01$) (Figure 2B). However, TGF-β1 increased the activity of caspase 3 in L-FKO hepatocytes (Figure 2B). These results suggest differential regulations of caspase 3 expression and activity in L-FKO hepatocytes. The underlying mechanisms of this observation remain to be determined. Consequently, apoptosis in hepatocytes induced by TGF-β1 was abrogated in L-FKO hepatocytes (Figure 2C). Thus, TGF-β1–induced apoptosis of primary hepatocytes requires Foxo1.

Induction of Apoptosis by TGF-β1 via Foxo1 Is Dependent on PKA

PKA phosphorylates Foxo1 at Ser273 to promote Foxo1 nuclear translocation and activate the transcription of target genes.[16] Therefore, the signaling pathway required for TGF-β1–activated Foxo1 was examined further, with a focus on PKA. TGF-β1 activated the phosphorylation of PKA substrates as well as CREB-S133 (Figure 3A). However, the pretreatment of PKA inhibitor H89 reduced the effects of TGF-β1 on p-Foxo1-S273 by 32% ($P = 0.037$) and Foxo1 stability by 19% ($P = 0.009$) (Figure 3C). TGF-β1–induced nuclear translocation of Foxo1 was also suppressed by H89, which was evident by the 35% decrease of nuclear Foxo1 (Figure 3B). More importantly, the effects of TGF-β1 on up-regulations of cleaved caspase 3 and Bim were diminished by H89 by 19% ($P = 0.0009$) and 21% ($P = 0.0082$), respectively (Figure 3C), which was followed by the alleviated apoptosis, as shown by the TUNEL staining (Figure 3D). Collectively, the current results indicated that signaling from PKA→Foxo1 is required for TGF-β1–stimulated apoptosis in hepatocytes.

Activation of PKA→Foxo1 Signaling by TGF-β1 Is Mediated by TβRI and Smad3

In response to stimulation of TGF-β1, TβRII interacts with and phosphorylates TβRI, which leads to the recruitment and phosphorylation of Smad2 and Smad3. The formation of the Smad complex promotes the expressions of TGF-β1 responsive genes.[12] Therefore, TβRI antagonist and Smad3 siRNA was used to investigate the upstream regulators in the TGF-β1 signaling that activate PKA-Foxo1 signaling. TβRI selective antagonist (SB431542) blocked the effects of TGF-β1 on the protein levels of PKA, p-Foxo1-S273, and total Foxo1 (Figure 4A). Specifically, the inhibition of TβRI led to the decrease of p-CREB-S133, p-Foxo1-S273, and total Foxo1 by 40%, 33%, and 26%, respectively (Figure 4A), which was coupled with a reduction in the caspase 3 activity by 35% (Figure 4B). Similar results were obtained by using Smad3 siRNA as the activity of caspase 3 induced by TGF-β1 was decreased by 56% ($P < 0.0001$) (Figure 4C). Moreover, Smad3 siRNA blocked the effects of TGF-β1 on p-Foxo1-S273 and total Foxo1, which was evident by a 68% and a 26.2% of reduction in p-Foxo1-S273 and Foxo1 levels, respectively (Figure 4D). Interestingly, Smad3 knockdown also resulted in reductions of basal levels of p-Foxo1-S273 and total Foxo1 protein without TGF-β1 treatment (Figure 4D), suggesting that Smad3 also regulates basal levels of Foxo1.

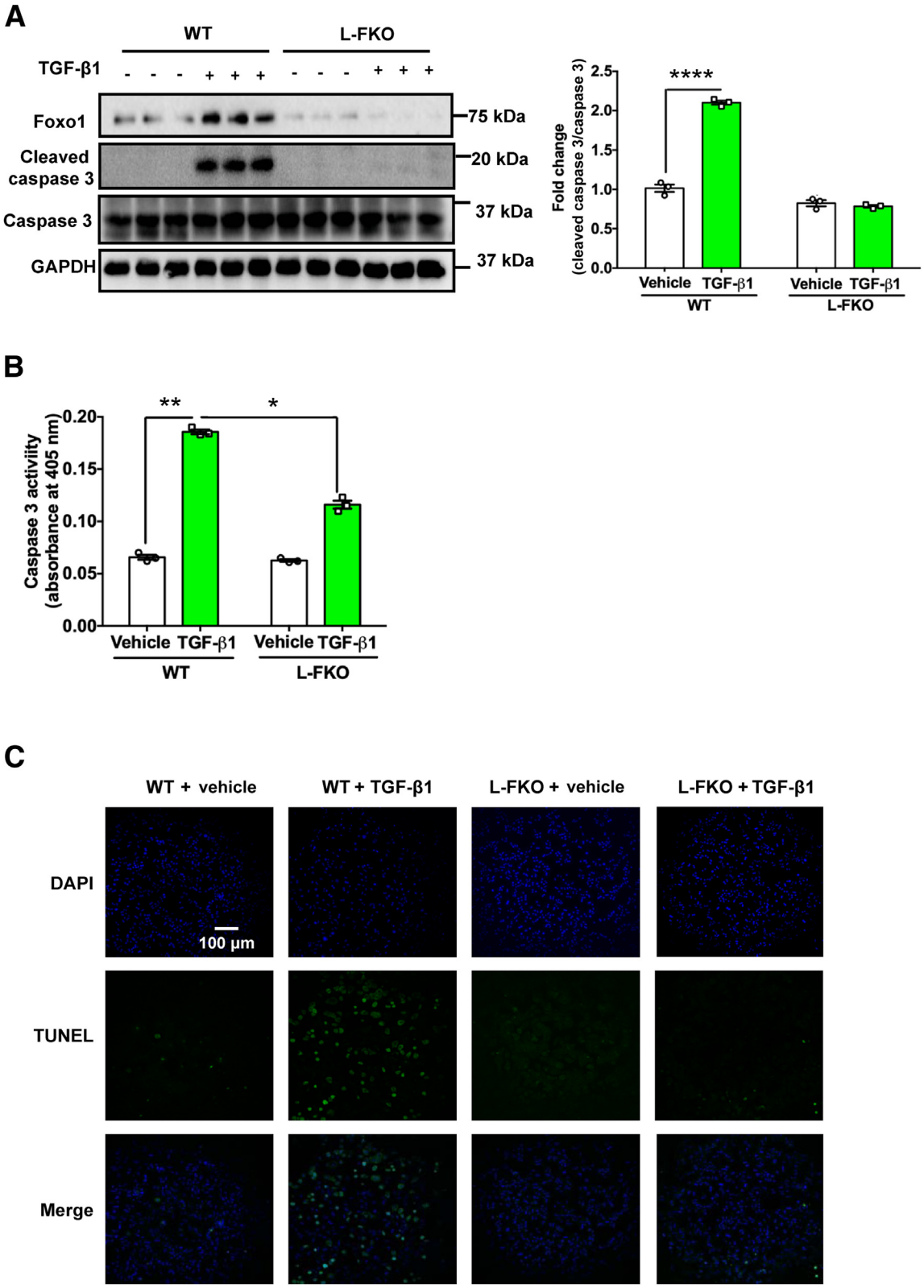

Figure 2 Transforming growth factor (TGF)-β1 induces apoptosis of primary hepatocytes in a forkhead box O1 (Foxo1)–dependent manner. Primary hepatocytes were isolated from wild-type (WT) control or liver-specific Foxo1 knockout (L-FKO) mice and then treated with 10 ng/mL of TGF-β1 for 24 hours. **A:** Protein levels of total Foxo1, cleaved caspase 3, and caspase 3 were analyzed by Western blot analysis and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **B:** Caspase 3 activity of the cells was analyzed by a colorimetric assay kit. **C:** Cell apoptosis was analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. Data are presented as the means ± SEM (**A** and **B**). $n = 3$ of each group (**A**–**C**). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$ between assigned groups using *t*-test (**A**) or one-way analysis of variance (**B**). Scale bar = 100 μm (**C**).

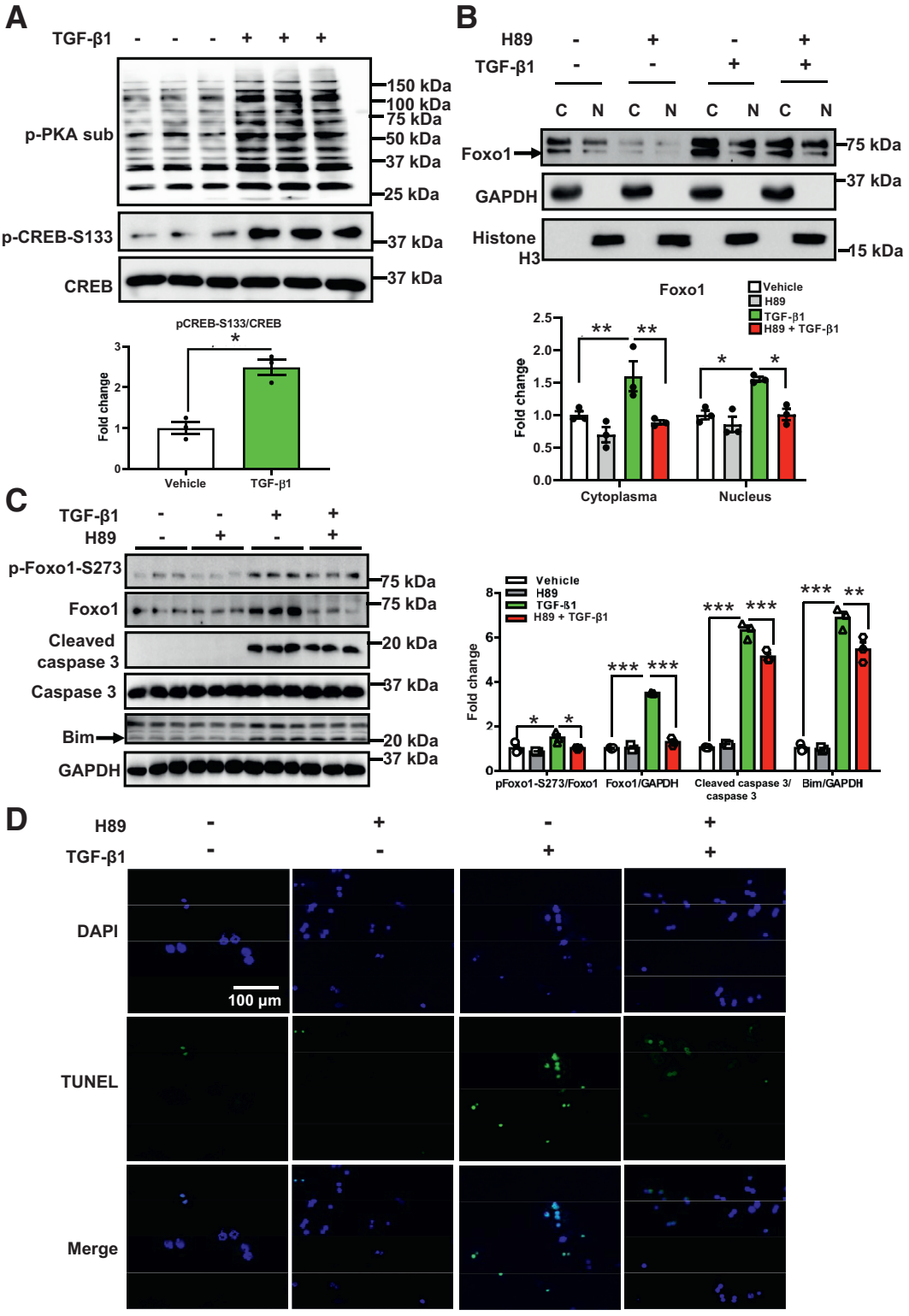

Figure 3 Activation of forkhead box 01 (Foxo1) and induction of apoptosis by transforming growth factor (TGF)-β1 require protein kinase A (PKA). Primary hepatocytes isolated from the wild-type control mice were treated with 10 μmol/L of PKA inhibitor H89 for 0.5 hours, followed by the treatment of TGF-β1 (10 ng/mL) for 24 hours. **A:** Protein levels of phosphorylated (p)-PKA substrates (sub), cAMP-response element binding protein (CREB) and p-CREB-S133 were analyzed by Western blot analysis. **B:** Proteins were extracted from cytosol (C) or nuclei (N) for the assessment of total Foxo1 by Western blot analysis. Cytoplasmic and nuclear Foxo1 were normalized by histone H3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. Representative images are shown. **C:** Protein levels of p-Foxo1-S273, Foxo1, cleaved caspase 3, caspase 3, and Bim were analyzed by Western blot analysis and normalized by GAPDH. **D:** Cell apoptosis was analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. Data are presented as the means ± SEM (**A**–**C**). $n = 3$ of each group (**A**–**D**). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ between assigned groups using *t*-test (**A**) or one-way analysis of variance (**B** and **C**). Scale bar = 100 μm (**D**).

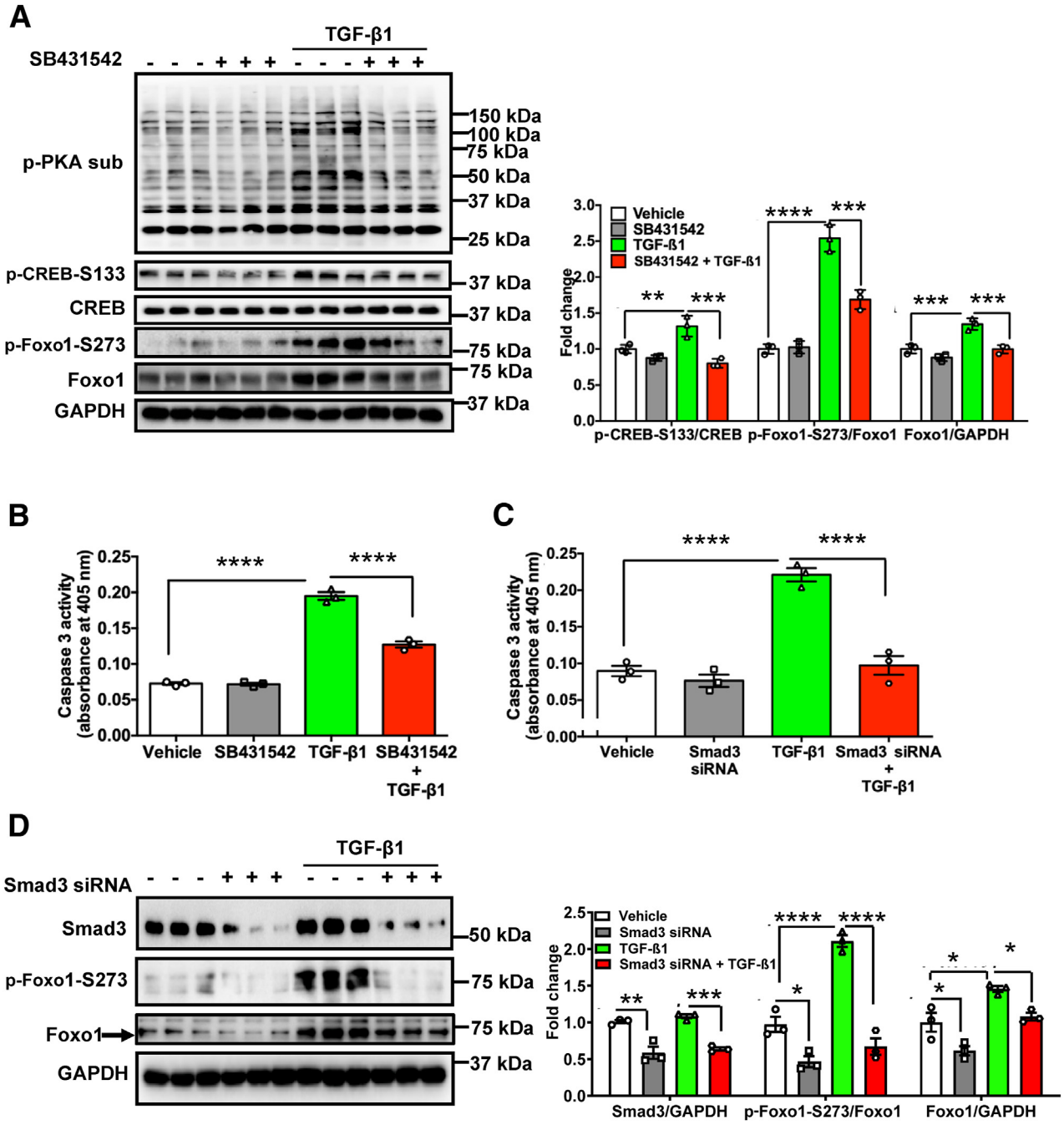

Figure 4 Activation of protein kinase A (PKA)→forkhead box O1 (Foxo1) signaling by transforming growth factor (TGF)-β1 is mediated by TGF-β receptor I (TβRI) and Smad3. Primary hepatocytes isolated from the wild-type control mice were treated with TβRI inhibitor SB431542 (20 μmol/L), followed by the treatment of TGF-β1 (10 ng/mL) for 24 hours. **A:** Protein levels of phosphorylated (p)-PKA substrates (sub), cAMP-response element binding protein (CREB), p-CREB-S133, p-Foxo1-S273, and total Foxo1 were analyzed by Western blot analysis. **B:** Caspase 3 activity of the cells was analyzed by a colorimetric assay kit. **C:** The cells were treated with Smad3 siRNA for 24 hours, followed by the treatment of TGF-β1 (10 ng/mL) for 24 hours. Caspase 3 activity was analyzed by a colorimetric assay kit. **D:** Protein levels of Smad3, p-Foxo1-S273, and Foxo1 were measured by Western blot analysis and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are presented as the means ± SEM (**A**–**D**). $n = 3$ of each group (**A**–**D**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ between assigned groups using one-way analysis of variance.

To further validate the role of Foxo1 in mediating the TGF-β signaling *in vivo*, Smad3 was overexpressed via a tail vein injection of the adenovirus. Overexpression of Smad3 dramatically increased the protein levels of p-Foxo1-S273 and total Foxo1 by 1.48- and 1.73-fold, respectively. Strikingly, in the liver of WT control mice, Smad3 overexpression increased protein levels of cleaved caspase 3 by 1.65-fold (Figure 5A). In addition, Smad3 overexpression remarkably increased both mRNA and protein levels of the Foxo1-target pro-apoptotic gene *Bim* by 1.50- and

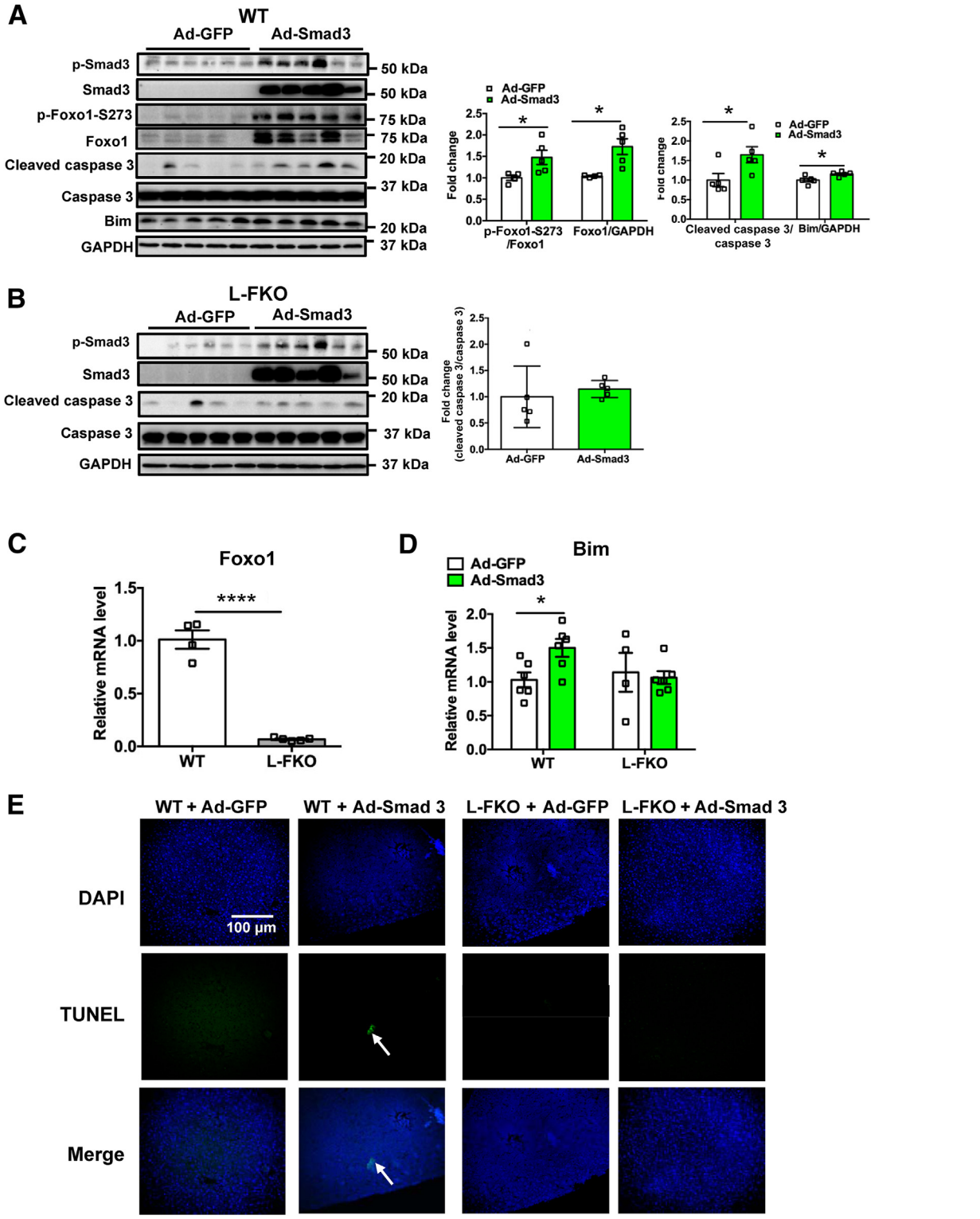

Figure 5 *In vivo* overexpression of Smad3 promotes cleaved caspase 3 and Bim in a forkhead box O1 (Foxo1)—dependent manner. Adenovirus-expressing green fluorescent protein (Ad-GFP) or adenovirus-expressing Smad3 (Ad-Smad3) was injected via tail vein to the recipient mice for 3 weeks. **A** and **B:** Protein levels of Smad3, phosphorylated (p)-Foxo1-S273, total Foxo1, cleaved caspase 3, caspase 3, and Bim in the liver of wild-type (WT) or liver-specific Foxo1 knockout (L-FKO) mice were analyzed by Western blot analysis, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control. **C** and **D:** mRNA levels of Foxo1 and Bim in the liver of WT or L-FKO mice were analyzed by quantitative RT-PCR. **E:** Liver tissues were fixed by formalin and embedded in paraffin. Hepatic apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. Positive signal is indicated by the **arrows**. Data are presented as the means ± SEM (**A**—**D**). $n = 4$ to 5 mice per group (**A**—**D**). $^{*}P < 0.05$, $^{****}P < 0.0001$ between assigned groups using *t*-test. Scale bar = 100 μm (**E**).

1.16-fold, respectively (Figure 5, A and D). However, the effects of Smad3 overexpression were diminished in the liver of L-FKO mice (Figure 5, B−D). In addition to the pro-apoptotic markers, hepatic apoptosis was also detected in Smad3-overexpressed livers. TUNEL staining was observed in the Smad3-overexpressed control WT liver, but not in the L-FKO liver (Figure 5E). Taken together, these results indicated that TGF-β1 activates PKA→Foxo1 signaling axis via TβRI→Smad3, which further promotes apoptosis in hepatocytes.

Phosphorylation of Foxo1-S273 Mediates the Pro-Apoptotic Effect of TGF-β1

Given that TGF-β1 stimulated the phosphorylation of Foxo1 at Ser273, the contribution of this specific phosphorylation site to TGF-β1−induced apoptosis was examined. Foxo1 knock-in mouse (273A/A) has Ser273 replaced by an alanine to mimic Foxo1 dephosphorylation.[16] The primary hepatocytes from the control mice exhibited markedly increased cleaved caspase 3 expression, whereas the 273A/A hepatocytes did not exhibit a significantly elevated level of cleaved caspase 3 on the treatment of TGF-β1 ($P = 0.2$) (Figure 6, A and B). Therefore Foxo1, via the phosphorylation at Ser273, may play a predominant role in mediating the pro-apoptotic effect of TGF-β1 in primary hepatocytes.

To confirm the pathologic role of p-Foxo1-S273 in mediating the pro-apoptotic effect of TGF-β1, L-TGF-β1OE mice were generated and bred with Foxo1-S273A/A mice to generate L-TGF-β1OE::S273A/A mice. These mice were maintained on NASH diet for 5 months, and then the liver tissues were collected. Compared with control mice, liver TGF-β1 overexpression exacerbated apoptosis in the liver of L-TGF-β1OE mice (Figure 6C). However, this effect was largely blocked in L-TGF-β1OE::S273A/A mice (Figure 6C), suggesting that p-Foxo1-S273 is a key player mediating TGF-β1−induced hepatocyte apoptosis.

Lipopolysaccharide (LPS) is another pro-apoptotic factor that activates PKA in macrophages.[25] Herein, LPS strikingly promoted PKA activation in primary hepatocytes (Figure 7A). To test whether the pro-apoptotic effect of LPS in hepatocytes is Smad3 dependent, LPS plus actinomycin D treatment (LPS/ActD) were used to induce apoptosis in primary hepatocytes with or without Smad3 knockdown by siRNA. Although LPS/ActD significantly increased caspase 3 activity, this was not affected by Smad3 knockdown (Figure 7B), suggesting that LPS induces hepatocyte apoptosis in an Smad3-independent manner.

Foxo1 mediates tumor necrosis factor (TNF)-α−induced fibroblast apoptosis.[26] To test whether Foxo1 plays a similar role of TNF-α−induced apoptosis in hepatocytes, WT and L-FKO hepatocytes were treated with TNF-α plus ActD for induction of apoptosis. TNF-α/ActD significantly enhanced caspase 3 activity in WT hepatocytes (Figure 7D). TNF-α barely affected PKA activation (Figure 7C), but TNF-α/ActD−stimulated caspase 3 activity was significantly reduced in L-FKO hepatocytes compared with that in WT cells (Figure 7D), suggesting that TNF-α/ActD promotes hepatocyte apoptosis independent of PKA and dependent on Foxo1.

Discussion

Liver is the major organ that executes the functions of endocrine hormones and controls glucose homeostasis. As an essential transcription factor, Foxo1 merges in the insulin and glucagon signaling via phosphorylation at Ser253 and Ser273, respectively,[15,16] which exerts a tight transcriptional control of hepatic glucose production in the fasting and feeding liver. Given the dual roles of Foxo1 in regulating hepatic glucose homeostasis, hepatic Foxo1 is believed to be a therapeutic target for insulin resistance and hyperglucagonemia, which are the hallmarks of the onset of type 2 diabetes.[27] However, the deficiency of hepatic Foxo1 not only ameliorates hyperglycemia, but also blocks liver failure in db/db mice,[28] which indicates the regulatory roles of Foxo1 in functions beyond hepatic glucose production. Because TGF-β1 signaling is well-known for its role in triggering apoptosis of hepatocytes that ultimately leads to liver failure,[12] the current study investigated whether Foxo1 was involved in the TGF-β1 signaling to induce hepatocyte apoptosis.

This study demonstrated that Foxo1 is a key mediator of the pro-apoptotic effect of TGF-β1 in primary hepatocytes, which is supported by several lines of evidence. First, the protein expression of Foxo1 was up-regulated by TGF-β1, which was coupled with an increased level of p-Foxo1-Ser273. Second, Foxo1 deficiency protected primary hepatocytes from TGF-β1−induced apoptosis. Foxo1 as a target of the TGF-β1 signaling was also evident by the overexpression of Smad3 *in vivo*. Both p-Foxo1-S273 and total Foxo1 levels were enhanced by Smad3 overexpression, which further contributed to the up-regulations of cleaved caspase 3 and Bim, coupled with the enhanced hepatocyte apoptosis. Moreover, dephosphorylation of Foxo1 at Ser273 by replacing the serine with alanine inhibited TGF-β1−induced apoptosis in primary hepatocytes and the liver of NASH diet fed mice.

The present study identified the pathway by which Foxo1 incorporates into the TGF-β1 signaling in the primary hepatocytes. By using the PKA inhibitor H89, TβRI selective antagonist, and Smad3 siRNA, it was found that PKA serves as the mediator that bridges the signal transduction between TGF-β1/Smad3 and Foxo1. This finding is consistent with existing literature reporting the interaction between Smad3 and PKA in the lung epithelial cells and hepatocytes, in which TGF-β1 promotes the binding between Smad3 protein and the regulatory subunits of PKA, followed by the release of the catalytic subunits of PKA from the PKA holoenzyme, which subsequently activates the downstream target genes.[29,30] Consistent with these

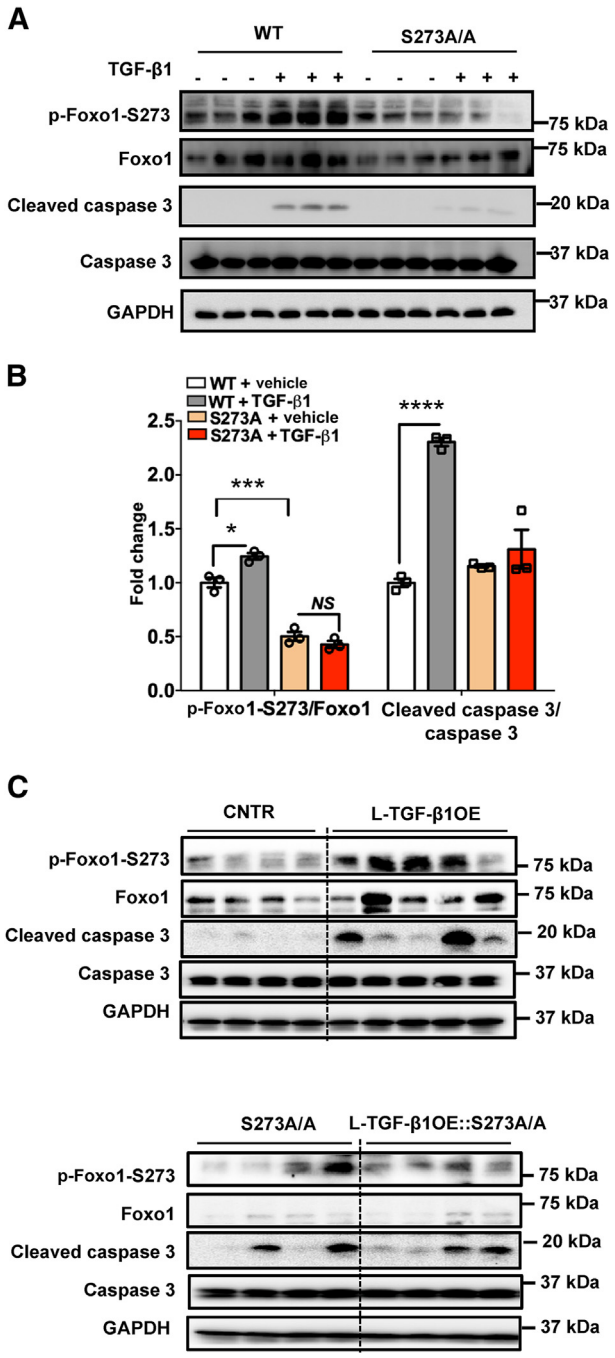

Figure 6 Phosphorylation of forkhead box O1 (Foxo1)—S273 mediates the pro-apoptotic effect of transforming growth factor (TGF)-β1. Primary hepatocytes isolated from the wild-type (WT) control or 273A/A mice were treated by 10 ng/mL of TGF-β1 for 24 hours. **A:** Protein levels of phosphorylated (p)-Foxo1-S273, Foxo1, cleaved caspase 3, and caspase 3 were analyzed by Western blot analysis. **B:** Quantitative results of **A**. **C:** Control (CNTR), L-TGF-β1OE, S273A/A, and L-TGF-β1OE::S273A/A mice were fed with nonalcoholic steatohepatitis diet for 5 months. Protein levels of p-PKA substrates, cAMP-response element binding protein (CREB), p-CREB-S133, p-Foxo1-S273, total Foxo1, cleaved caspase3, and caspase3 in the liver of these mice were analyzed by Western blot analysis. Data are presented as the means ± SEM (**B**). $n = 3$ of each group (**B**). $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ between assigned groups using one-way analysis of variance. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS, no statistical significance.

studies, the current study demonstrated that Smad3 phosphorylation is required for TGF-β1—induced PKA activation. We speculate that Smad3 may act in a similar manner in activating PKA to exert the pro-apoptotic effect of TGF-β1 through Foxo1 in primary hepatocytes. TGF-β1 signaling induces hepatic gluconeogenesis via Foxo1 through the protein phosphatase 2A (PP2A)—AMP-activated protein kinase (AMPK) pathway.[31] Given the essential role of PKA in glucagon signaling to control glucose homeostasis, the cross talk between TGF-β1/Smad3 and PKA established in this study impels us to investigate the regulatory role of the TGF-β1 signaling in glucose production in future research.

The specific phosphorylation site of Foxo1 in mediating the pro-apoptotic effect of TGF-β1 in the primary hepatocytes was also characterized in the current study. Akt directly interacts with Smad3 to regulate the sensitivity of the TGF-β1—induced apoptosis.[32] However, the present study indicates that Ser273, a novel phosphorylation site of Foxo1 regulated by glucagon,[16] mediates the pro-apoptotic effect of TGF-β1. This finding could further support that PKA is downstream of TGF-β1/Smad3 to promote the transcriptional activity of Foxo1. The functions of Foxo1 in addition to regulating glucose homeostasis have been recognized, which include regulations of oxidative stress, lipid metabolism, as well as autophagy and adaptation to starvation. However, these studies only discussed the roles of the insulin-regulated Foxo1.[33] The present study identified the potential of PKA-regulated Foxo1 phosphorylation site Ser273 in controlling hepatocyte apoptosis, which advances our knowledge on the role of Foxo1 in metabolic control.

Hepatocyte apoptosis is associated with liver diseases, including NASH and liver fibrosis.[34] Engulfment of the apoptotic bodies by Kupffer cells enhances their expression of cytokines and reactive oxygen species levels, which activates hepatic stellate cells, driving liver fibrosis progression.[35] Here, hepatic Foxo1 was demonstrated to play a role in up-regulating Bim and inducing hepatocyte apoptosis by involving in the TGF-β1 signaling. Moreover, hepatic TGF-β1 signaling—induced cell death was shown to promote NASH development.[13] Similarly, in the NASH mouse model, liver TGF-β1 overexpression exacerbated apoptosis in the NASH liver, and such an effect was largely abolished in Foxo1-S273A/A mice, underlining the key role of p-Foxo1-S273 in control of TGF-β1—regulated hepatocyte apoptosis in NASH development. We speculate that hepatic Foxo1 may regulate the development of NASH and liver fibrosis. However, Foxo1 relies heavily on cell type and tissue context to exert different functions.[36] In hepatic stellate cells, Foxo1 reduces liver fibrosis via inhibiting the proliferation and transdifferentiation of hepatic stellate cells.[37] In macrophages, Foxo1 has been recently shown to promote NASH development via stimulating macrophage inflammatory response.[38]

Foxo1 is a putative tumor suppressor, and its expression is dysregulated in several tumor types, including prostate

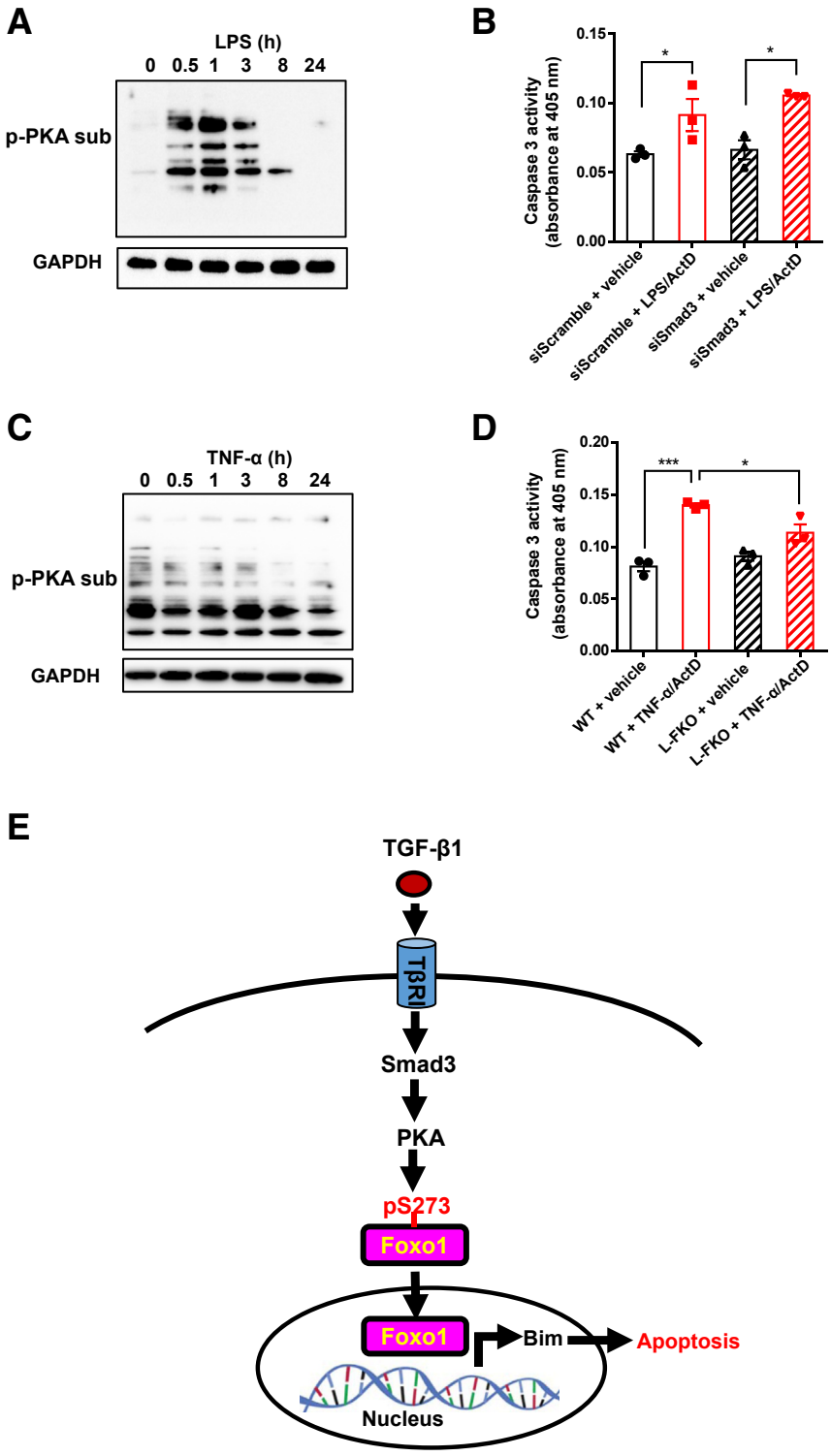

Figure 7 Lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β1–induced hepatocyte apoptosis. **A:** Wild-type (WT) primary hepatocytes were treated with LPS (5 μg/mL) for indicated hours, and phosphorylated protein kinase A (p-PKA) substrates (sub) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels were analyzed by Western blot analysis. **B:** WT primary hepatocytes were transfected with small interfering RNA targeting Smad3 (siSmad3) or small interfering RNA targeting scramble (siScramble) for 16 hours and then treated with LPS (5 μg/mL) plus actinomycin D (1 μg/mL; LPS/ActD), and caspase 3 activity was determined. **C:** WT primary hepatocytes were treated with TNF-α (25 ng/mL) for indicated hours, and p-PKA substrates and GAPDH protein levels were analyzed by Western blot analysis. **D:** WT and liver-specific forkhead box O1 (Foxo1) knockout (L-FKO) primary hepatocytes were treated with TNF-α (25 ng/mL) plus actinomycin D (1 μg/mL), and then caspase 3 activity was determined. **E:** In primary hepatocytes, TGF-β1 activates TGF-β receptor I (TβRI)/Smad3, followed by activating PKA and inducing Foxo1 phosphorylation at Ser273, which drives Foxo1 nuclear translocation to activate the transcription of pro-apoptotic genes, triggering apoptosis. Data are presented as the means ± SEM (**B** and **D**). $*P < 0.05$, $***P < 0.001$ between assigned groups using one-way analysis of variance.

cancer, breast cancer, and leukemia.[36] One of the major mechanisms by which Foxo1 prevents cancer growth is by inducing apoptosis and cell cycle arrest of cancer cells. Previously, the correlation between Foxo1 and development of cancers was linked by the phosphatidylinositol 3-kinase–AKT signaling, which is overactivated in human cancers as oncogenes. However, recent studies highlight the key role of PKA signaling in control of Foxo1 activity via p-Foxo1-S273.[16,39,40] Interestingly, PKA activity is dysregulated in several types of human cancer, and pharmaceutical

activation of PKA activity ameliorates progression of some cancers, including lung cancer and myeloid leukemia.[41] Thus, PKA→Foxo1 signaling-mediated apoptosis identified by the current study is a potential mechanism by which PKA controls cancer development. Further studies investigating the role of p-Foxo1-S273 in the progression of cancer may bring new insights into our knowledge of cancer development.

In summary, the present study established the pro-apoptotic TGF-β1→PKA→Foxo1 signaling axis in hepatocytes and highlighted the role of Foxo1, particularly via p-Foxo1-S273 (Figure 7E). Given the key role of hepatocyte apoptosis in liver diseases, such as NASH and fibrosis, further studies are under investigation to explore the potential roles of TGF-β1→PKA→Foxo1 axis in control of liver disease development.

Author Contributions

Y.C. designed the study, performed the research, interpreted the results, and wrote the manuscript; Q.P. performed research and wrote the manuscript; W.L. and W.A. performed research and reviewed the manuscript; S.Y. reviewed the manuscript; and S.G. designed the study, analyzed the data, and reviewed and revised the manuscript, and is responsible for the integrity of this work.

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