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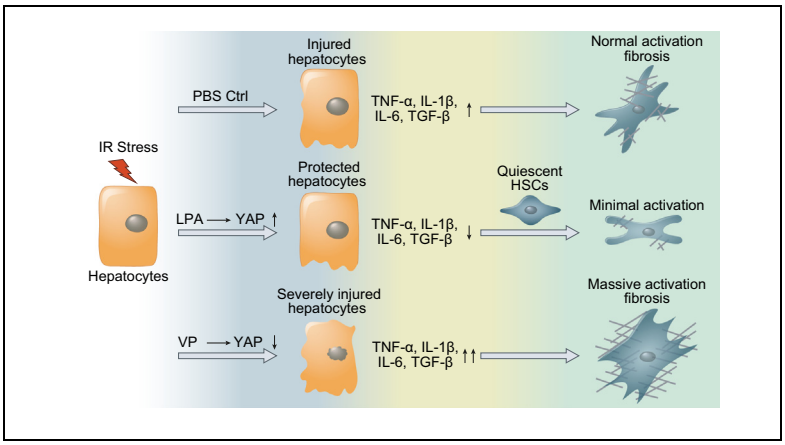
Activation of YAP attenuates hepatic damage and fibrosis in liver ischemia-reperfusion injury

Graphical abstract



Highlights

- High graft YAP expression was correlated with well-preserved histology and improved hepatocellular function in human OLT.
- YAP activation promoted downstream regenerative/anti-oxidative gene induction.
- YAP activation diminished oxidative stress, necrosis/apoptosis, and suppressed the innate inflammatory response.
- YAP activation suppressed extracellular matrix synthesis and hepatic stellate cell activation, and stopped fibrogenesis.
- YAP activation failed to protect *Nrf2*-deficient mouse livers against IR-mediated tissue damage.

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Lay summary

In the clinical arm, graft YAP expression negatively correlated with liver function and tissue damage after human liver transplantation. YAP activation attenuated hepatocellular oxidative stress and diminished the innate immune response in mouse livers following ischemia-reperfusion injury. In the mouse model, YAP inhibited hepatic stellate cell activation, and abolished injury-mediated fibrogenesis up to 7 days after the ischemic insult.

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Activation of YAP attenuates hepatic damage and fibrosis in liver ischemia-reperfusion injury

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Background & Aims: Hepatic ischemia-reperfusion injury (IRI) is a major complication of hemorrhagic shock, liver resection and transplantation. YAP, a key downstream effector of the Hippo pathway, is essential for determining cell fate and maintaining homeostasis in the liver. We aimed to elucidate its role in IRI.

Methods: The role of YAP/Hippo signaling was systematically studied in biopsy specimens from 60 patients after orthotopic liver transplantation (OLT), and in a mouse model of liver warm IRI. Human biopsy specimens were collected after 2–10 h of cold storage and 3 h post-reperfusion, before being screened by western blot. In the mouse model, the role of YAP was probed by activating or inhibiting YAP prior to ischemia-reperfusion.

Results: In human biopsies, high post-OLT YAP expression was correlated with well-preserved histology and improved hepatocellular function at postoperative day 1–7. In mice, the ischemia insult (90 min) triggered intrinsic hepatic YAP expression, which peaked at 1–6 h of reperfusion. Activation of YAP protected the liver against IR-stress, by promoting regenerative and anti-oxidative gene induction, while diminishing oxidative stress, necrosis/apoptosis and the innate inflammatory response. Inhibition of YAP aggravated hepatic IRI and suppressed repair/anti-oxidative genes. In mouse hepatocyte cultures, activating YAP prevented hypoxia-reoxygenation induced stress. Interestingly, YAP activation suppressed extracellular matrix synthesis and diminished hepatic stellate cell (HSC) activation, whereas YAP inhibition significantly delayed hepatic repair, potentiated HSC activation, and enhanced liver fibrosis at 7 days post-IRI. Notably, YAP activation failed to protect *Nrf2*-deficient livers against IR-mediated damage, leading to extensive fibrosis.

Conclusion: Our novel findings document the crucial role of YAP in IR-mediated hepatocellular damage and liver fibrogenesis, providing evidence of a potential therapeutic target for the management of sterile liver inflammation in transplant recipients.

Lay summary: In the clinical arm, graft YAP expression negatively correlated with liver function and tissue damage after human liver transplantation. YAP activation attenuated hepatocellular oxidative stress and diminished the innate immune response in mouse livers following ischemia-reperfusion injury. In the mouse model, YAP inhibited hepatic stellate cell activation, and abolished injury-mediated fibrogenesis up to 7 days after the ischemic insult.

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Introduction

Orthotopic liver transplantation (OLT) is considered the standard treatment for end-stage liver disease. Following organ retrieval, cold preservation and warm ischemia-reperfusion injury (IRI) can lead to impaired graft function, including primary graft non-function, which may predispose patients to acute and chronic rejection. Indeed, by contributing to a shortage of available donor organs, IRI represents one of the most challenging problems in transplantation.[1–4] Thus, novel therapeutic concepts to combat IRI are needed to improve OLT outcomes and expand the donor organ pool.

Hepatic IRI, initiated by hypoxic stress, is an exogenous antigen-independent local inflammatory response. IR-insulted hepatocytes then produce damage-associated molecular patterns, which trigger innate immune responses and inflammation.[3] Hepatocellular death is mainly characterized by reactive oxygen species (ROS)-induced hepatocellular necrosis and innate pro-inflammatory cytokine-mediated apoptosis in liver IRI.[2]

Liver fibrosis is a highly conserved and coordinated protective response to tissue injury, which disrupts the normal liver architecture, and causes hepatocellular dysfunction and portal hypertension.[5–8] Hepatic stellate cells (HSCs) are considered the major source of liver myofibroblasts.[9–11] The interaction between macrophage-mediated innate immunity and HSC activation/proliferation is essential in liver fibrogenesis.[12–14]

Keywords: Fibrogenesis; Liver ischemia-reperfusion injury; Orthotopic liver transplantation; YAP; Inflammation; Hippo; Immune response.
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Prolonged ischemia time was considered one of the most important risk factors related to post-transplant hepatic fibrosis in pediatric liver transplantation.[7] However, the mechanism of IR-mediated fibrosis/cirrhosis and chronic graft non-function remains to be determined.

The Hippo pathway and its downstream effector YAP (Yes-associated protein) control organ size in animals through the regulation of cell proliferation and apoptosis.[15,16] Hippo signaling sequesters YAP to the cytoplasm via phospho-specific and cell-tension based mechanisms.[17] YAP primarily binds to the TEAD family of transcription factors to activate target gene expression.[17] In the mammalian liver, YAP determines cell fate and maintains homeostasis.[18] Its high-to-low gradient expression from the portal vein area to the central vein contributes to the heterogeneity of hepatocytes and liver zonation.[19,20] Loss of YAP in the liver leads to defects in both hepatocyte survival and biliary epithelial cell development,[21] whereas its ectopic activation results in hepatocyte dedifferentiation and hepatomegaly, and may promote carcinogenesis.[15,16,22] After hepatectomy, YAP activation was also found to be essential for liver repair and regeneration.[23,24]

Herein, we evaluated the role of the YAP/Hippo pathway in hepatocellular protection and hepatic fibrosis induction during liver IRI. This study examined whether and how YAP signaling might affect hepatocyte function, the innate immune response, and HSC-mediated tissue repair/fibrosis in a well-established murine liver IRI model. Our clinical arm reinforced the pivotal role of YAP in hepatocellular protection in human OLT. By providing evidence for a previously unrecognized role of YAP in hepatic protection, repair/regeneration, and fibrosis, this study provides new insights into a potential therapeutic intervention against sterile inflammatory liver injury and fibrosis.

Materials and methods

Clinical liver transplantation study

Sixty adult OLT recipients were recruited under an institutional research board protocol (#ChiCTR-IIR-16008040: March 2018-November 2018, Ren Ji Hospital, Shanghai, China). All organ donations recorded in the electronic database were contributed voluntarily, and no donor organs were obtained from executed prisoners or other institutionalized persons. Informed consent was obtained from all individuals. Routine standard pre-OLT evaluation and immunosuppression therapy were administered. Non-fatty donor organs, procured according to standardized techniques, were perfused with and stored in cold University of Wisconsin (UW) solution. Cold ischemia time was defined as the time between the perfusion of the donor liver with UW solution and its removal from the cold storage. Hepatocellular injury was evaluated by serum alanine aminotransferase (sALT) at postoperative days (PODs) 1–7. Liver biopsies were obtained from the left lobe during back-table preparation (pre-OLT: prior to implantation), and at 3 h after portal reperfusion (post-OLT: prior to the abdominal closure).

Mouse warm liver IRI model

Male, 6–8-week-old, wild-type (WT) mice (Jackson Laboratory, Bar Harbor, ME) or *Nrf2*-deficient mice (breeding pairs provided by Dr. Thomas Kensler, Johns Hopkins University) on a C57BL/6 background (backcrossed for at least 12 generations) were used in a model of partial warm hepatic IR, as described.[1–3] In general, the arterial/portal vessels to the cephalad lobes were clamped for 90 min. There was no vascular occlusion in sham-controlled mice. In the treatment groups, animals were infused at 1 h prior to the onset of liver ischemia with a single dose of YAP activator (1-oleoyl lysophosphatidic acid [LPA]: 0.8 μmol/kg i.v., Tocris Bioscience, Minneapolis, MN) or YAP inhibitor (verteporfin [VP]: 0.8 μmol/kg i.v., MilliporeSigma, St. Louis, MO) dissolved in PBS. Mice were sacrificed at various time-points after reperfusion; liver and serum samples were collected for analysis. All animal experiments were approved by the UCLA Animal Research Committee.

ALT measurement

Serum and culture medium ALT levels were measured by ALT kit (ThermoFisher, Waltham, MA) according to the manufacturer's instructions.

Western blot

Liver proteins (30 μg/sample) were separated by gel electrophoresis and transferred to a nitrocellulose membrane. YAP, phospho-YAP (Ser127), Nrf2 and β-actin monoclonal antibodies (mAbs) (Cell Signaling Technology, Danvers, MA) were used as probes. To compare protein expression in multiple human OLT samples, densitometry quantification was conducted. The YAP protein value was normalized according to β-actin intensity.

Histopathology

Liver specimens (5 μm), stained with H&E, Masson's trichrome and Sirius red were analyzed blindly. Primary mAbs against YAP (Cell Signaling Technology), macrophages (CD68) (AbD Serotec, Raleigh, NC), neutrophils (Ly6G) (BD Biosciences, San Jose, CA), and α-SMA (Abcam, Cambridge, MA) were used. Superoxide levels in hepatic tissue were detected with ROS-sensing dye dihydroethidium (DHE; AnaSpec, Fremont, CA). Liver sections were evaluated blindly by counting labeled cells in 10 high-power fields (HPF).

Quantitative RT-PCR

Quantitative PCR was performed with platinum SYBR green quantitative PCR kit (ThermoFisher) by the QuantStudio3 (ThermoFisher). Primers to amplify specific gene fragments were published.[1–3] Target gene expressions were calculated by their ratios to the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

DNA fragments in liver sections, resulting from necrosis/apoptosis, were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method (Klenow-FragEL DNA Fragmentation Detection Kit; MilliporeSigma). TUNEL-positive cells were counted in 10 HPF/section.

Hepatocyte culture

Mouse hepatocytes were isolated by *in situ* collagenase perfusion followed by Percoll gradient centrifugation, before being cultured with complete William's E medium plus maintenance supplement (ThermoFisher) for 24 h. For the hypoxia-reoxygenation experiment, culture medium, supplied with LPA (10 μM), VP (10 μM) or PBS and equilibrated in 95%N_2-5%CO_2 or 95%O_2-5%CO_2, was used as hypoxia or reoxygenation

medium, respectively. Cells were cultured with hypoxia medium at 37 °C for 6 h in a humidity airtight chamber equilibrated with 95%N_2-5%CO_2. The medium was then replaced with reoxygenation medium, and cells were further incubated at 37 °C in a 95%O_2-5%CO_2 atmosphere for 8 h. Cells were processed for immunofluorescence staining (YAP, Cell Signaling Technology), caspase-3/7 detection (CellEvent caspase-3/7 detection; ThermoFisher) and JC-1 assay (MitoProbe JC-1 assay; ThermoFisher), and supernatants were assessed for ALT.

Hepatic stellate cell culture

Mouse HSCs were isolated by *in situ* collagenase perfusion followed by Nycodenz gradient centrifugation from mice at 7 days post IRI.[25] Immunofluorescence staining of YAP and α-SMA were processed 10 h after planting.

Statistical analysis

Group comparisons were performed using a Student's *t* test for mouse experiments, and Mann-Whitney *U* test for human data. Linear regression (R^2) was used to evaluate the strength of linear relationship between variables. *P* values <0.05 were considered statistically significant.

Results

Post-OLT YAP expression negatively correlated with liver function and tissue damage in human OLT

We first aimed to evaluate the peri-operative graft expression of YAP and its correlation with liver damage in a human OLT cohort (n = 60). Pre-OLT hepatic biopsies were collected during back-table preparation after 2–10 h cold storage (prior to implantation) and post-OLT biopsies were obtained at 3 h after reperfusion (prior to abdominal closure). Western blot-assisted pre- and post-OLT YAP expression profiles are shown in Fig. 1A (representative of 6 cases). As pre-OLT YAP was at basal levels, post-OLT YAP expression increased after reperfusion. Interestingly, the post-OLT YAP levels correlated negatively with sALT values at POD1 (Fig. 1B: $R^2 = 0.1560$, $p = 0.0018$), indicating that increased post-OLT YAP expression was essential for hepatic cytoprotection. There was no significant correlation between post-OLT YAP ratio and cold ischemia time, or intraoperative blood loss (surgical stress factors, Fig. S1A).

To evaluate the impact of post-OLT YAP levels for clinical outcomes, 60 human OLTs were divided into low post-OLT YAP group (Low YAP: n = 33) and high post-OLT YAP group (High YAP: n = 27), using a post-OLT YAP/β-actin ratio = 0.95 as the threshold (Fig. 1C). There was no significant correlation between post-OLT YAP classification and donor/recipient background, *e.g.*, cold ischemia time, model for end-stage liver disease score, age, gender, weight/body mass index or serum triglyceride/cholesterol values (Table S1, Fig. S1B). Unlike the Low YAP group, OLT recipients characterized by high post-OLT YAP levels exhibited lower sALT at POD1 (Fig. 1D, $p < 0.01$), as well as rapid recovery of sALT from POD1 to POD7 (Fig. 1E). Moreover, post-OLT YAP expression in grafts (green fluorescence indicative of YAP) negatively correlated with liver histology and hepatocellular death in liver biopsies (at 3 h after portal reperfusion), as demonstrated by H&E staining (pathologic features such as sinusoidal congestion, edema, vacuolization were observed in the Low YAP group), TUNEL staining (grey color indicative of necrosis/apoptosis in the Low YAP group) and DHE staining (bright red fluorescence indicative of high intracellular oxidation in the Low YAP group) (Fig. 1F).

Local YAP expression profile in murine liver IRI

We then determined whether IR triggered the expression of the endogenous *Yap* gene in mouse livers subjected to 90 min of warm ischemia, followed by reperfusion. Unlike in sham controls, *Yap* mRNA levels increased progressively after the ischemic insult, peaking by 1–6 h of reperfusion and decreasing thereafter (Fig. 2A; $p < 0.001$).

YAP activation ameliorated liver IRI

We analyzed hepatocellular damage after modulation of the YAP pathway in our mouse model of 90 min of warm ischemia followed by 6 h of reperfusion. Treatment with YAP activator (LPA) at 1 h prior to the onset of ischemia promoted YAP gene and protein induction (Fig. 2B,E). Unlike in the control PBS group, mice pre-treated with LPA were resistant to liver IRI, demonstrated by reduced sALT levels (Fig. 2C; $p < 0.001$) and well-preserved hepatic architecture, without sinusoidal congestion, edema, vacuolization or necrosis (Fig. 2D). To confirm that the beneficial effect was YAP dependent, a separate group of WT mice was pre-treated with YAP inhibitor (VP). Compared to controls, YAP inhibition exacerbated liver IRI, demonstrated by increased sALT levels (Fig. 2C; $p < 0.001$) and more severe lobular edema, widespread hemorrhage, congestion/hepatocellular necrosis (Fig. 2D).

YAP activation attenuated oxidative stress in liver IRI

To assess the putative regulatory role of YAP activation, we screened IR-induced oxidative stress and hepatic oncotic necrosis/apoptosis. Pre-treatment with LPA activated downstream regenerative (*Ctgf [Ccn2], Cyr61 [Ccn1], Ankrd1*) and anti-oxidative (*MnSOD [Sod2]*, catalase [*Cat*]) gene programs (Fig. 2F; $p < 0.001$), decreased intracellular oxidative stress (Fig. 3A), prevented hepatocellular necrosis/apoptosis and reduced frequency of $TUNEL^+$ cells (Fig. 3B: black arrow; Fig. S2A). By targeting YAP-TEAD signaling,[21] pre-treatment with VP diminished ($p < 0.001$) downstream gene induction (*Ctgf, Cyr61, Ankrd1, MnSOD,* Catalase; Fig. 2F). Strikingly, VP-assisted YAP inhibition significantly enhanced liver oxidative stress (Fig. 3A) and hepatocellular necrosis/apoptosis (Fig. 3B: black arrow; Fig. S2A).

YAP activation diminished innate immune responses in liver IRI

LPA-facilitated YAP activation suppressed macrophage ($CD68^+$) and neutrophil ($Ly6G^+$) infiltration (Fig. 3C: white arrow; Fig. S2B), abolishing the pro-inflammatory cytokine expression pattern (Fig. 3D: *Tnf-α, Il1b, Il6, p* <0.001). In contrast, YAP inhibition amplified macrophage/neutrophil sequestration (Fig. 3C: white arrow; Fig. S2B), and increased cytokine expression (Fig. 3D: *Tnf-α, Il1b, Il6, p* <0.001).

YAP activation prevented hepatocyte death

We then analyzed the modulatory function of YAP activation in well-controlled hypoxia-reoxygenation stressed mouse primary hepatocyte cultures. LPA treatment enhanced YAP accumulation in hepatocyte nuclei (Fig. 4C, Fig. S2C), promoted downstream regenerative/anti-oxidative gene expression (Fig. 4A: *Ctgf, Cyr61, Ankrd1* and *MnSOD*) and protected hepatocytes against oxidative stress, as demonstrated by decreased ALT release

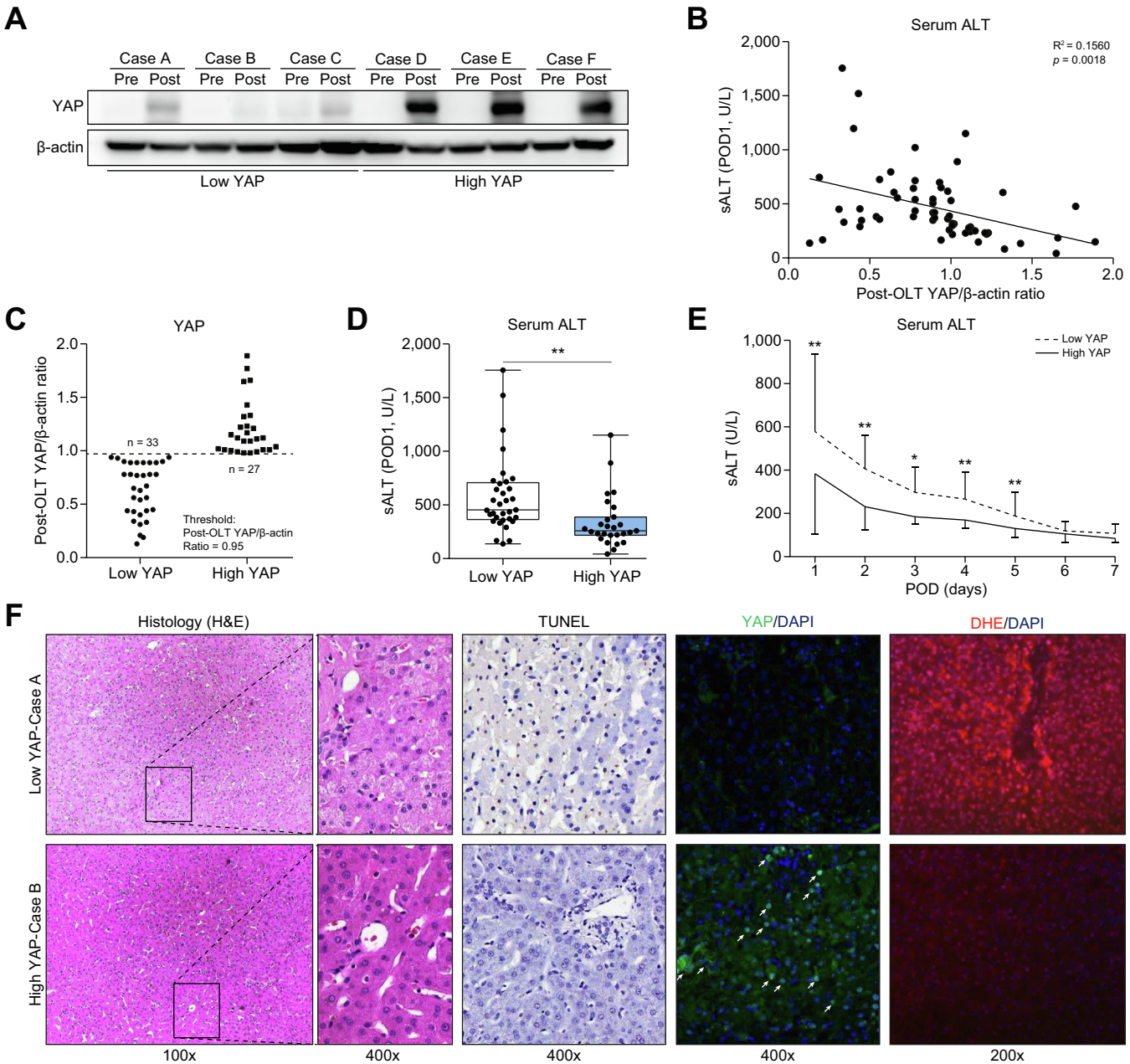

Fig. 1. Peri-operative hepatic YAP expression and its correlation with liver damage in human OLT. N = 60. (A) Western blot-assisted pre- and post-OLT YAP expression profiles in OLT biopsies (representative 6 cases). (B) The ratio of post-OLT YAP/β-actin correlated negatively with sALT at POD1. (C) Sixty human OLTs were divided into low ratio of post-OLT YAP/β-actin group (Low YAP: n = 33) and high ratio of post-OLT YAP/β-actin group (High YAP: n = 27) by using a post-OLT YAP/β-actin ratio = 0.95 as a threshold. (D) sALT values in both Low YAP and High YAP groups in OLT recipients at POD1. (E) sALT decreasing curve in Low YAP and High YAP groups in OLT recipients (POD1-7). (F) Histology (H&E staining), TUNEL staining, YAP staining, DHE staining in OLT biopsies (representative 2 cases for Low YAP and High YAP groups) (magnification ×100, ×200 and ×400; $^{**}p$ <0.01, $^{*}p$ <0.001 by Student's *t* test). OLT, orthotopic liver failure; POD, postoperative day; sALT, serum alanine aminotransferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. (This figure appears in colour on the web.)

(Fig. 4B) and caspase-3/7 activity (Fig. 4D, Fig. S2C), and intact mitochondrial membranes (Fig. 4E, Fig. 2C), compared to the control group. In contrast, VP facilitated YAP repression in hepatocyte nuclei (Fig. 4C, Fig. S2C) and suppressed YAP-related downstream gene induction (Fig. 4A). As VP mainly blocked the YAP-TEAD axis, basal levels of YAP were observed in the cytoplasm (Fig. 4C, Fig. S2C). VP-mediated YAP inhibition further exacerbated hepatocellular damage, as shown by higher ALT release (Fig. 4B), abundant caspase-3/7 activity (Fig. 4D, Fig. S2C), and ROS-stressed mitochondria (Fig. 4E, Fig. S2C).

YAP activation abolished liver IR-mediated fibrosis

Although serum ALT levels progressively decreased from 6 h to 1 day post-reperfusion in IR-stressed livers, and returned to normal by day 3 (Fig. 5A), liver fibrosis was steadily developing from 1 day to 7 days, as shown by gross morphology (fibrotic change), H&E staining (fibrous portal expansion and aggregated collagen fibers), Masson's trichrome staining (blue color was indicative of collagen deposition), and Sirius red staining (red color was indicative of collagen deposition) (Fig. 5B, Fig. S2D,3A). Interestingly, IR-stressed livers in mice

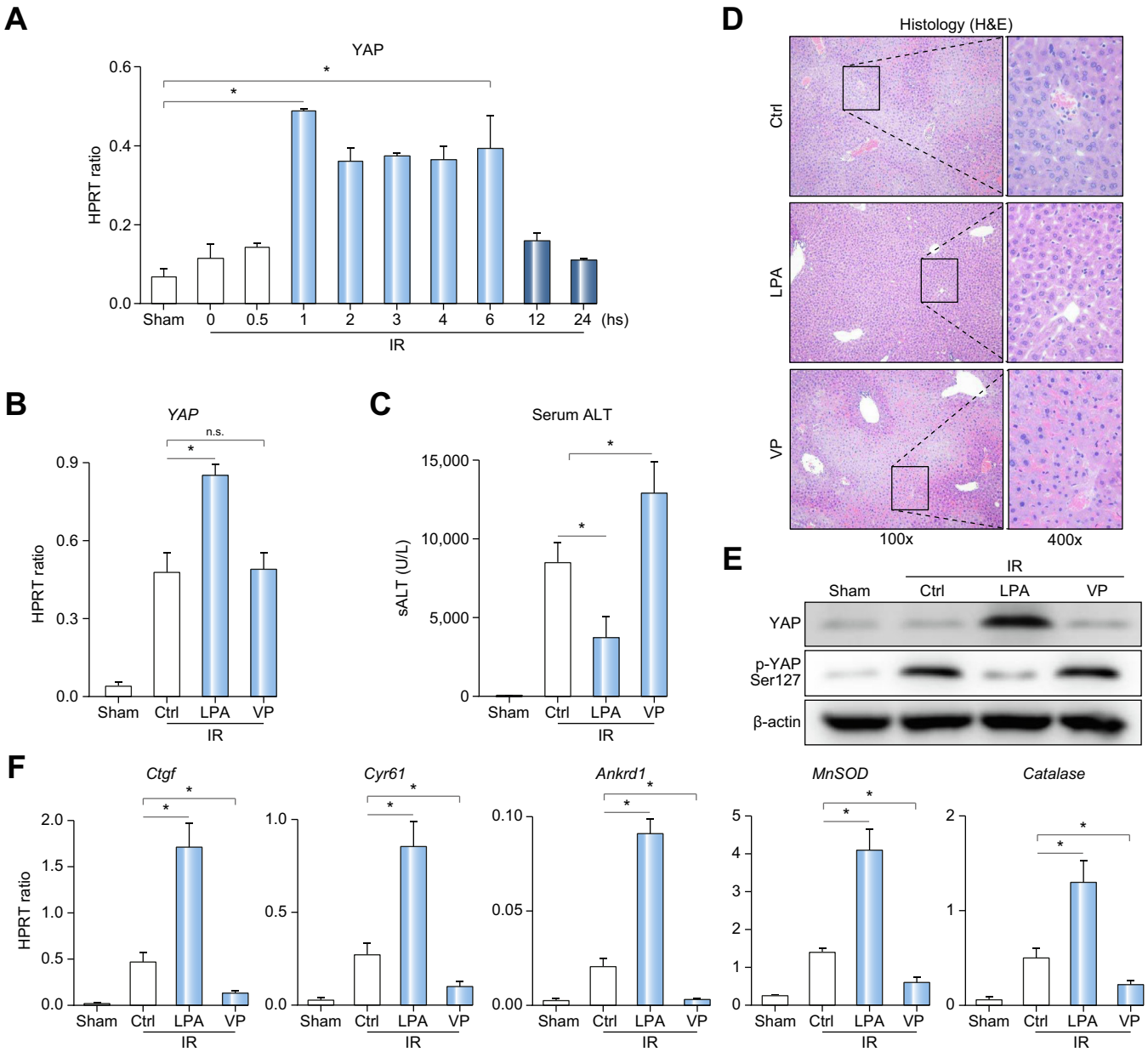

Fig. 2. YAP activation ameliorated liver IRI. (A) Kinetics of hepatic YAP gene expression in murine liver IRI. Liver samples harvested from B6 mice that were sham-operated or subjected to 90 min of partial warm ischemia, followed by various lengths of reperfusion. WT mice were pre-treated with PBS (Ctrl), a YAP activator (LPA) or a YAP inhibitor (VP) at 1 h before ischemia. Liver and serum samples were collected at 6 h post reperfusion for: (B) YAP gene induction, (C) sALT levels, (D) liver histology (representative H&E staining; magnification ×100 and ×400), (E) YAP and p-YAP (Ser127) protein induction and (F) YAP downstream gene expression (*p <0.001 by Student's t test; n = 10–12/group). HPRT, hypoxanthine-guanine phosphoribosyl transferase; IRI, ischemia-reperfusion injury; LPA, 1-oleoyl lysophosphatidic acid; sALT, serum alanine aminotransferase; VP, verteporfin; WT, wild-type. (This figure appears in colour on the web.)

pre-treated with LPA maintained intact hepatic architecture, diminished fibrosis formation during the recovery (Fig. 5B, Fig. S2D,3B), and suppressed extracellular matrix (ECM) synthesis (Fig. 5C: *α-Sma* [*Acta2*], *Col1a1* and *Timp1*, p <0.001). In contrast, VP pre-treatment delayed liver recovery despite serum ALT returning to normal levels by day 7 (Fig. 5A), as demonstrated by severe fibrosis (Fig. 5B, Fig. S2D,3C), and the enhanced fibrosis-related gene expression program (Fig. 5C).

YAP inhibition potentiated hepatic stellate cell stimulation

Stimulation of HSCs is responsible for ECM deposition and fibrosis development.[9] VP treatment elevated *Tgf-β* and pro-inflammatory cytokines (*Tnf-α, Il1b, Il6*) in IR-livers from 6 h to 7 days post-reperfusion (Fig. 6A). We then cultured HSCs harvested from ischemic livers at 7 days post-reperfusion. Interestingly, HSCs from VP-treated livers were fibrogenic and contractile with abundant YAP and α-SMA, while LPA-treated livers had a rounded shape and minimal YAP or α-SMA (Fig. 6B). We also found increased α-SMA expression in VP-treated livers, compared to the LPA-conditioned group using *in vivo* α-SMA immunofluorescence staining (Fig. 6C).

Nrf2 is required for LPA-facilitated hepatocellular protection in liver IRI

Given the previously defined anti-oxidative role of Nrf2 in IRI,[26] we then determined the level of hepatocellular damage after

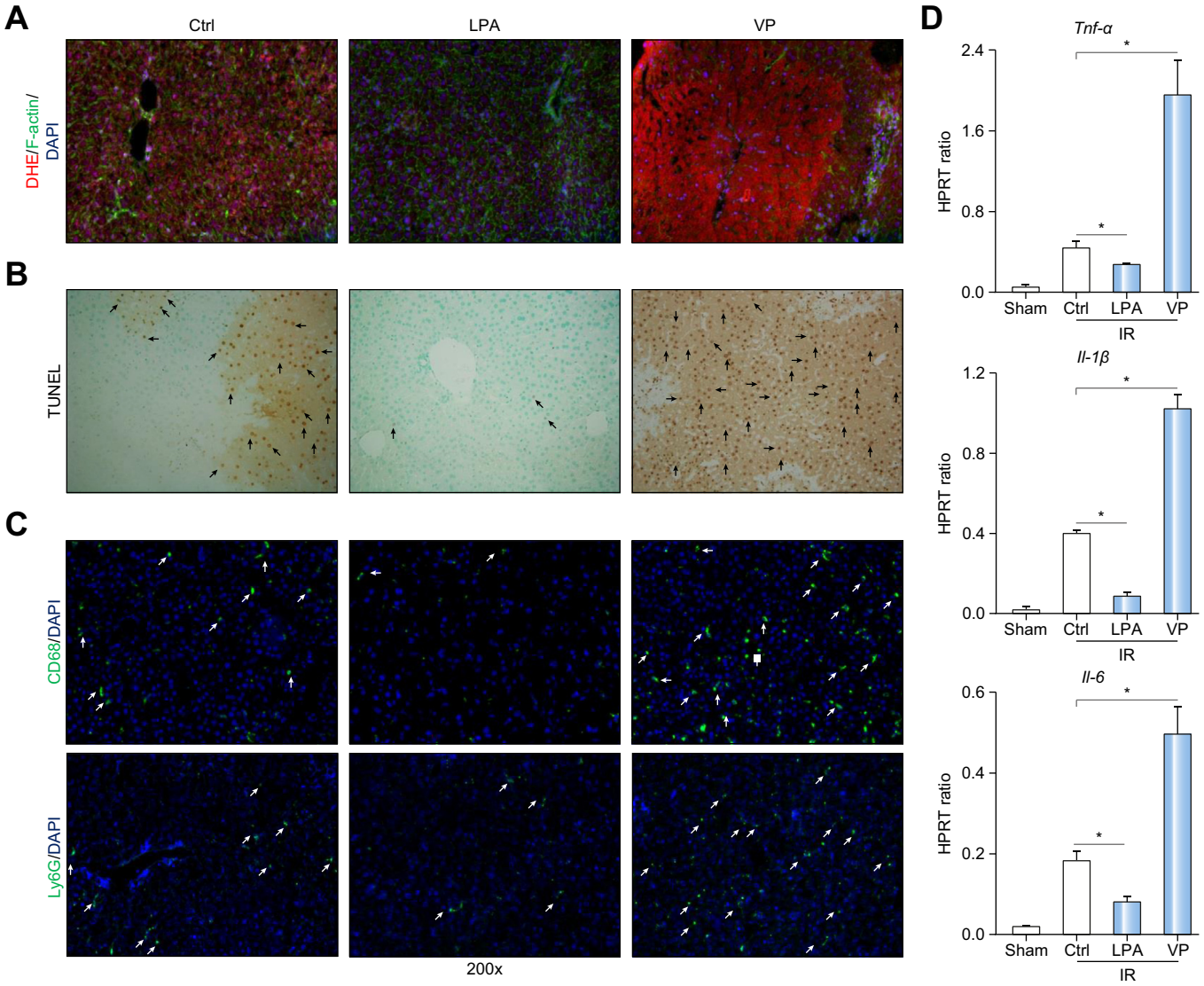

Fig. 3. YAP activation prevented hepatocyte death and suppressed pro-inflammatory response in liver IRI. Mice pre-treated with PBS, LPA or VP were subjected to 90 min of warm ischemia and analyzed at 6 h of reperfusion for: (A) ROS-sensing dye DHE staining (red fluorescence indicating ROS production). (B) TUNEL-assisted detection of hepatic necrosis/apoptosis (black arrows) in ischemic liver lobes. (C) CD68$^+$ macrophages and Ly6G$^+$ neutrophils in IR liver lobes (white arrow indicated positive staining). (D) qRT-PCR assisted detection of *Tnf-α*, *Il1b* and *Il6* (magnification ×200, *p <0.001 by Student's t test; n = 4–6/group). DHE, dihydroethidium; HPRT, hypoxanthine-guanine phosphoribosyl transferase; IRI, ischemia-reperfusion injury; LPA, 1-oleoyl lysophosphatidic acid; ROS, reactive oxygen species; qRT-PCR, quantitative reverse transcription PCR; TUNEL, TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; VP, verteporfin. (This figure appears in colour on the web.)

infusion of a YAP activator to *Nrf2*-deficient mice, who were subjected to 90 min of warm ischemia followed by 6 h of reperfusion. As LPA treatment, but not VP treatment, induced hepatic Nrf2 protein expression in WT liver IRI (Fig. 7A), LPA administration failed to prevent liver IRI in *Nrf2*-deficient mice, as indicated by comparable sALT levels (Fig. 7B) and elevated gene induction of pro-inflammatory cytokine profiles (Fig. 7C). Consistent with *in vivo* findings, LPA failed to protect *Nrf2*-deficient hepatocytes against hypoxia-reoxygenation stress (Fig. 8A-C). This may imply that YAP-mediated hepatocellular protection was Nrf2 dependent. After 7 days of reperfusion, both LPA and control groups in *Nrf2*-deficient mice represented massive fibrosis development, as shown by gross morphology (fibrotic change), H&E staining (fibrous portal expansion and aggregated collagen fibers), Masson's trichrome staining (dense blue color indicated collagen deposition), and Sirius red staining (dense red color indicated collagen deposition) (Fig. 7D, Fig. S2E). HSCs from LPA-treated *Nrf2*-deficient livers were fibrogenic/contractile with abundant YAP and α-SMA (Fig. 8D).

Discussion

The present study is the first to demonstrate that post-OLT YAP expression in human liver transplants correlated with clinical outcomes. Indeed, high peri-operative YAP expression was associated with better preserved liver histopathology and hepatocellular function in OLT recipients. Our parallel study in a well-established murine liver IRI model revealed the crucial role of YAP signaling in hepatocellular protection and HSC-mediated fibrosis *in vivo* and *in vitro*. As liver IR triggered endogenous local YAP induction, a single intravenous infusion of LPA, a YAP activator, conferred hepatic cytoprotection against IR

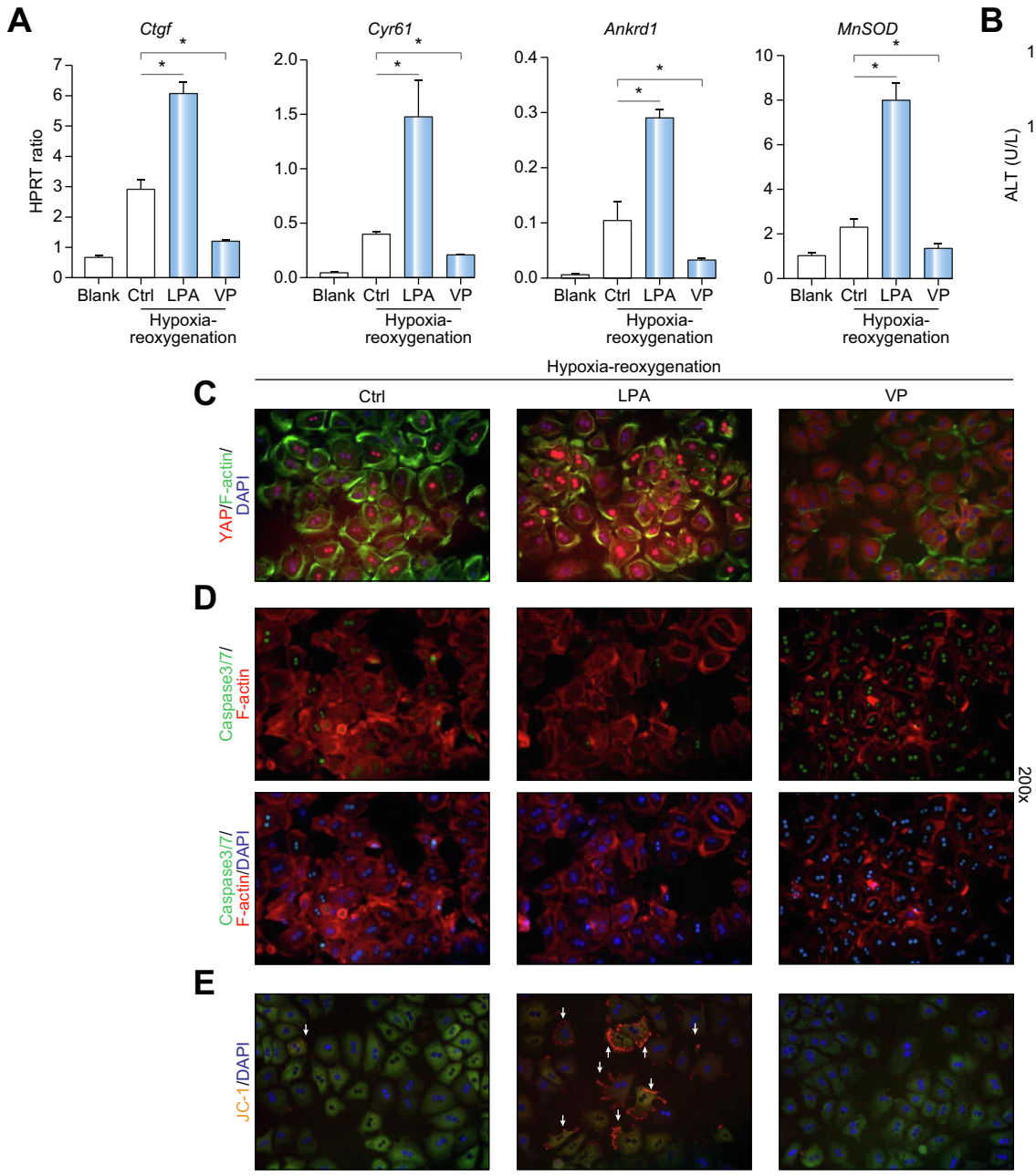

Fig. 4. YAP activation protected primary hepatocytes against hypoxia-reoxygenation insult *in vitro*. WT mouse hepatocyte cultures were supplemented with PBS, LPA or VP. After 6 h of hypoxia and 8 h of reoxygenation, the cell cultures were analyzed for: (A) YAP downstream gene expressions. (B) Supernatant ALT levels. (C) Hepatocellular YAP staining (red fluorescence indicating YAP accumulation in nucleus). (D) Caspase-3/7 detection (green fluorescence indicating positive caspase-3/7 in nucleus). (E) JC-1 staining (green fluorescence indicating mitochondria depolarization, red fluorescence indicating intact mitochondria membrane) (magnification ×200, *p <0.001 by Student's t test; n = 4–6/group). ALT, alanine aminotransferase; HPRT, hypoxanthine-guanine phosphoribosyl transferase; LPA, 1-oleoyl lysophosphatidic acid; VP, verteporfin; WT, wild-type. (This figure appears in colour on the web.)

insult, evidenced by diminished sALT levels, preserved hepatic architecture, amplified regenerative/anti-oxidative gene program, attenuated hepatocellular necrosis/apoptosis, and abolished innate immune responses. These findings were confirmed in hypoxia-reoxygenation stressed primary mouse hepatocyte cultures, where activation of YAP ameliorated cellular damage and necrosis/apoptosis by augmenting regenerative/anti-oxidative gene programs. As IR-activated HSCs contribute to liver fibrosis, YAP inhibition exacerbated the hepatocellular damage in response to HSC activation, which resulted in prolonged recovery and massive fibrosis. However, YAP activation prevented IRI, which suspended HSC activity and ultimately fibrogenesis. Interestingly, LPA failed to protect the liver against IR insult, and the resulting massive fibrosis, in *Nrf2*-deficient mice. Hence, this translational study highlights the pivotal modulatory role of the YAP pathway in combating IR-hepatocellular damage and maintaining homeostasis in IR-stressed OLTs.

In the clinical arm of our study, post-OLT YAP expression was dramatically increased in 45% (27/60, post-OLT YAP/β-actin >0.95) of human OLTs (exclusion of fatty liver, Fig. S1B), which associated with well-preserved liver histology and improved hepatocellular function. It may imply that YAP signaling is required for self-repair and the maintenance of homeostasis under cellular stress. In agreement with human data, we first

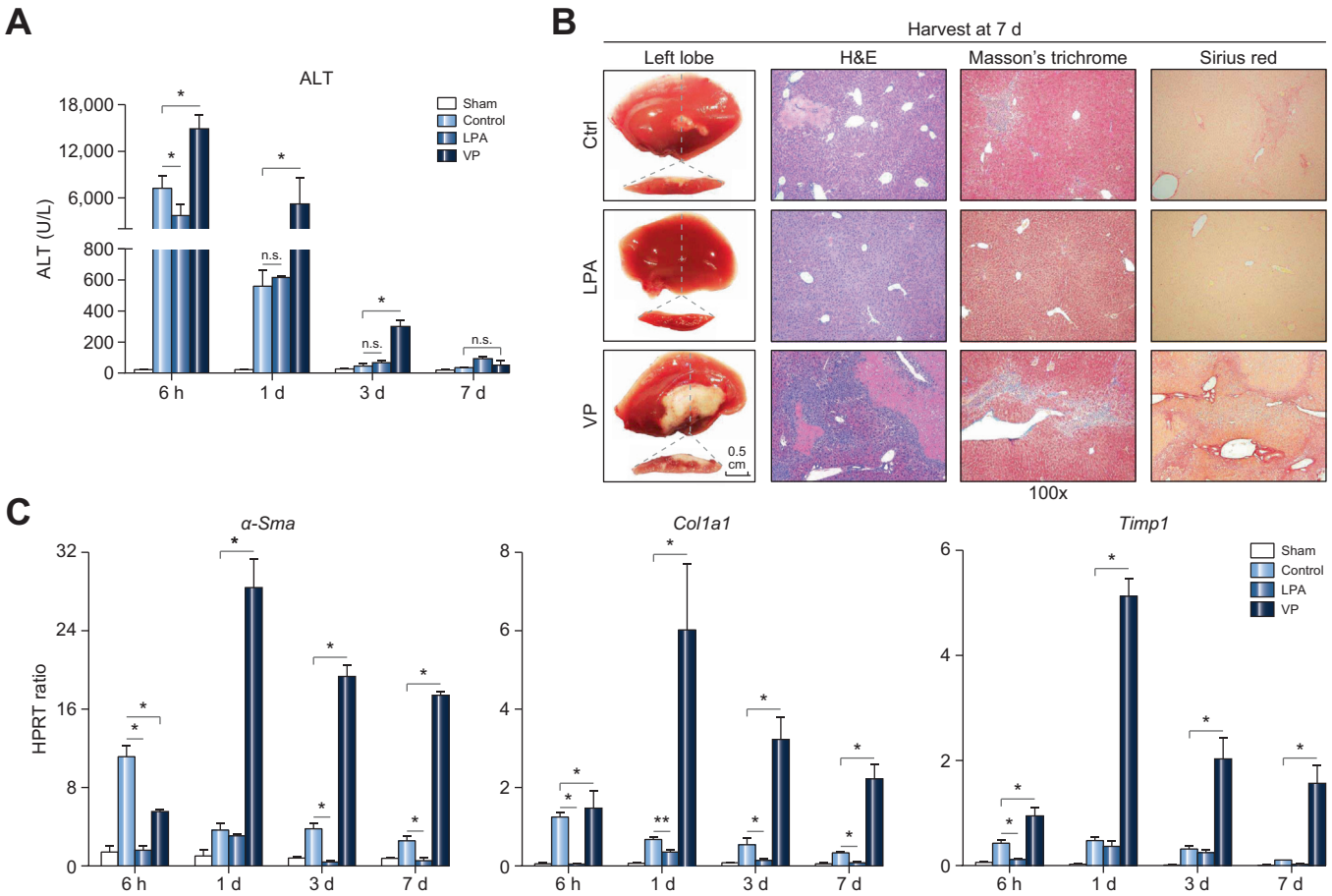

Fig. 5. YAP activation diminished fibrogenesis development after liver IRI. Mice pre-treated with PBS, LPA or VP were subjected to 90 min of warm ischemia, followed by various lengths of reperfusion. Serum and liver samples were collected for: (A) ALT. (B) Histology (7 days after reperfusion): gross morphology (fibrotic change); H&E staining; Masson's trichrome staining (blue color indicating collagen deposition); and Sirius red staining (red color indicating collagen deposition). (C) Fibrosis-related gene expression. (magnification ×100, n.s. represented no significance, $^{**}p$ <0.01, $^{*}p$ <0.001 by Student's *t* test; n = 4–6/group). ALT, alanine aminotransferase; HPRT, hypoxanthine-guanine phosphoribosyl transferase; IRI, ischemia-reperfusion injury; LPA, 1-oleoyl lysophosphatidic acid; VP, verteporfin. (This figure appears in colour on the web.)

found that warm ischemia insult triggered hepatic endogenous YAP expression in mouse livers, the levels of which were elevated between 1–6 h of reperfusion (damage phase). It is plausible that YAP induction progresses in parallel with IR-mediated hepatocellular damage. To mimic liver IRI *in vivo*, we employed hypoxia-reoxygenation stressed primary hepatocyte cultures in which cellular stress mediated intrinsic accumulation of YAP in hepatocyte nuclei. Strikingly, enhancing YAP expression with exogenous LPA attenuated liver IRI, evidenced by diminished sALT levels and well-preserved histology in WT mice, while promoting nuclear YAP production to protect hepatocytes against hypoxia-reoxygenation stress *in vitro*. In marked contrast, treatment with a YAP inhibitor abolished regenerative/anti-oxidative gene expression programs, exacerbated hepatic IRI, and potentiated hypoxia-reoxygenation mediated hepatocellular damage *in vitro*.

Liver IRI combines ischemia-triggered hypoxic cellular stress, and inflammation-mediated reperfusion injury. Endogenous ROS-inflicted tissue damage initiates circulatory disturbances and a cascade of inflammation responses, leading to hepatocyte death.[1–3] Hepatocyte oncotic necrosis and apoptosis, which lead to parenchymal cytodestruction, proceed via DNA degradation detected by the TUNEL assay.[27] We observed mild positive TUNEL^{+} cells and moderate red DHE staining in human OLT recipients in the Low YAP group, which is consistent with relatively high post-OLT sALT levels. To further evaluate the putative role of YAP in liver IRI, we demonstrated that YAP activation facilitated downstream regenerative (*Ctgf, Cyr61,* and *Ankrd1*) and anti-oxidative (*MnSOD* and Catalase) gene programs, diminished ROS production, abolished hepatocyte oncotic necrosis/apoptosis, and decreased CD68^{+} macrophage/Ly6G^{+} neutrophil infiltration and function (IRI signature markers). Conversely, VP-assisted YAP inhibition enhanced hepatic ROS production, increased frequency of TUNEL^{+} cells and augmented CD68^{+} macrophage/Ly6G^{+} neutrophil hepatic sequestration and pro-inflammatory cytokine expression. It is plausible that YAP activation prevents hepatocellular damage by enhancing hepatocellular regeneration and anti-oxidation activity while maintaining hepatocyte integrity against oncotic necrosis and apoptosis. We have previously shown that TIM-4^{+} macrophage-mediated hepatocyte phagocytosis is the initial stimulus of innate immunity in liver IRI.[3] Here, YAP activation prevented hepatocellular damage, which further abolished innate immune responses in liver IRI. In agreement with our *in vivo* data, YAP activation ameliorated ALT release, diminished hepatocyte death, and maintained mitochondrial integrity in hypoxia-reoxygenation insulted primary hepatocyte cultures. These results reinforce the critical role of YAP in promoting

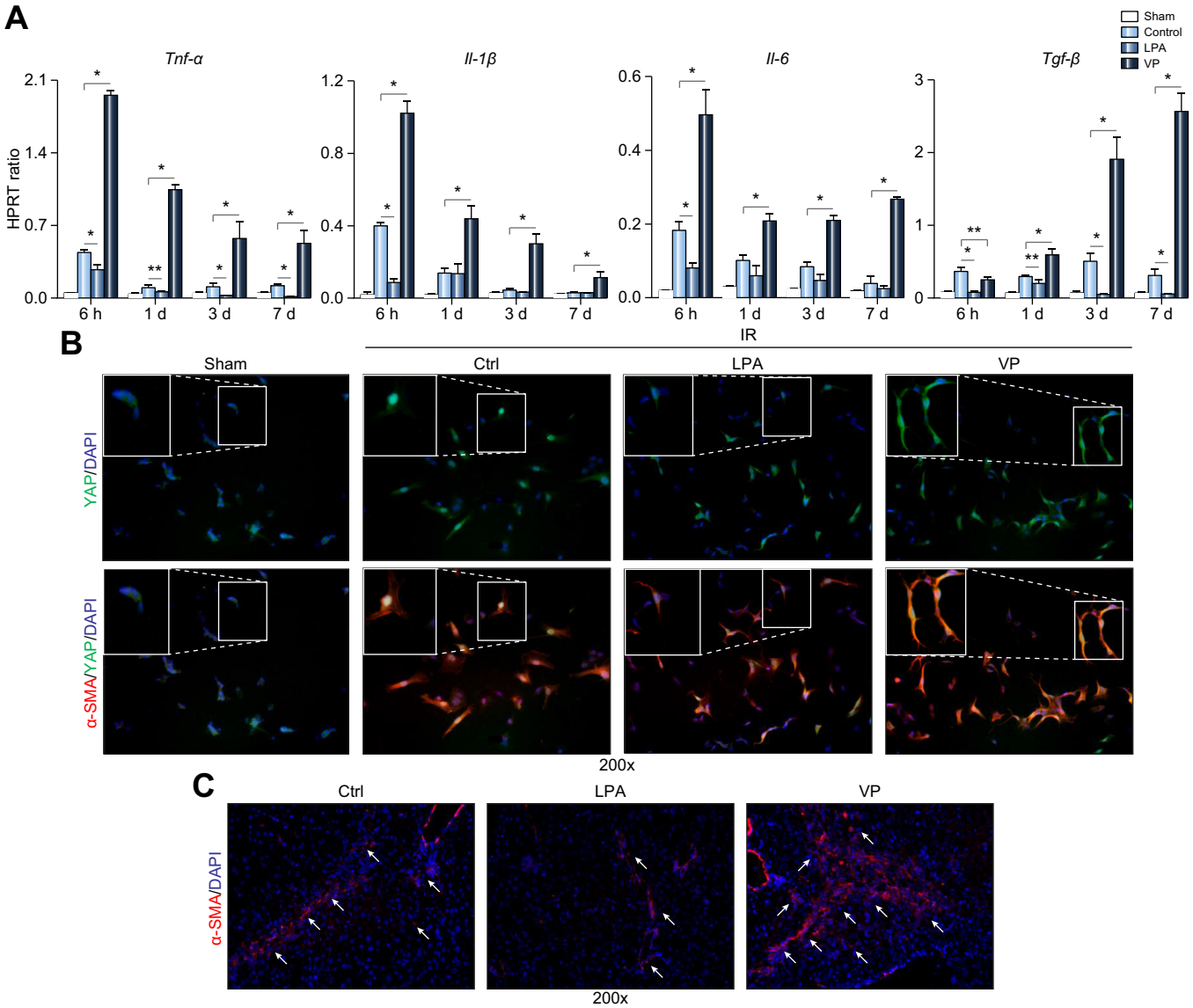

Fig. 6. YAP inhibition exacerbated inflammation and activated HSCs in liver IRI. Mice pre-treated with PBS, LPA or VP were subjected to 90 min of warm ischemia, followed by various lengths of reperfusion. (A) Liver samples were harvested serially between 6 h to 7 days after reperfusion to measure pro-inflammatory cytokine gene inductions. (B) HSCs were isolated from IR-livers at 7 days after reperfusion, and stained for YAP (green fluorescence) and α-SMA (red fluorescence). (C) IR-livers at 7 days after reperfusion stained for α-SMA (red fluorescence) (magnification ×200, $^{**}p$ <0.01, $^{*}p$ <0.001 by Student's *t* test; n = 4–6/group). IRI, ischemia-reperfusion injury; HPRT, hypoxanthine-guanine phosphoribosyl transferase; HSCs, hepatic stellate cells; LPA, 1-oleoyl lysophosphatidic acid; VP, verteporfin. (This figure appears in colour on the web.)

hepatocellular anti-oxidation and regeneration against IR insult, with resultant improvements in liver function. Furthermore, YAP inhibition exacerbated hepatocyte death, confirming that this regulation was YAP dependent.

Fibrosis after liver transplantation is associated with impaired liver function and poor graft survival.[5,7,28] Acute liver injury also leads to fibrosis, which is as an attempt to repair damaged tissue and trigger self-limited protection.[29] In our study, inhibiting YAP resulted in delayed post-IRI liver recovery, while triggering abundant ECM synthesis, massive collagen deposition and fibrosis development. It has been shown that monocyte-derived macrophages are the predominant pro-fibrogenic macrophage population in the liver.[30] In fibrotic liver, macrophages were consistently shown to localize in close proximity to activated myofibroblasts while their depletion attenuated carbon tetrachloride inflicted liver fibrosis.[31,32] TGF-β, produced by macrophages, has been shown to activate myofibroblasts and promote ECM synthesis.[33,34] A recent study showed that macrophage-derived pro-inflammatory TNF-α and IL-1β were essential to maintain myofibroblast survival through NF-κB.[13,14] Here, we found that the severity of IR-mediated liver fibrosis increased along with hepatocellular damage. Indeed, YAP activation prevented hepatocyte damage in liver IRI, limiting fibrosis and suppressing macrophage function. Meanwhile, YAP inhibition exerted opposite effects, *i.e.*, exacerbated IR-hepatocyte and was associated with amplified pro-inflammatory cytokine expression between 6 h and 7 days after reperfusion. In addition, TGF-β was further enhanced, along with fibrogenesis development.

Fate tracing has revealed that HSCs are dominant contributors to liver fibrosis, regardless of the etiology.[9] Stimulated by pro-inflammatory cytokines (TGF-β, TNF-α, IL-1β and IL-6), HSCs

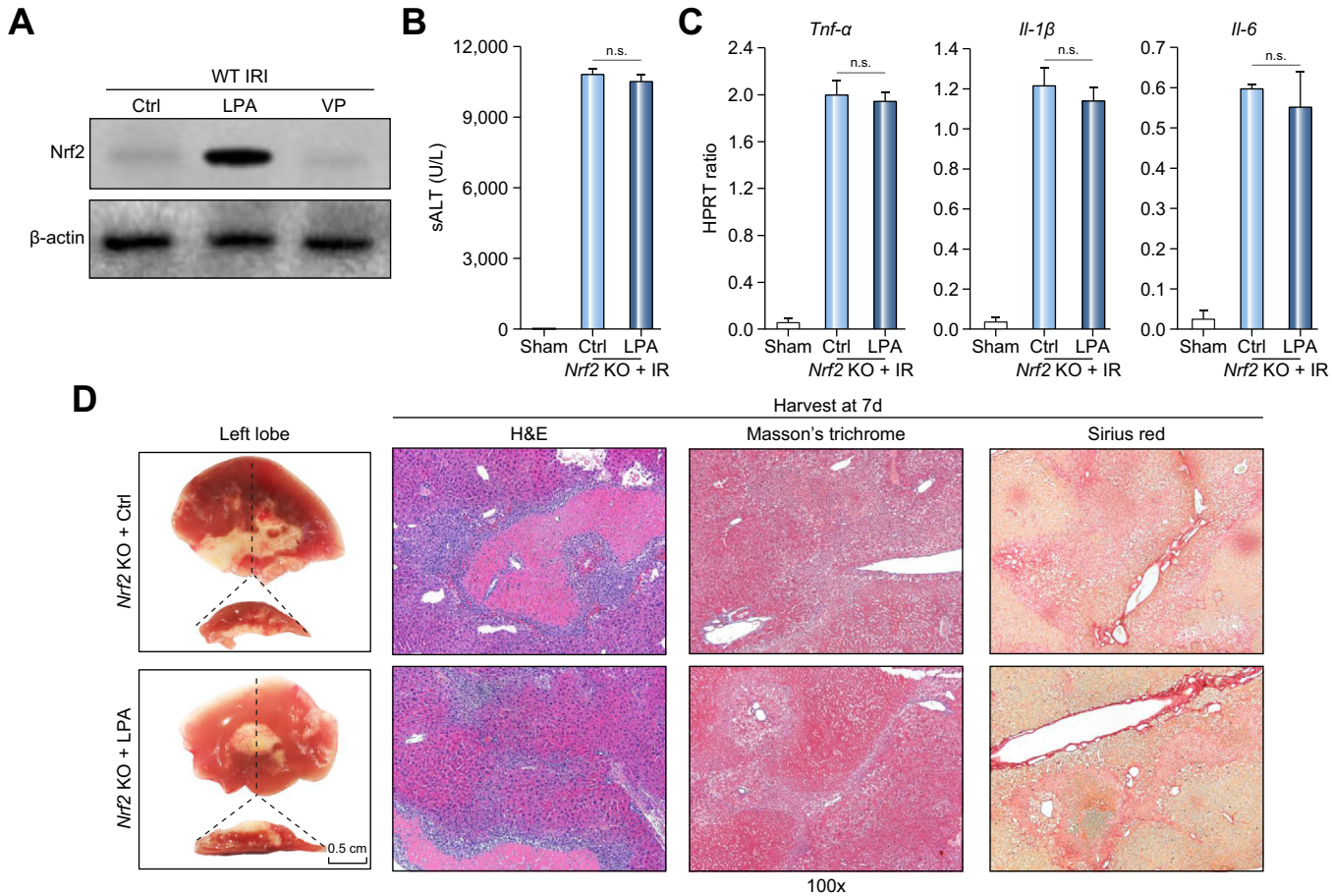

Fig. 7. YAP activation failed to protect *Nrf2*-deficient livers against IR-damage. WT and *Nrf2*-deficient mice pre-treated with PBS, LPA or VP were subjected to 90 min of warm ischemia followed by 6 h or 7 d of reperfusion. Liver and serum samples were collected for analysis. (A) Western blot-assisted Nrf2 expression in WT liver after IRI. In *Nrf2*-deficient mice: (B) sALT levels. (C) qRT-PCR detected gene induction of *Tnf-α*, *Il1b* and *Il6*. (D) Liver histology (7 days after reperfusion): gross morphology (fibrotic change); H&E staining; Masson's trichrome staining (blue color indicating collagen deposition); and Sirius red staining (red color indicating collagen deposition). (magnification ×100, n.s. represented no significance by Student's *t* test; n = 4–6/group). HPRT, hypoxanthine-guanine phosphoribosyl transferase; IRI, ischemia-reperfusion injury; LPA, 1-oleoyl lysophosphatidic acid; qRT-PCR, quantitative reverse transcription PCR; sALT, serum alanine aminotransferase; VP, verteporfin; WT, wild-type. (This figure appears in colour on the web.)

become activated and transdifferentiate to myofibroblast cells, which are characterized by loss of lipid droplets, increased migration and excessive ECM synthesis.[25] A recent study has shown that VP-mediated YAP inhibition attenuated HSC activity in liver IRI.[35] In our murine liver IRI model, HSCs were activated and transferred to myofibroblasts, along with hepatic pro-inflammatory cytokine induction at very early post-reperfusion stage (6 h). Recent studies have shown that YAP was a core regulator in HSC activation/differentiation into myofibroblasts.[36,37] Upregulating YAP in HSCs/myofibroblasts prolonged their activation and promoted ECM synthesis, which led to more severe fibrosis.[36,37] Intriguingly, we found that YAP activation largely protected IR-stressed hepatocytes, by reducing inflammation and inactivating HSCs. Unlike hepatocytes, quiescent HSCs were insensitive to YAP activation (Fig. S4), which resulted in minimal liver fibrosis. On the other hand, YAP inhibition exacerbated hepatocyte damage, with a strong pro-inflammatory response, HSC activation, and liver fibrogenesis. These results imply that HSC-mediated liver fibrosis requires 2 signals: i) the initial stimulation of quiescent HSCs by IR-induced pro-inflammatory cytokines; ii) regulation by YAP activation.

The transcription factor Nrf2, regulating cellular resistance against oxidative stress, has been considered as a "master switch" of intracellular redox homeostasis.[38] We have shown that *Nrf2*-deficient liver grafts were susceptible to IR-mediated damage.[26] It has been shown that crosstalk between Nrf2 and YAP facilitated anti-oxidation in bladder cancer cells.[39] Interestingly, LPA infusion, but not VP preferentially heighten Nrf2 protein production in IR-stressed WT livers. By employing *Nrf2*-deficient mice, we further determined that LPA administration failed to protect the liver against IR-damage, as evidenced by raised sALT levels and a strong innate immune response. YAP activation also failed to attenuate IR-mediated fibrogenesis in *Nrf2*-deficient mice. Hence, Nrf2 signaling is required for YAP activation mediated cytoprotection in liver IRI.

In summary, this study systemically evaluated the pivotal role of YAP in hepatic protection and fibrosis development in liver IRI. Harnessing cytoprotective and fibrogenesis mechanisms by YAP may be essential in the maintenance of hepatic homeostasis *in vivo* by minimizing local organ damage and depressing HSC-dependent fibrosis. As YAP-mediated hepatocellular protection and fibrosis induction was *Nrf2*-dependent,

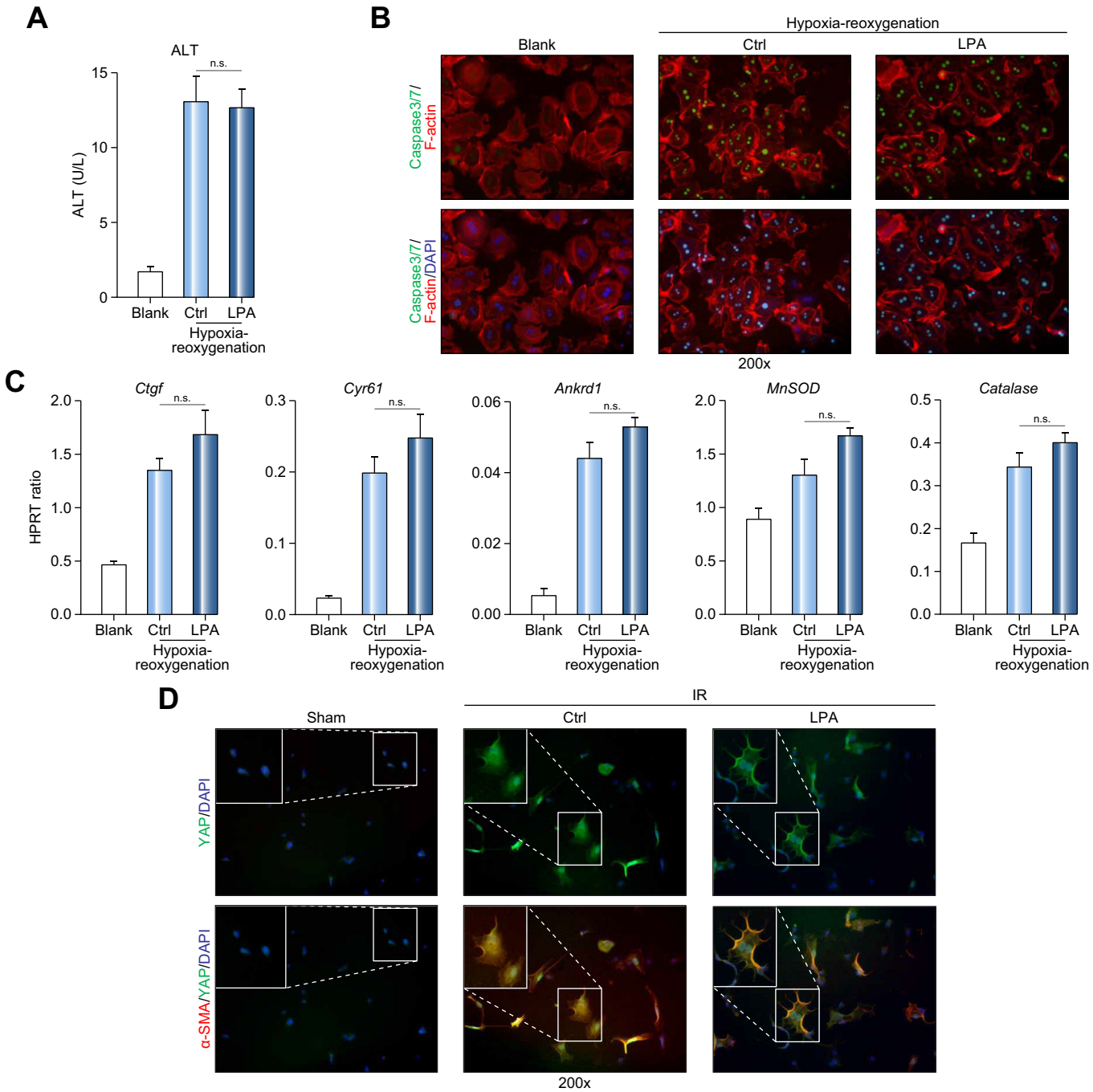

Fig. 8. YAP activation failed to protect *Nrf2*-deficient primary hepatocytes against hypoxia-reoxygenation insult *in vitro*, and failed to suppress *Nrf2*-deficient HSCs in liver IRI. *Nrf2*-deficient mouse hepatocyte cultures were supplemented with PBS or LPA. After 6 h of hypoxia and 8 h of reoxygenation, the cell cultures were analyzed for: (A) Supernatant ALT levels. (B) Caspase-3/7 detection (green fluorescence indicating positive caspase-3/7 in nucleus). (C) YAP downstream gene expression. (D) HSCs were isolated from PBS or LPA-treated IR-*Nrf2*-deficient livers at 7 days after reperfusion, and stained for YAP (green fluorescence) and α-SMA (red fluorescence). (magnification ×200, n.s. represented no significance by Student's *t* test; n = 4–6/group). ALT, alanine aminotransferase; HSCs, hepatic stellate cells; LPA, 1-oleoyl lysophosphatidic acid; VP, verteporfin. (This figure appears in colour on the web.)

our findings provide new insights into the pathogenesis of sterile inflammatory liver damage and fibrosis, while identifying potential novel molecular targets for therapeutic interventions.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work. Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contribution

YL, TL, and CZ participated in the performance of the research and data analysis. JX and ZX participated in the performance of the research. RWB and JWKW participated in article revision.

NX, QX and HJ participated in the research design, discussion and article writing.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.05.029.

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Author names in bold designate shared co-first authorship

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