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Running title: The role of miR-1224 in acute liver failure

Keywords: miR-1224, acute liver failure, cell death, proliferation, biomarker, Nfib, oxidative stress

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Abbreviations

ALF, Acute liver failure; I/R, Ischemia-reperfusion; miRNA, microRNA; APAP, acetaminophen; H_2O_2, Hydrogen peroxide; CCl_4, Carbon tetrachloride; BrdU, 5-bromo-2'-deoxyuridine; AUC, Area under curve; SR, spontaneous recovery; NSR, non-

spontaneous recovery; siRNA, small interfering RNA; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; AU, arbritary units; H/R, hypoxia/regeneration; TNF, tumor necrosis factor; MTT, methyl thiazol tetrazolium; cl. CASP-3, cleaved Caspase-3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; TUNEL, terminal deoxynucleotide transferase nick end labeling; ROS, reactive oxygen species

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Authors Contributions

S.R., C.R. and T.L. conceived and designed the experiments; S.R. performed and analyzed most of the experiments; H.B. and F.W. analysed ALF patient data and performed immunohistochemistry experiments; AT.S., J.G. and M.V. contributed to the performance and analyses of experiments; S.R., C.R. and T.L. wrote the manuscript; C.R., T.L., H.B., F.T., and C.T.. provided intellectual input; H.B. organized patient recruitment and collected human serum and tissue samples.

Conflict of Interest

The authors declare that they have no competing interests.

Abstract

Background and Aims

Patient outcome in acute liver failure (ALF) is crucially determined by the appropriate balance between cell death and compensatory cell proliferation. MicroRNAs (miRNAs) – small non-coding RNAs that function as guide molecules in RNA silencing – have evolved as crucial mediators of nearly all developmental and pathological processes including physiology and disease of the liver. We investigated the role of miR-1224 during ALF.

Methods

We measured miR-1224 in livers of mice in various acute liver disease murine models and in ALF patients using quantitative real-time polymerase chain reaction. We studied the regulation of miR-1224 in AML12 cells and primary hepatocytes upon H_2O_2 stimulation. Cell proliferation and cell death were analysed by BrdU and TUNEL stainings, respectively.

Results

We found that miR-1224 was up-regulated in hepatocytes upon I/R *in vivo* and *in vitro* accompanied by impaired proliferation and elevated apoptosis. This function of miR-1224 was mediated by repressing the anti-apoptotic gene *Nfib* in hepatocytes. Strikingly, miR-1224 was also up-regulated in human livers and serum of ALF patients and indicated an unfavourable prognosis with an excellent prognostic value compared to other known serum markers in this clinical setting.

Conclusions

miR-1224 is a previously unrecognized regulator of proliferation after ALF in hepatocytes and represents a novel and specific biomarker of liver injury with prognostic value in

ALF. Thus, miR-1224 may represent a target for novel therapeutic and diagnostic strategies in the context of ALF and warrants further testing as a biomarker in prospective trials.

Lay summary

In acute liver failure, miR-1224 expression is modulated by oxidative stress that leads to a decrease in hepatocyte cell proliferation and increase in apoptosis. Increase in miR-1224 in serum of ALF patients offers a promising candidate for diagnostic purposes.

Introduction

Acute liver failure (ALF) represents a life-threatening condition associated with high morbidity and mortality. In ALF, liver cell death normally induces compensatory cell proliferation to restore liver mass and avoid loss of organ function [1, 2]. While the balance of liver regeneration and hepatocyte death plays a critical role in the outcome of ALF [3, 4], the underlying regulatory mechanisms linking cell death with compensatory proliferation are still only poorly understood.

MicroRNAs (miRNAs) are small non-coding RNAs of approximately 21-23 nucleotides in length that negatively regulate gene expression at the posttranscriptional or translational level [5]. In the recent past, the number of known miRNAs has grown exponentially, and currently over 2000 miRNAs are identified to be encoded by the human genome [6]. Importantly, it is estimated that about 60 % of all mammalian genes are regulated by miRNAs [7]. Consequently, miRNAs also play an important role in diverse highly regulated processes such as cell differentiation, proliferation and cell death [8]. Since miRNAs can be detected in clinical samples, they also represent promising diagnostic or prognostic biomarkers for various diseases [9].

Altered expression of miRNAs has also been implicated in a variety of liver diseases [10, 11]. It has been demonstrated that miRNAs can regulate proliferation during liver regeneration after partial hepatectomy [12]. Thus, we hypothesized that miRNAs might be involved in the complex regulation of hepatocyte death and subsequent compensatory cell proliferation during ALF. In this study, we demonstrate that several miRNAs are specifically regulated in livers of mice after hepatic ischemia and reperfusion (I/R) injury. Among those, miR-1224 was significantly up-regulated in *in vivo* (I/R, CCl_4, APAP) and *in vitro* (H_2O_2, serum starvation, TNF stimulation) models of liver

failure. In humans, up-regulation of miR-1224 was specific for patients with an unfavourable clinical course and correlated with elevated serum concentrations of miR-1224 in patients that succumbed to death from ALF. Functionally, we show that miR-1224 inhibited expression of *Nfib* in hepatocytes leading to an impaired cell proliferation and increased cell death. Thus, our data suggest that miR-1224 might not only be a candidate for novel treatment strategies in ALF but also might have potential as a biomarker for diagnosis of ALF and guiding therapeutic decisions in the treatment of patients with liver failure.

Materials and methods

Animals

Male C57BL/6J wild type mice (6-8 weeks old) were obtained from the Charles River (Wilmington, MA, USA). Animals were maintained in a pathogen-free facility. All animal experiments were approved by the Federal Ministry for Nature, Environment and Consumers' Protection of the state of North Rhine-Westphalia and were performed in accordance to the respective national, federal, and institutional regulations.

Liver ischemia and reperfusion (I/R) model

We used a well-established mouse model of warm hepatic ischemia followed by reperfusion [13]. Reperfusion was initiated after 75 min of liver ischemia. Sham animals underwent anesthesia and exposure of the portal triad without hepatic ischemia. Mice were sacrificed at the indicated time points and specific samples were taken for further analysis. Only sex-matched animals were compared. To separate serum, mouse blood was collected in EDTA containing tubes and centrifuged at 10,000 rpm for 5 min at room temperature.

CCl_4-induced damage and APAP –induced toxicity

Acute liver damage in mice by CCl_4 and APAP was induced as recently described [14, 15].

Microarray anaylsis

The *mir*Vana™ miRNA isolation kit (Applied Biosystems, Carlsbad, CA, United States) was applied to isolate total RNA according to the manufacturer's protocol, and

subsequently analyzed with a Geniom Real-Time Analyzer (GRTA) (Febit GmbH, Heidelberg, Germany) [16] using the Geniom Biochip miRNA *Mus Musculus* . The total RNA quality was done with the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, United States) using RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, United States). The 260/280 nm absorbance values were consistently above 1.9. The array contained ten replicates of each mouse miRNA and miRNA star (*) sequences as annotated in the Sanger miRBase v.14.0. Sample labelling with biotin was carried out by microfluidic-based enzymatic chip labeling of miRNAs (MPEA) [17]. Following hybridization for 16 h at 42 °C, the biochip was washed as indicated by the supplier and signal enhancement was processed with the GRTA. For each array, signal intensities were calculated using the Geniom Wizard Software (Febit GmbH, Heidelberg, Germany).

Cell culture

Mouse AML12 hepatocyte cell line was kindly provided by Prof. Dr. Steven Dooley (Medizinische Fakultät Mannheim, Mannheim, Germany). AML12 cells were cultured in DMEM/F-12 medium supplemented with 10 % FBS (Invitrogen, CA, USA), 1x insulin-transferrin-selenite (Pan Biotech, Aidenbach, Germany), dexamethasone (Pan Biotech, Aidenbach, Germany), penicillin/streptomycin (Pan Biotech, Aidenbach, Germany). Cells were grown at 37 °C in a humidified atmosphere with 5 % CO_2. For serum starvation, cells were incubated in serum-free medium for the indicated time points. Cells were stimulated with 100 ng/ml TNFα for indicated times and treated with 1 mM H_2O_2 either for indicated times or for 3 h.

Hepatocyte isolation

Hepatocytes were isolated from mouse liver as previously described [18]. Briefly, male C57BL/6 wild type mice (6-8 weeks old) were anesthetized with ketamine-xylazine solution and the liver was perfused with saline solution for 3 min followed by digestion with collagenase and trypsin inhibitor in EBSS (Pan Biotech, Germany) for 10 min. Hepatocytes were centrifuged at 500 rpm for 2 min and washed for several times. Hepatocytes were cultured in DMEM medium supplemented with 10 % FBS and 1 % penicillin/streptomycin at 37 °C in a humidified atmosphere with 5 % CO_2.

Quantitative RT-PCR

For mRNA: Total RNA from tissues and cells was prepared using Trizol reagent according to manufacturer's protocol (Invitrogen, CA, USA). Reverse transcription of total RNA into cDNA was performed using first strand cDNA synthesis kit (Roche Diagnostics, IN, USA). Quantitative Real–Time PCR analysis was performed using SYBR Select Master Mix (Thermofischer Scientific, Waltham, MA, USA) and gene specific primers (Eurofins MWG Operon, Ebersberg, Germany) on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). mRNA expression data were normalized to GAPDH. Primer sequences were listed in Supplementary CTAT.

For miRNA: Total RNA along with miRNA was isolated with Trizol reagent by Direct-zol™ RNA Miniprep (Zymo Research, Irvine, CA). RNA was reverse transcribed using miScript Reverse Transcription kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. A quantitative PCR was performed using miScript SYBR Green PCR kit (Qiagen, Germany) and miScript Primer assays (Qiagen, Germany) for miR-1224 with RNU6 as an internal control. miRNAs was detected on ABI 7500 Fast

real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative expression values were normalized to the internal control by using $2^{-\Delta\Delta Ct}$. Primer sequences were listed in Supplementary CTAT.

miRNA isolation from serum

Isolation of miRNA from serum samples of mice was performed and normalized against SV40 as described [14].

Patients

We investigated sera from 39 ALF patients (mean age 44.7 ± 2.9 years; 30.8 % male). The 39 ALF patients included 18 with spontaneous recovery (SR), and 21 with non-spontaneous recovery (NSR) that died for miRNA-1224 expression (Table 1A). Causes of ALF included drug-induced toxicity (NSR n=6, SR n=9), Budd-Chiari Syndrome (NSR n=3, SR n=0), viral hepatitis (NSR n=2, SR n=5), autoimmune hepatitis (NSR n=1, SR n=2), and unknown etiology (NSR n=9, SR n=2). Sera were collected within days 1 to 2 of hospital admission and stored at -20 °C. In addition, liver tissues from 10 ALF patients (mean age 42.0 ± 5.4 years; 0% male) with SR (n=4) or NSR who revealed liver transplantation (n=3) or died (n=3) were analyzed for miRNA-1224 expression (Table 1B).

Cell transfection

miR-1224 mimic (miR-1224) (MSY0005460), miR-1224 antagomiR (1224ag) (MIN0005460) and AllStars negative control siRNA (1027280) or miScript inhibitor negative control (1027271) (Neg Ctrl) were purchased from Qiagen (Hilden, Germany).

Nfib plasmid was obtained from Biocat (BC014290). AML12 cells or primary hepatocytes were cultured for 24 h and transfected with 50 nM mimics or 100 nM antagomiR using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions for 24 h. To knockdown the expression of *Nfib*, small interfering RNA (siRNA) from Qiagen (Hilden, Germany) was transfected in AML12 cells for 24 h according to the manufacturer's instructions.

Luciferase assay

AML12 cells were seeded in 24-well plate for 24 h and transiently transfected with luciferase constructs (psiCheck-2^{TM}, Promega, Madison, USA) containing 3'UTR of *Nfib* or Insr along with miR-mimics using Lipofectamine 2000 (Invitrogen, CA, USA). After 24 h of transfection, cells were harvested and lysed with 100 µl of passive lysis buffer (Promega, Madison, WI). Lysates were analyzed using dual luciferase assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The luciferase activities were measured and ratios of Firefly luciferase luminescence to renilla luciferase luminescence were calculated. lKKβE/E and IkBα R/R plasmids were gifts from Gilles Courtois, INSERM U1038, iRTSV, CEA, Grenoble, France). Cells were transfected with NF-κB-dependent reporter plasmid (Igκ-luc) as described [19].

Western Blot

Cells were homogenized with NP-40 lysis buffer to obtain protein lysates. Proteins were separated on SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. The membranes were probed with the following antibodies: Cyclin A2 , Cyclin E1, Cyclin D1 , anti-GAPDH.

Cell viability assay

Cell viability was measured by MTT (Thermofischer Scientific, Waltham, MA, USA). MTT according to the manufacturer's instructions.

FAS-induced apoptosis

For *in vitro* apoptosis induction, a final concentration of 0.5 µg/mL Fas/Jo2 antibody was added to the AML12 culture medium.

Caspase activity assay

Caspase activity assay was performed in AML12 cells as described [20]. To measure the activity of Caspase-3 or Caspase-8, 10 µg of protein lysates were subjected to 0.1 µM/ml Ac-DEVD-AFC (Biomol, Hamburg, Germany) or 0.1 µM/ml Ac-IETD-AFC, respectively.

Immunofluorescence

BrdU (B5002, Sigma-Aldrich, St. Louis, MO, USA) was incorporated in AML12 cells and stained with anti-BrdU antibody as described [21]. Cleaved Caspase-3 and Ki-67 staining was performed according to manufacturer's protocol. Coverslips were stained with the secondary antibodies anti-mouse A488 and anti-rabbit A555 and mounted onto slides with VECTASHIELD antifade mounting medium with DAPI

Cell death was measured by terminal deoxynucleotide transferase (dUTP) nick end labelling (TUNEL) staining using *in situ* cell death detection kit (Roche Diagnostics, IN, USA) according to the manufacturer's protocol.

Image acquisition was performed at a magnification of 20x with a Zeiss Axio Imager.Z1 microscope, Axiocam MRm and HRc cameras using Axiovision 4.8 software (Carl Zeiss, Inc., Oberkochen, Germany).

Detection of intracellular ROS

2',7'-Dichlorodihydrofluorescein diacetate (DCHF-DA) dye (D6883, Sigma-Aldrich, St. Louis, MO**,** USA) diluted with DMEM medium was added to AML12 cells or primary hepatocytes and cells were incubated in 5 % CO_2 at 37 °C for 50 min. Cells were fixed with 3 % PFA and washed with PBS and analyzed using fluorescence microscopy (Leica, Germany). ROS fluorescence was quantified with a microplate cell imaging imaging multi-mode reader (Cytation3, Biotek Instruments, Germany) at wavelengths of 488 nm for excitation and 525 nm for emission. Cells were pre-treated with 100 μM BHA for 30 min followed by addition of 1 mM H_2O_2 for 3 h.

Immunohistochemical analysis

Paraffin sections were obtained from the Department of Pathology at Hannover Medical School. Sections were deparaffinized in xylene, followed by rehydration in an ethanol gradient. Endogenous peroxidase was blocked with 3 % hydrogen peroxide in distilled water. For antigen retrieval, slides were boiled for 5 min in antigen-unmasking solution (Vector Laboratories). After washing with 0.05 % Tween-20 in Tris-buffered saline (TBST) nonspecific binding sides were blocked with blocking solution (1 % BSA with horse serum in TBST; VECTASTAIN ABC kit, Vector Laboratories) for 1 h at room temperature. Afterwards, sections were incubated overnight at 4 °C with primary antibodies against NFIB (1:300, sab1402289, Sigma-Aldrich, St. Louis, MO**,** USA). After

repeated washings with TBST, sections were incubated with biotinylated secondary antibody Ab solution (VECTASTAIN ABC kit, Vector Laboratories) for 30 min and covered with avidin-biotin complex reagent (VECTASTAIN ABC Kit, Vector Laboratories) for 1 h at room temperature. Sections were stained in aminoethylcarbazole substrate solution (AEC chromogen kit, Sigma-Aldrich, St. Louis, MO**,** USA) and counterstained with hematoxylin. Finally, sections were covered with mounting medium (Aquamount, VWR International GmbH, Germany). Ki-67 (1:500, Vector Laboratories, #VP-K451) was stained as previously described [2, 11].

Bioinformatic analysis

Predicted targets of miR-1224 are obtained from TargetScan (www.targetscan.org). Gene ontology was obtained from DAVID database (https://david.ncifcrf.gov). The microarray data were obtained from National Center for Biotechnology Information Gene Expression Omnibus database (accession number GSE38941).

Statistical analysis

Differences between groups were assessed by an unpaired two-sample t-test or Mann-Whitney test and multiple comparisons between more than two groups have been conducted by one-way ANOVA with Kruskal-Wallis test for post hoc analysis. Statistical analysis was performed by using Graph Pad Prism version 5.0. Data represent means ± standard error of the mean (SEM). A p-value < 0.05 was considered significant.

Results

miR-1224 is up-regulated in murine acute liver disease models and patients with acute liver failure

To systematically identify miRNAs involved in acute liver failure (ALF) pathophysiology, we applied the well-established model of hepatic ischemia and reperfusion (I/R) injury in mice. We compared miRNA expression profiles in livers from mice after I/R to the expression profiles in livers from control mice by performing microarray analysis on RNA extracts from these livers. Out of 710 miRNAs tested on the microarray, 30 were differentially regulated upon induction of acute liver damage (Fig. 1A). Unsupervised hierarchical clustering analysis among the 30 differentially expressed miRNAs yielded 2 major clusters, with respective sham and I/R samples closely clustering together (Fig. 1A). Out of 30 miRNAs, 27 miRNAs were up-regulated and 3 miRNAs were down-regulated (Fig. 1B) in livers of I/R-mice compared to control animals. The regulation of exemplary miRNAs identified in the array analysis was confirmed by qPCR. As shown in Supplementary Fig. 1A, down-regulation of miR-125b-5p and miR-720 as well as up-regulation of miR-1196, miR-762, miR-1224, miR-705 and miR-223, could be confirmed. Thus, by applying a systematic array-based approach we identified subsets of miRNAs that are differentially regulated during I/R-induced ALF.

Among the miRNAs that were differentially regulated in I/R, miR-1224 fulfilled the criteria of a strong regulation above a fold change of 4 (Fig. 1B) and a high conservation among human and mouse (Supplementary Fig. 1B). Moreover, miR-1224 showed a significant time dependent up-regulation in I/R-induced ALF (Fig. 1C) and correlated with the degree of liver injury according to aspartate transferase (AST) levels as a surrogate for hepatic cell death (Supplementary Fig. 1C). Based on these data, we chose miR-1224

for further investigation in our study. Furthermore, we also tested miR-1224 expression in two different acute liver damage models : CCl_4 and APAP intoxication. In both models, miR-1224 expression was time dependently up-regulated (Fig. 1D and Supplementary Fig. 1D), indicating the specificity of miR-1224 in ALF.

Based on the striking regulation of miR-1224 in murine liver failure, we next attempted to correlate these findings with data from patients. We therefore used samples from explanted livers of patients with ALF from different disease entities as well as samples from healthy patients [11, 14, 22]. Quantitative polymerase chain reaction (qPCR) analysis of miRNA extracts showed a significant up-regulation of miR-1224 in injured livers compared to controls (Fig. 1E). To investigate a potential role of miR-1224 in determining ALF outcome, we used a second cohort of ALF patients that were separated into patients that recovered spontaneously (SR, n= 4) and patients without spontaneous recovery (NSR, n=6) who underwent liver transplantation (n=3) or died (n=3). Compared to SR patients, those with NSR showed a significantly higher expression of miR-1224 (Fig. 1F), suggesting a specific function of miR-1224 in the pathophysiology of ALF in mice and human.

Elevated serum levels of miR-1224 indicate poor survival in patients with ALF

There is an ample evidence that circulating miRNAs can serve as biomarkers for organ damage such as liver failure [23]. We therefore hypothesized that the up-regulation of miR-1224 in injured livers might be associated with the corresponding alterations in miR-1224 serum levels. To test this hypothesis, we measured miR-1224 concentrations in sera from mice after I/R- or sham-surgery. As shown in Fig. 2A, serum levels of miR-1224 were significantly higher in mouse serum upon induction of I/R when

compared to sham-operated mice. Elevated levels of miR-1224 strongly correlated with the established markers of liver failure such as aspartate or alanine transaminase (AST/ ALT) (Fig. 2B), suggesting that miR-1224 might represent a previously unrecognized serum marker for liver cell death. To confirm the hypothesis that injury of hepatocytes is associated with a release of miR-1224 into the extracellular space, we performed *in vitro* hypoxia and reoxygenation experiments (H/R) with primary hepatocytes and analysed miR-1224 concentrations in the supernatants. As seen in Fig. 2C, miR-1224 expression levels in supernatant were significantly higher in cells after induction of H/R compared to controls.

We next isolated miRNA from sera of patients with ALF and analyzed miR-1224 levels of patients that spontaneously recovered (SR, n=18) and patients without SR who died (NSR, n=21). Compared to SR patients, patients who succumbed to death showed significantly higher levels of miR-1224 (Fig. 2D), highlighting that elevated miR-1224 serum levels are associated with an unfavourable disease course in patients with ALF. Furthermore, we compared miR-1224 levels in serum of ALF patients with those of previously published miRNAs ([11]; miR-21, miR-122 and miR-221) and those of differentially regulated miRNAs in the array analysis (Fig. 1A; miR-451, miR-135a* and miR-150). As shown in Supplementary Fig. 2A, there was no significant change in the expression of miR-451, miR-135a* and miR-150 among SR and NSR patients, while (in line to previous results [11]) levels of miR-21, miR-122 and miR-221 were lower in patients that did not recover spontaneously. Of note, no correlation between miR-1224 and any other miRNAs became apparent (Supplementary Fig. 2B), highlighting the specificity of alterations in miR-1224 serum levels in this context. Finally, we performed receiver operating characteristic (ROC) curve analyses to determine the predictive value

of miR-1224 serum concentrations in patients with ALF and calculated an area under curve (AUC) value of 0.861 (Fig. 2E), suggesting that miR-1224 represent a new biomarker to predict the outcome of patients with ALF.

Induction of miR-1224 expression in ALF is dependent on increased oxidative stress

To explore the molecular mechanism involved in the regulation of miR-1224 in ALF, we characterized the expression of this miRNA in different mouse tissues. Low expression of miR-1224 was detected in kidney, lung and spleen, whereas in heart, brain and liver, miR-1224 was expressed at higher levels (Supplementary Fig. 3A). To further dissect the cell-specific regulation of miR-1224 during ALF, we isolated primary hepatocytes and $CD45^+$ immune cells from livers of mice subjected to I/R or sham. Upon I/R, miR-1224 was significantly up-regulated in hepatocytes, whereas no regulation was found in $CD45^+$ immune cells (Fig. 3A), suggesting a specific role of miR-1224 in hepatocytes during ALF.

I/R is associated with a dramatic increase in the release of reactive oxygen species (ROS) [3]. As a common ROS inducer, H_2O_2 is used to mimic I/R injury *in vitro*. In both primary hepatocytes and immortalized murine hepatocytes (AML12), H_2O_2 stimulation resulted in a strong increase of DCF fluorescence as a surrogate for intracellular oxidative stress (Fig. 3B and Supplementary Fig. 3B), accompanied by an increase in miR-1224 expression (Fig. 3C). Similar results were obtained when these cells were serum-starved, representing another *in vitro* model mimicking ROS induction in ALF (Fig. 3D). To confirm that ROS represent the main inducer of miR-1224 expression in hepatocytes, we treated both primary hepatocytes and AML12 cells with the ROS

scavenger BHA (Butylated hydroxyanisole). Indeed, the ROS-dependent increase in miR-1224 expression after H_2O_2 treatment could be completely abolished by pre-treatment with BHA, compared to H_2O_2 -treated cells (Fig. 3E and Supplementary Fig. 3C). Using another antioxidant N-acetylcysteine (NAC), APAP-induced ROS and miR-1224 expression were inhibited in primary murine hepatocytes when compared with APAP-stimulated hepatocytes (Supplementary Fig. 3D).

Tumour necrosis factor (TNF) is one of the well-known pro-inflammatory mediators released during I/R [24] that is confirmed by strong up-regulation of TNF expression in murine livers upon I/R (Supplementary Fig. 4A). TNF is also known to induce ROS production in liver cells [25]. In order to investigate that ROS induce the expression of miR-1224 up-regulation, we stimulated AML12 cells with TNF for different time points. Indeed, the increase in ROS in TNF-treated AML12 hepatocytes is correlated with the up-regulation of miR-1224 at 6 h after TNF stimulation (Supplementary Fig. 4B). This regulation could be prevented by pre-treatment with BHA (Supplementary Fig. 4C), which is in line to our previous result [14] demonstrating that BHA pre-treatment abolished TNF induced ROS formation in hepatocytes. . Since TNF is known to activate the ROS-dependent transcription factor NF-κB [25], we finally investigated whether miR-1224 expression is dependent on NF-κB activation. Therefore, we transfected AML12 cells with dominant kinase mutant IKKβ E/E (constitutively activation of the NF-κB pathway) and the super-repressor IκBa R/R (repression of the NF-κB pathway) (Supplementary Fig. 4D) and found that miR-1224 expression was up-regulated and down-regulated respectively (Supplementary Fig. 4E), suggesting the dependence of miR-1224 expression on NF-κB activity. Collectively, these data suggest that ROS

produced by both H_2O_2 and TNF in injured livers play a driving factor in the activation of miR-1224 expression.

miR-1224 up-regulation decreases cell proliferation in ALF

To investigate the effect of miR-1224 in regulating cell survival, primary hepatocytes and AML12 cells were transfected with miR-1224 mimic for 24 h and treated with H_2O_2 for 3 h (Supplementary Fig. 5A). MTT assay on these cells revealed that overexpression of miR-1224 alone did not alter cell viability, whereas cells overexpressing miR-1224 showed an impaired viability upon H_2O_2 treatment compared to All stars negative control (Neg ctrl) transfected cells (Fig. 4A). In line with the previous result, knockdown of miR-1224 expression (Supplementary Fig. 5B) was associated with a increased cell viability (Supplementary Fig. 5C). It is known that ALF-induced compensatory cell proliferation is correlated with an impaired clinical outcome. Immunohistological analysis of liver biopsies from patients with ALF showed significantly lower numbers of $Ki67^+$ cells in those patients that did not recover compared to patients with a more favourable clinical course (Fig. 4B). Similarly, lower numbers of $Ki67^+$ cells became apparent upon miR-1224 transfection in H_2O_2-treated cells compared to control (Neg Ctrl) cells (Fig. 4C), providing a mechanistic link between elevated miR-1224 expression and impaired patients prognosis in the context of ALF. To determine how miR-1224 affects cell cycle progression in this context, we performed pulse labelling studies using BrdU in H_2O_2-treated AML12 cells. The percentage of $BrdU^+$ cells was dramatically decreased in miR-1224 transfected H_2O_2-treated cells, supporting the involvement of miR-1224 in regulating the cell cycle transition from G1 to S phase (Fig. 4D). Moreover, expressions of Cyclin D1, Cyclin A2 and Cyclin E1 were reduced in miR-1224 overexpressing in

combination with H_2O_2-treated AML12 cells (Fig. 4E and Supplementary Fig. 5D). The expression of cyclins was increased and rescued by treating cells with miR-1224 antagomiR only or in combination with H_2O_2, respectively when compared to All stars negative control (Neg ctrl) transfected cells (Supplementary Fig. 5D). Collectively, these data demonstrate a role of miR-1224 in the regulation of hepatocyte proliferation during ALF.

To reinforce the role of miR-1224 in regulating liver cell injury during ALF, we next investigated whether these above results could be reproduced in a second model for induction of in vitro liver cell injury. We therefore treated both AML12 cells and primary hepatocytes with acetaminophen (APAP), leading to a decrease in cell viability (Supplementary Fig. 6A), associated with an increase in miR-1224 expression (Supplementary Fig. 6B). In line to our previous data on H_2O_2, inhibition of miR-1224 prevented from APAP induced cell death, while up-regulation of miR-1224, in turn, caused a further decrease in cell viability, both in AML12 cells and primary hepatocytes (Supplementary Fig. 6C). Thus, similar to H_2O_2, miR-1224 antagonism protects against APAP-induced hepatocyte toxicity, as a clinically relevant model of induction of ALF.

miR-1224 up-regulation promotes apoptosis in ALF

We hypothesized that miR-1224 might also be involved in the regulation of apoptosis during ALF. Therefore, we transfected AML-12 hepatocytes with miR-1224 or control vector and stimulated them with H_2O_2. TUNEL stainings were performed to measure the amount of cell death. In these analysis, the percentage of $TUNEL^+$ cells was significantly increased in miR-1224 transfected, H_2O_2-treated cells in comparison to H_2O_2-treated control-transfected (Neg Ctrl) cells (Fig. 5A), suggesting a role of miR-1224 in the

regulation of cell death during ALF. We next analysed the cleavage of Caspase-8 and its downstream effector Caspase-3 to deduce the influence of miR-1224 in this process. Notably we found significantly higher numbers of cl. Caspase-3^+ cells in miR-1224 transfected-H_2O_2-treated cells compared to control-transfected (Neg Ctrl) cells (Fig. 5B). Similarly, activities of both Caspase-3 and Caspase-8 were increased upon transfection with miR-1224 in combination with H_2O_2 treatment (Fig. 5C), suggesting a yet unknown function of miR-1224 in the regulation of cell death during ALF. Recently, an up-regulation of miR-1224 in livers of mice after treatment with a Fas antibody (anti-CD95, clone Jo2) as an inducer of pure apoptosis was reported [26]. In line, we found higher levels of miR-1224 in Jo2- treated AML12 hepatocytes (Fig. 5D). Strikingly, miR-1224 promoted Fas-induced apoptosis in these cells, suggesting a specific role of miR-1224 in the pathophysiology of apoptosis during ALF (Fig. 5E), which is in line to our previous results that indicate an impaired prognosis of patients with a strong up-regulation of miR-1224 during ALF (Fig. 1E).

Nfib is a direct target of miR-1224

Next we tested whether miR-1224 is able to modulate the expression of genes involved in the regulation of cell proliferation and death. Possible miR-1224 targets (Supplementary Table 1) were identified by using the publicly available algorithm TargetScan that resulted in 43 unique targets common to both human and mice. In order to find proliferation-related target of miR-1224, gene ontology terms were searched on these 43 targets. Furthermore, proliferation-related 13 targets were examined for their expression in ALF patients (GSE38941) (Supplementary Fig. 7A). Out of 13 targets, 5 genes were downregulated in ALF patients that were further tested in RNA extracts from

livers of mice after I/R or sham surgery. Out of these, only *Nfib* and Insr were found to be signifcaintly down-regulated in injured livers, while all other putative target genes were not regulated (Fig. 6A).

To verify that *Nfib and Insr* are direct targets of miR-1224, we generated a luciferase reporter plasmid containing the 3'UTR of *Nfib* or *Insr* flanking the putative miR-1224 binding sites. This dual luciferase reporter assay revealed that miR-1224 significantly reduced the luciferase activity of *Nfib* construct when compared to miR-neg-transfected cells (Fig. 6B and Supplementary Fig. 7B). Moreover, mRNA and protein level of *Nfib* were dramatically down-regulated in cells transfected with miR-1224 compared to control-transfected cells (Fig. 6C). Consistent with the data from mice after I/R surgery, treatment with serum starvation (Supplementary Fig. 8A) as well as H_2O_2 (Supplementary Fig. 8B) significantly down-regulated *Nfib* level in AML12-hepatocytes and a further reduction in *Nfib* expression was observed when cells were transfected with miR-1224 in combination with H_2O_2 (Fig. 6D). To translate these data to the human situation, we analysed expression of *NFIB* in liver samples from patients with ALF or healthy controls. Both genes were down-regulated in ALF patients, suggesting that miR-1224 negatively regulates *NFIB* in humans (Fig. 6E). We hypothesized that, similar to miR-1224, *Nfib* might also be predictive for the clinical course in patients with ALF. Therefore, we evaluated the protein level of NFIB in liver tissues of ALF patients with SR or NSR. In agreement with increased miR-1224 levels (Fig. 1E), we found decreased levels of NFIB (Fig. 6F) in liver tissues of patients with an unfavourable outcome (NSR) when compared to patients that recovered (SR). Taken together, *Nfib* and *Sp1* are new targets of miR-1224 and determine the prognosis of patients with ALF.

Nfib regulates cell proliferation and cell death in H_2O_2 –treated hepatocytes

We next assessed whether down-regulation of *Nfib* in hepatocytes would be sufficient to recapitulate the anti-proliferative and pro-apoptotic effects of miR-1224 in ALF. To test this, we used siRNA to knockdown *Nfib* gene in AML12 cells, leading to decreased RNA and protein level of Nfib (Fig. 7A). Knockdown of Nfib significantly reduced the cell viability in H_2O_2-treated AML12 cells when compared with control-transfected (Neg Ctrl) cells (Fig. 7B). To confirm that the effect of miR-1224 was specifically mediated by *Nfib,* cells were transfected with miR-1224 antagomir (miR-1224ag) and siRNA for *Nfib*. Strikingly, co-transfection of siNfib abolished the increase in cell viability upon miR-1224 down-regulation (Fig. 7C). Furthermore, to confirm the function of Nfib, we transfected Nfib plasmid in AML12 cells alone or co-transfected with miR-1224 and cell viability was measured. As shown in Fig. 7D, cell viability was increased on addition of Nfib plasmid that was rescued by overexpressing miR-1224 in Aml12 cells. These results confirm that miR-1224 acts on cell viability mediated by Nfib.

On a functional level, these results were further confirmed by Ki67- and BrdU-staining experiments (Fig. 7E), which revealed a significant inhibition in cell proliferation in cells transfected with *Nfib* and treated with H_2O_2. Next, we tested whether knockdown of *Nfib* is involved in the regulation of cell apoptosis. As shown in Fig. 7F, inhibition of the genes increased the number of $TUNEL^+$ cells in H_2O_2-treated AML12 cells. In addition, *Nfib* siRNA led to an increase in Caspase activity (Fig. 7E and Supplementary Fig. 9), implying that high expression level of miR1224 inhibit cell proliferation and cause cell death by regulating *Nfib* in ALF.

Discussion

This study provides the first evidence on a functional role of miR-1224 in murine and human acute liver failure. We demonstrate that miR-1224 is up-regulated in both livers and serum of patients with liver failure and directly correlates to the degree of liver injury and hepatic cell death. On a cellular level, we showed that up-regulation of miR-1224 within the liver is restricted to hepatocytes. Moreover, miR-1224 acts as a novel regulator of cell death and proliferation by targeting *Nfib*, representing established regulators of the cell cycle. Thus, miR-1224 holds clinical promise as a new therapeutic target and biomarker in the context of ALF.

Deregulation of different miRNAs including miR-1224 was previously shown in liver tissues affected by acetaminophen (APAP) exposure [27] as well as in livers from mice fed with methionine-choline-deficient (MCD) diet [28]. These studies support our observation on a previously unknown role of miR-1224 in the pathogenesis of acute and chronic liver diseases. Besides the liver, up-regulation of miR-1224 was also found in experimental models of acute kidney injury [29] as well as in patients after cerebral ischemia [30]. Together with our results, these previous data shed new light on a possible common paradigm regarding how miR-1224 regulates cell injury in different organs. I/R is a complex process which involves release of several mediators including TNF and ROS which are responsible for liver injury. Therefore, not one mediator but a cascade of autocrine and paracrine signals are the drivers of liver injury [24]. A direct evidence of ROS production by H_2O_2 , TNF and APAP has been provided by DCF fluorescence and co-treatment with antioxidants. Inspite of the low sensitivity of primary hepatocytes to TNF stimulation, we could show that TNF has an impact on miR-1224 expression as shown previously [31]. I/R-related liver damage induces different forms of

cell death, namely apoptosis and necrosis. By using an array-based approach, we identified a panel of 30 miRNAs deregulated in the context of I/R. This set of miRNAs demonstrated a striking overlap with a recently published panel of miRNAs deregulated in hepatocytes from mice that were subjected to Fas Ligand-stimulation as an apoptotic model of cell death in the liver ([26]; Supplementary Fig. 10). Notably both arrays were performed using the same platform leading to a high level of comparability between the both studies. Within this subset of miRNA with a likely role in cell death and more specifically apoptosis, miR-1224 was also up-regulated after treatment with H_2O_2 in hepatocytes. miR-1224 expression directly correlates with AST/ALT serum levels in mice and the amounts of $TUNEL^+$ and $cl.Casp\text{-}3^+$ cells in hepatocytes as surrogate for apoptotic cell death. Increased amounts of apoptotic cells were recently described in patients with an unfavourable clinical outcome compared to other patients [32]. In line, extensive TUNEL reactivity was observed in liver samples of NSR patients [33] as well as in a model of APAP-induced liver damage [34], further arguing for a role of miR-1224 in promoting cell death. In our study, NSR patients with higher miR-1224 expression demonstrated a significant impaired prognosis compared to other patients.

To further explore the functional relevance of our findings, we investigated whether differences in miRNA expression are reflected by a different expression of selected target genes in liver tissue of ALF patients. We identified *Nfib* as a direct target of miR-1224 in ALF. NFIB is involved in the regulation of cell death, especially apoptosis in various cancers [35-37]. In line with increased miR-1224 expression, we found significantly decreased *Nfib* expression in liver tissues of spontaneously recovered (SR), compared to nonspontaneously recovered (NSR) patients. Since miRNAs can regulate hundreds of target genes, and each gene can be regulated by several miRNAs we

cannot exclude that additional miRNAs or other mechanisms might be involved regulating liver cell death and regeneration. Nevertheless, our data strongly argue for a role of miR-1224 in signalling pathways integrating liver cell death and regeneration in ALF.

Recently, we demonstrated that serum miRNA levels might reflect pathophysiological processes with a direct influence on the prognosis of patients with liver diseases. In this present study, we detected significantly elevated serum levels of miR-1224 in patients with ALF compared to healthy controls. Notably, within the group of ALF patients, elevated miR-1224 levels were restricted to those patients with an unfavourable clinical course, while patients that recovered from ALF demonstrated almost normal miR-1224 levels. While concordant changes of miR-1224 in serum and tissue were previously shown in a mouse model of kidney injury [29], plasma miR-1224 was down-regulated in contrast to elevated levels in tissue subjected to APAP intoxication [27]. These divergent studies suggest that the plasma/ tissue equilibrium of miRNAs might vary with distinct pathogeneses of liver diseases. Despite the currently unknown mechanism of miRNA regulation in the serum, the striking regulation of miR-1224 in the serum of ALF patients might have implications for clinical aspects of ALF. Of note, a prognostic function of elevated miR-122, miR-21, and miR-221 concentrations in patients with ALF was recently demonstrated [11]. In our study, miR-1224 showed an outstanding performance in predicting patients prognosis (AUC, 0.861) (Fig. 2E), which was even superior compared to these recently published markers (AUC, 0.6-0.7). Nevertheless, since serum miR-1224 concentrations were significantly different but still showed some overlap SR and NSR patients, it is likely that not one miRNA but detection of a whole panel might provide the necessary sensitivity

and specificity for discriminating between both groups. Thus, when considering miR-1224 as a new prognostic marker in ALF, analysis on larger patient cohorts with distinct hepatic disease-causes and differential clinical states will have to be analyzed to further test the potential of miR-1224 levels in the serum as biomarkers for detection or monitoring of ALF. Moreover, *in vivo* analysis using miR-1224 knock-out mice applying transient knock-down of miR-1224 would further reinforce the role of miR-1224 in the pathophysiology of liver injury.

Together, our data show that intra- and extracellular miR-1224 is up-regulated in human patients with ALF and in mice after I/R mimicking ALF. This translational impact might help to make a rational selection of the suitable candidates for liver transplantation.

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Figure legends

Fig. 1. Expression profiles of miRNAs deregulated in I/R

(A) Microarray analysis for miRNA was performed with RNA extracts from livers of mice after I/R- or SHAM surgery (n = 4 per group). Hierarchical cluster analysis of the significantly regulated miRNAs bright green = low expression; black = no difference in expression; bright red = high expression. (B) Table of deregulated miRNAs from dispalyed microarray analysis. (C) Expression of miR-1224 in livers from C57Bl/6 mice after I/R or sham surgery for different time points was analysed by qRT-PCR (n = 6 per group). (D) Expression levels of miR-1224 in livers from C57Bl/6 mice after 24 hCCl_4 and 24 h APAP treatment was analysed by qRT-PCR (n=5 per group) (E) Expression levels of miR-1224 in explanted livers from patients with ALF (n = 5) and healthy controls (n = 5) were analysed by qRT-PCR. (F) Expression of miR-1224 was analysed by qRT-PCR in patients that recovered from ALF (SR; n = 4) compared to non-recovered patients (NSR; n=6). Results are shown as mean ± SEM, *p < 0.05; **p < 0.01, ns = not significant, Student's *t* test.

Fig. 2. Elevated serum levels of miR-1224 indicate poor survival in patients with ALF.

(A) Expression levels of miR-1224 in serum were determined by qRT-PCR in samples from mice after I/R or sham surgery (n = 6 per group), ANOVA with Kruskal-Wallis test (B) Spearman rank correlation analysis of miR-1224 expression and serum AST or ALT concentrations in serum from mice (n = 6). (C) miR-1224 expression were determined by qRT-PCR in supernatants of primary hepatocytes after hypoxia and reoxygenation

(H/R) and control cells (n = 6 per group). (D) miR-1224 expression in serum was determined by qRT-PCR in patients with ALF and are depicted with respect to patients' fate [spontaneous recovery (SR) (n = 18), death (NSR) (n = 21)]. (E) Receiver operating curve analysis displaying the diagnostic power of miR-1224 serum concentrations in predicting patients fate (area under the curve/AUC 0.861). Results are shown as mean ± SEM, *$p < 0.05$; **$p < 0.01$, Student's *t* test.

Fig. 3. miR-1224 expression is up-regulated under oxidative stress.

(A) Relative expression of miR-1224 was determined by qRT-PCR in hepatocytes and $CD45^+$ cells isolated from livers of I/R or sham-treated C57Bl/6 mice (n = 4 per group). (B) Intracellular ROS production was determined in primary hepatocytes and AML12 cells after 3 h H_2O_2 treatment by DCF fluorescence (n = 3 per group). (C) miR-1224 expression was analysed by qRT-PCR in primary hepatocytes and AML12 cells treated with H_2O_2 for different time points (n = 3 per group). (D) miR-1224 expression was measured by qRT-PCR in serum-starved primary hepatocytes and Aml12 cells for different time points (n = 3 per group). (E) miR-1224 was measured by qRT-PCR in primary hepatocytes and AML12 cells pre-treated with BHA for 30 min and incubated with or without H_2O_2 for 3 h (n = 3 per group). Results are shown as mean ± SEM, *$p< 0.05$; **$p < 0.01$, ***p<0.001, ns = not significant, ANOVA with Kruskal-Wallis test.

Fig. 4. Elevated expression of miR-1224 is associated with impaired cell survival and proliferation in ALF.

(A) Primary hepatocytes and AML12 cells were transfected with miR-1224 or negative control (Neg Ctrl) for 24 h and treated with or without H_2O_2 for 3 h. Cell viability was

measured by MTT assay. (B) Immunohistochemical (IHC) analysis of $Ki67^+$ cells in liver tissues of ALF patients with SR or NSR. (n = 3 per group). (C,D) IHC analysis and quantification of $Ki67^+$ cells (pink) (C) and BrdU (green) (D) in AML12 cells transfected with or without miR-1224 and treated with H_2O_2 compared to negative control (Neg Ctrl) cells. (E) Western blot analysis on extracts from AML12 cells transfected with or without miR-1224 followed by treatment with H_2O_2 compared to negative control (Neg Ctrl) cells. All experiments are performed in triplicates. Results are shown as mean ± SEM, * $p< 0.05$; ** $p < 0.01$, ***p<0.001, ns = not significant, ANOVA with Kruskal-Wallis test.

Fig. 5. Overexpression of miR-1224 promotes apoptosis in AML12 cells.

(A,B) IHC analysis and quantification of $TUNEL^+$ (green) (A) and cl. $Casp\text{-}3^+$ (red) in AML12 cells transfected with or without miR-1224 and treated with H_2O_2 compared to negative control (Neg Ctrl) cells. (C) Caspase activity in AML12 cell lysates was determined using the Caspase-3 substrate DEVD-AMC and the Caspase-8 substrate DEVD-AFC. (D) AML12 cells were treated with Fas Agonist for 12 h and relative expression of miR-1224 was measured by qRT-PCR (n = 3 per group). (E) AML12 cells were transfected with control (Neg Ctrl), miR-1224 or miR-1224ag and treated with Fas Agonist. Cell viability was measured with MTT assay. All experiments are performed in triplicates. Results are shown as mean ± SEM, * $p < 0.05$; ** $p < 0.01$, ns = not significant, ANOVA with Kruskal-Wallis test.

Fig. 6. *Nfib* is a direct target of miR-1224.

(A) Schematic diagram illustrating the selection of putative targets of miR-1224 whose expression was analysed by qRT-PCR RNA extracts from livers of mice after I/R or

sham surgery (n = 6 per group). (B) Luciferase assay performed on AML12 cells transfected with *Nfib*-3'UTR reporter plasmid together with miR-1224 or negative control (Neg Ctrl) (n = 3 per group). (C) Expression of *Nfib* was analysed by qRT-PCR and Western Blot from AML12 cells and Huh7 cells, respectively transfected with miR-1224 or negative control (Neg Ctrl). (D) Expression of *Nfib* was analysed by qRT-PCR in AML12 cells transfected with or without miR-1224 and treated with H_2O_2 compared to negative control (Neg Ctrl) cells (n = 3 per group). (E) Expression of *NFIB* was measured by qRT-PCR from liver samples of patients with ALF (n = 6) as well as in healthy controls (n = 4). (F) IHC analysis and quantification of *NFIB* in liver tissues of ALF-patients with SR or NSR (n = 3 per group). Results are shown as mean ± SEM, $*p < 0.05$; $**p < 0.01$, ***p < 0.001, ns = not significant, Student's *t* test.

Fig. 7. Nfib modulates cell proliferation and cell death in AML12 cells.

(A) Expression of *Nfib* was measured by qRT-PCR and Western Blot in AML12 cells and Huh7 cells, respectively after transfection with the respective siRNAs for 24 h. (B) AML12 cells were transfected with Nfib siRNA alone or treated with H_2O_2 for 3 h. Cell viability was assessed by MTT assay after treatment. (C) AML12 cells were transfected with miR-1224ag in combination with Nfib siRNA. Cell viability was measured by MTT assay. (D) Cell viability was measured in cells transfected with Nfib plasmid along with miR-1224. (E) IHC analysis and quantification of $Ki67^+$ (pink), $BrdU^+$ (green), $TUNEL^+$ (green) and cl. Casp-3^+ (red) in AML12 cells transfected with siRNAs against *Nfib* and treated with H_2O_2 compared to negative control (Neg Ctrl) cells. All experiments are performed in triplicates. (F) IHC analysis and quantification of $TUNEL^+$ (green) and cl. Casp-3^+ (red) in AML12 cells transfected with siRNAs against *Nfib* and treated with H_2O_2

compared to negative control (Neg Ctrl) cells. All experiments are performed in triplicates. Results are shown as mean ± SEM, $*p < 0.05$; $**p < 0.01$, ***p < 0.001, ns = not significant , ANOVA with Kruskal-Wallis test.

Table

Table 1A. Demographics and Clinical Features of ALF Patients (Serum Cohort)

Characteristics	ALF Total	ALF NSR	ALF SR
No. of patients (n)	39	21	18
Age (y)	44.9 ± 2.9	55.5 ± 3.1	32.4 ± 3.1**
Male sex (%)	30.8	28.6	33.3
AST (U/l)	4107.1 ± 1154.0	4713.0 ± 2074.0	3400.3 ± 700.7
ALT (U/l)	3269.2± 377.9	2687.3 ± 471.9	3948.1± 579.5
Bilirubin (µmol/L)	181.1 ± 24.0	219.6 ± 36.9	136.3 ± 26.6

** $p<0.01$. Abbreviations: ALF, acute liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NSR, non-spontaneous recovery; SR, spontaneous recovery

Table 1B. Demographics and Clinical Features of ALF Patients (Liver Tissue Cohort)

Characteristics	ALF Total	ALF NSR	ALF SR
No. of patients (n)	10	6	4
Age (y)	42.0 ± 5.4	49.2 ± 7.5	31.3 ± 3.7
Male sex (%)	0.0	0.0	0.0
AST (U/l)	1589.1 ± 285.5	1236.0 ± 390.6	2118.8 ± 272.5
ALT (U/l)	1885.0 ± 369.4	1691.2 ± 548.0	2175.8 ± 475.0
Bilirubin (µmol/L)	223.7 ± 55.3	240.5 ± 73.0	198.5 ± 96.2

Abbreviations: ALF, acute liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NSR, non-spontaneous recovery; SR, spontaneous recovery

Figure 1

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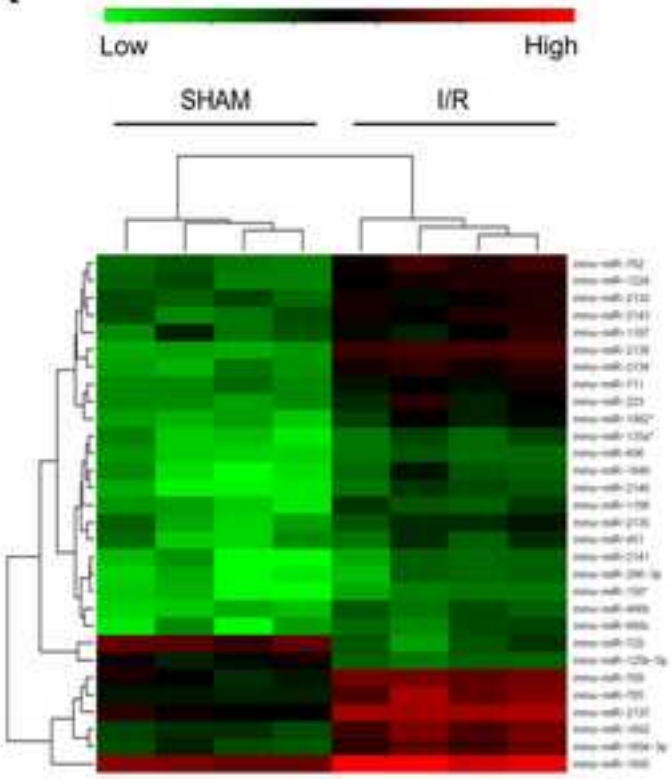
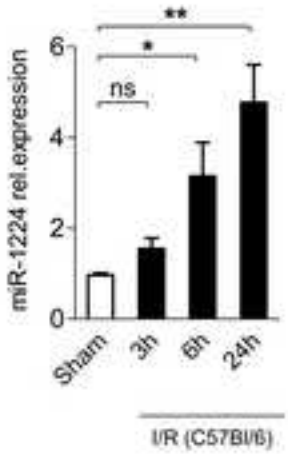
A



B

miRNAs dysregulated in hepatic I/R					
Upregulated miRNAs (n=27)			Downregulated miRNAs (n=3)		
miRNA	Fold change	p-value	miRNA	Fold change	p-value
miR-2134	17,26	0,01	miR-125b-5p	0,23	0,05
miR-762	11,15	0,02	miR-2135	0,17	0,03
miR-1892	10,70	0,05	miR-720	0,07	0,01
miR-1894-3p	10,19	0,04			
miR-2137	9,57	0,001			
miR- 2146	9,51	0,03			
miR-1982*	8,33	0,03			
miR-711	7,78	0,03			
miR-709	7,78	0,002			
miR-1224	7,72	0,01			
miR- 2143	6,82	0,01			
miR-1895	6,02	0,02			
miR-2141	5,77	0,04			
miR-296-3p	5,61	0,05			
miR-2132	5,24	0,02			
miR-1196	5,02	0,02			
miR-135a*	4,95	0,05			
miR-466h	4,85	0,01			
miR-669c	4,75	0,05			
miR-150*	4,72	0,03			
miR-696	4,62	0,01			
miR-451	4,14	0,09			
miR-705	9,00	0,12			
miR-223	6,77	0,13			
miR-1940	6,72	0,16			
miR-1187	4,25	0,12			

C



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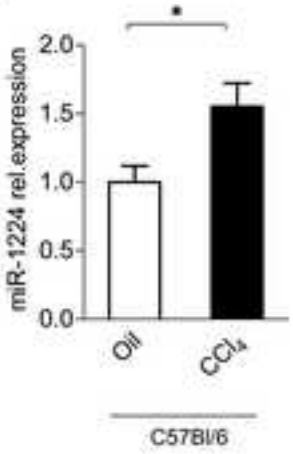

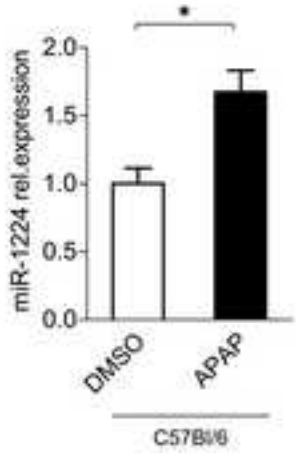

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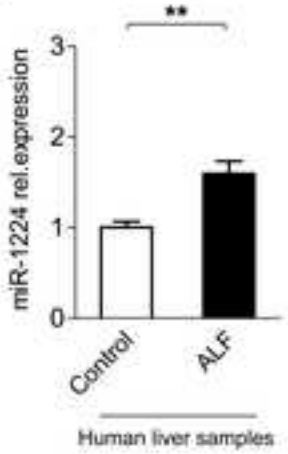

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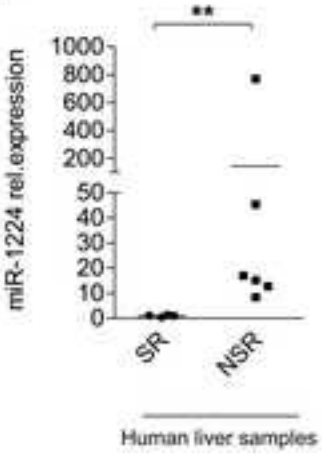

Figure 2

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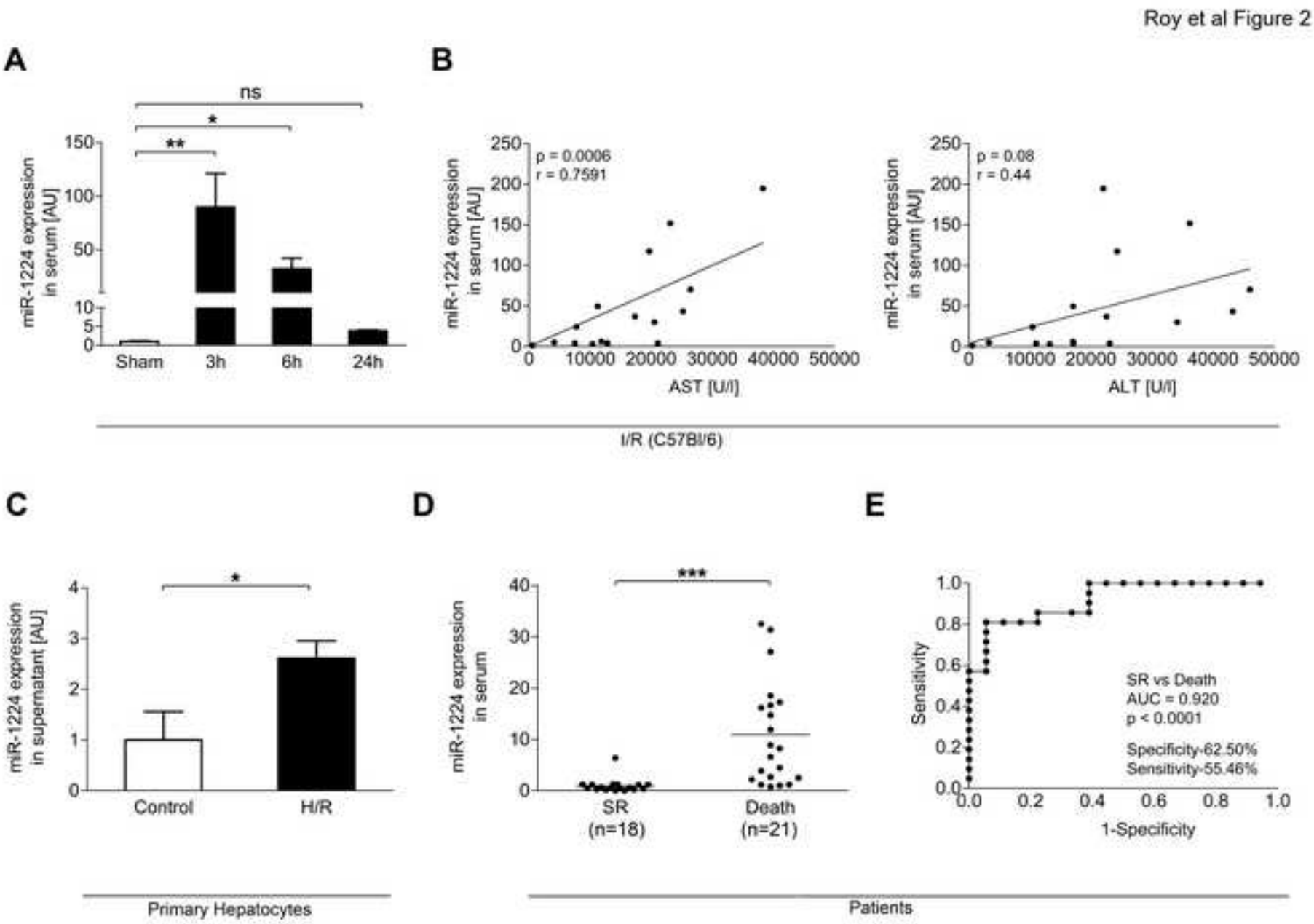

Figure 3

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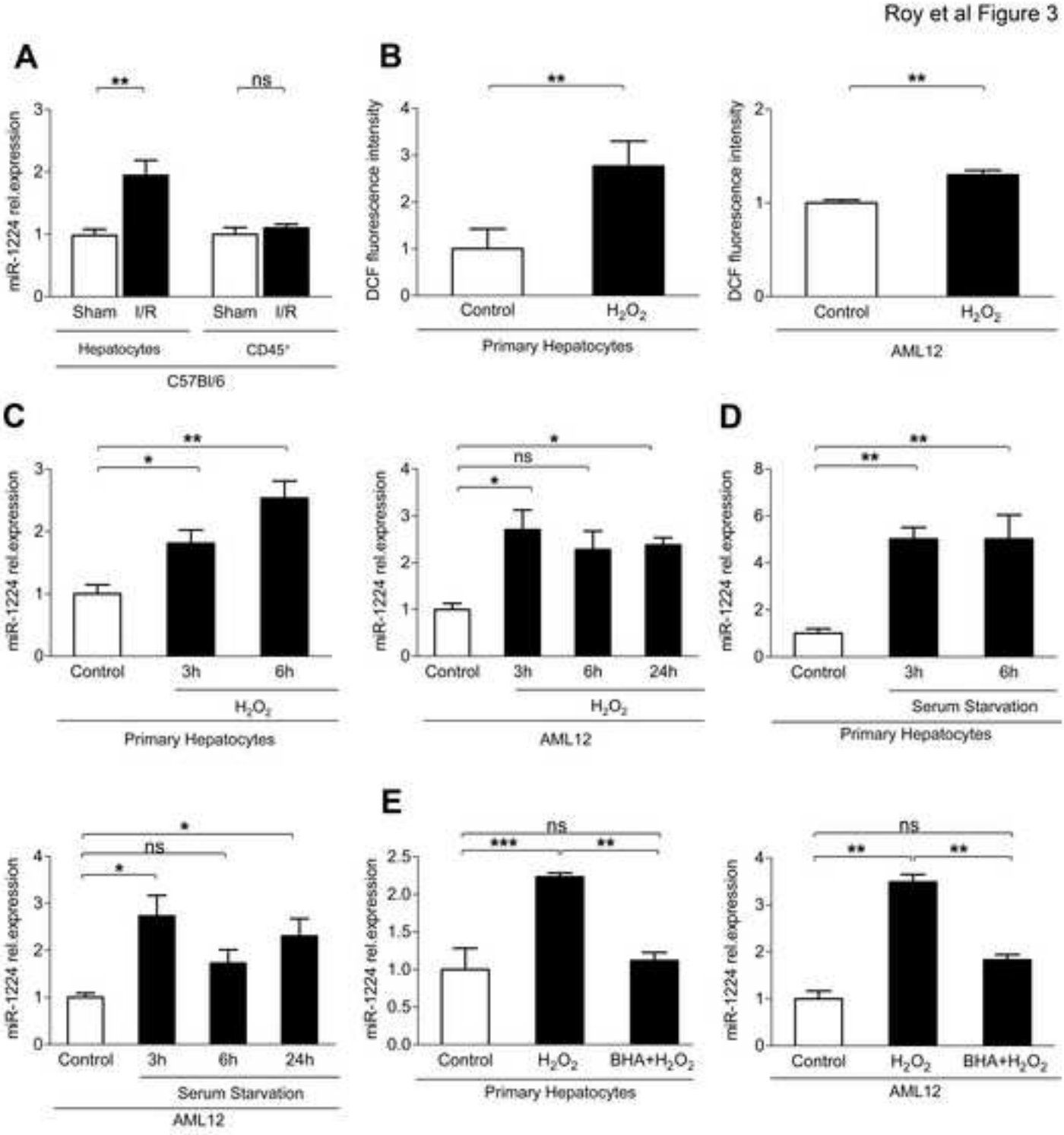

Figure 4

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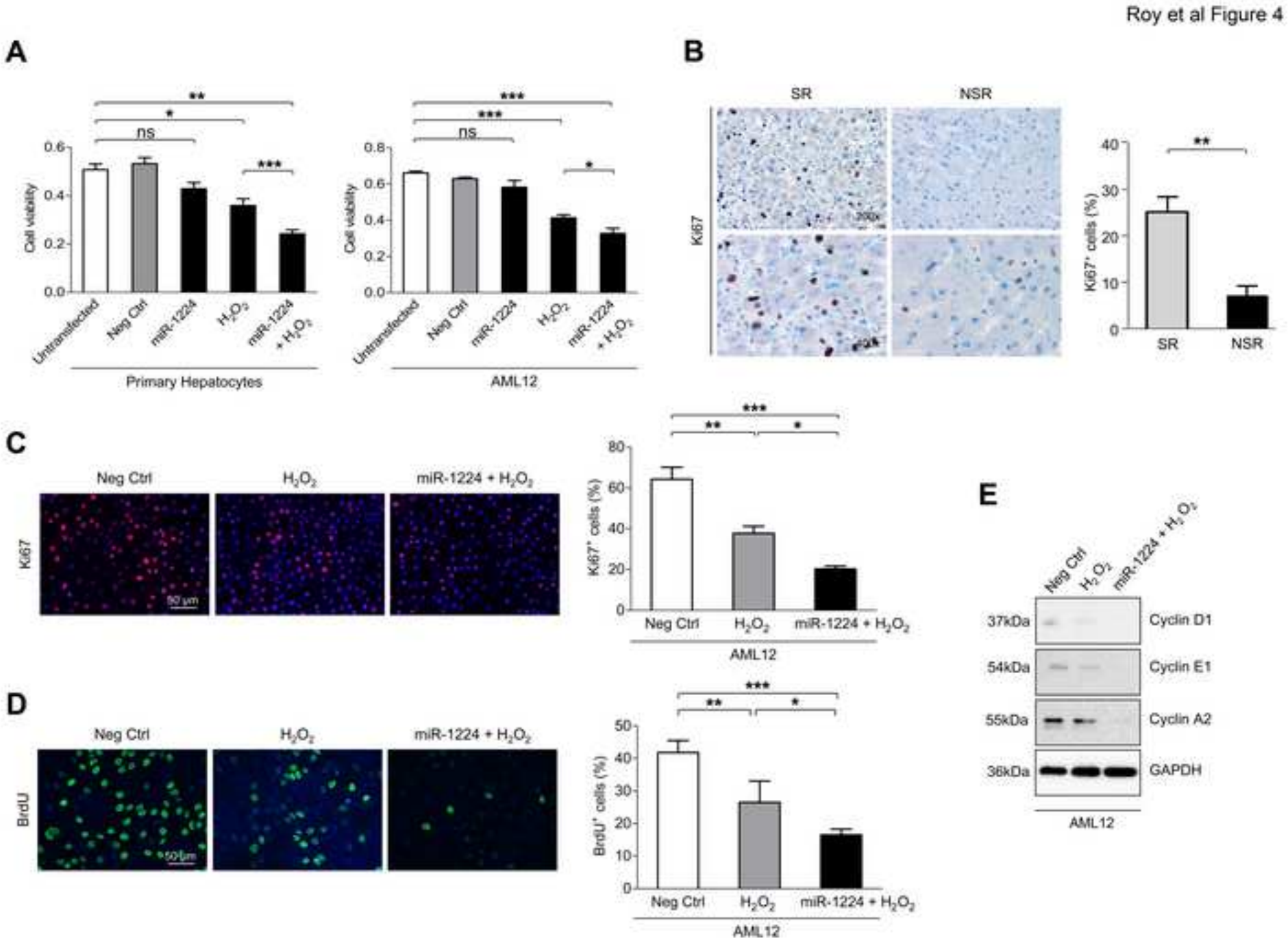

Figure 5

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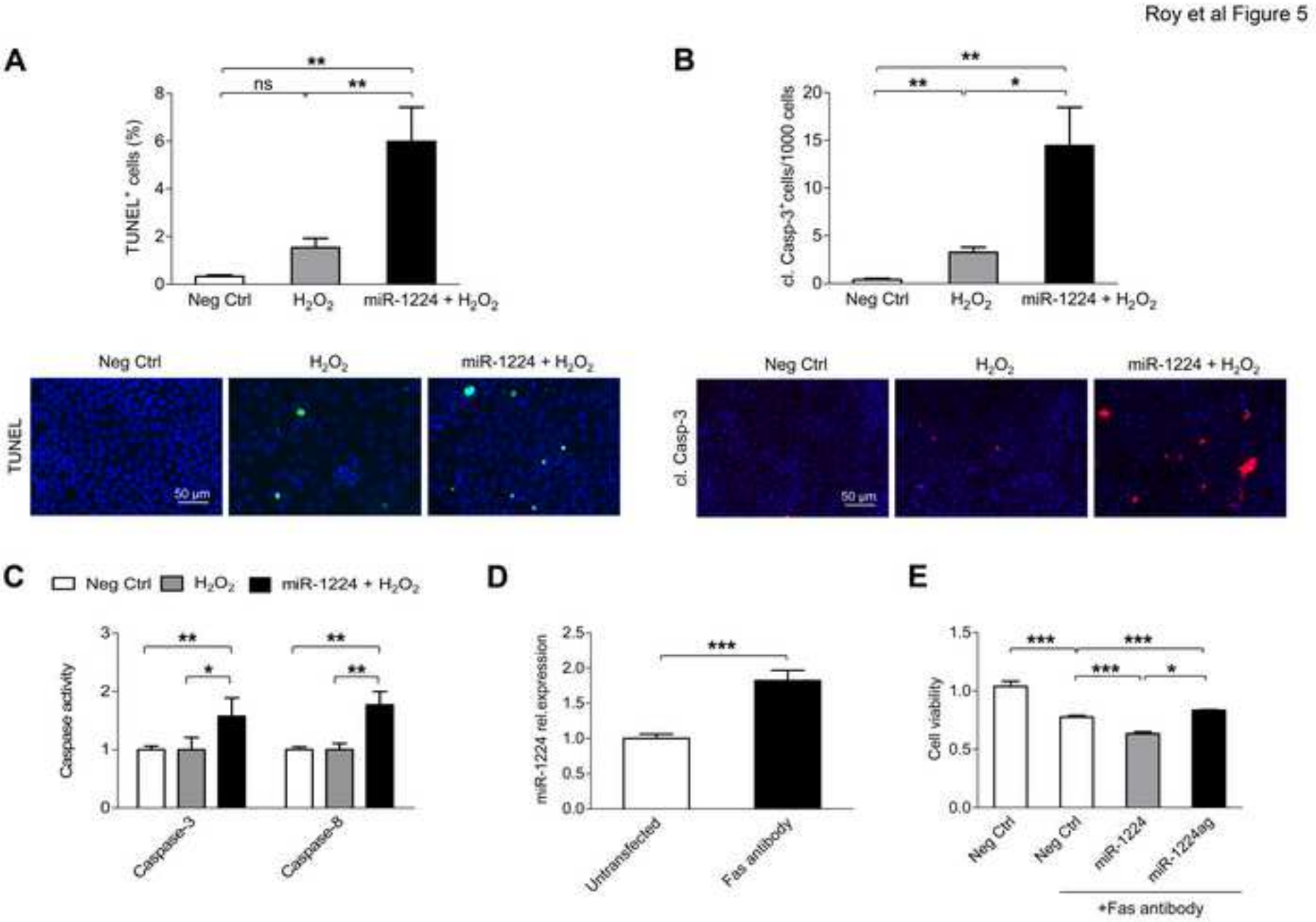

Figure 6

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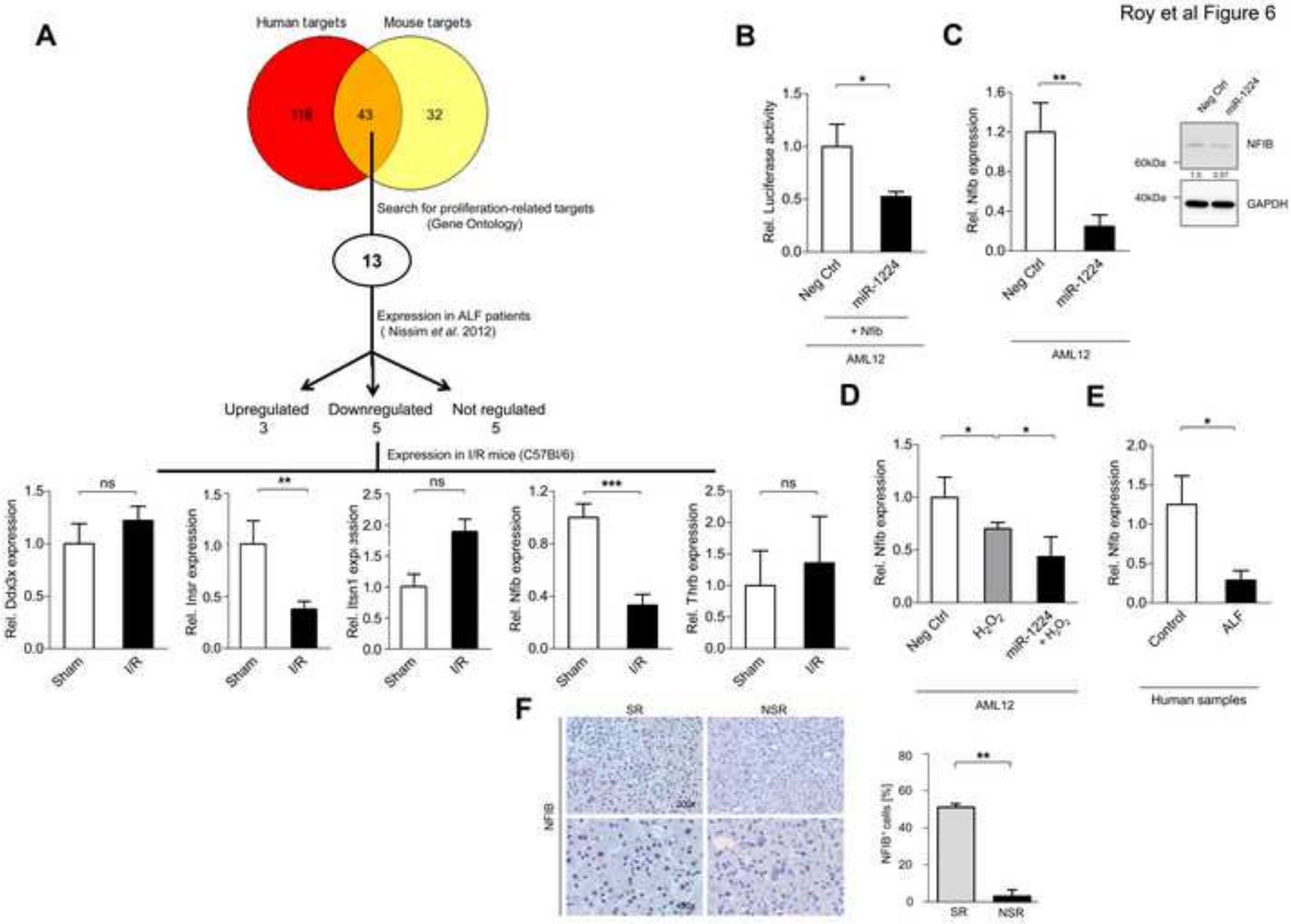

Figure 7

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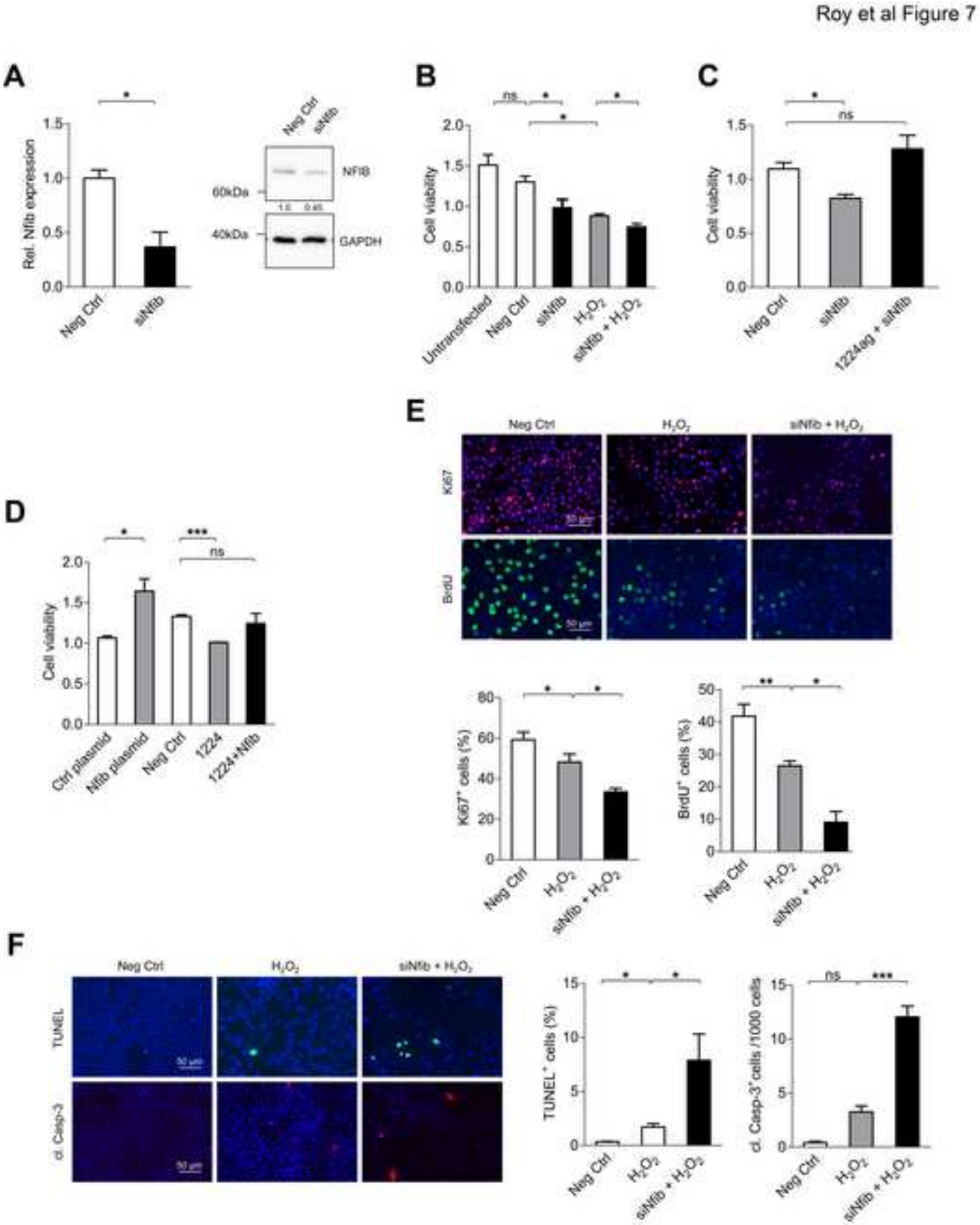
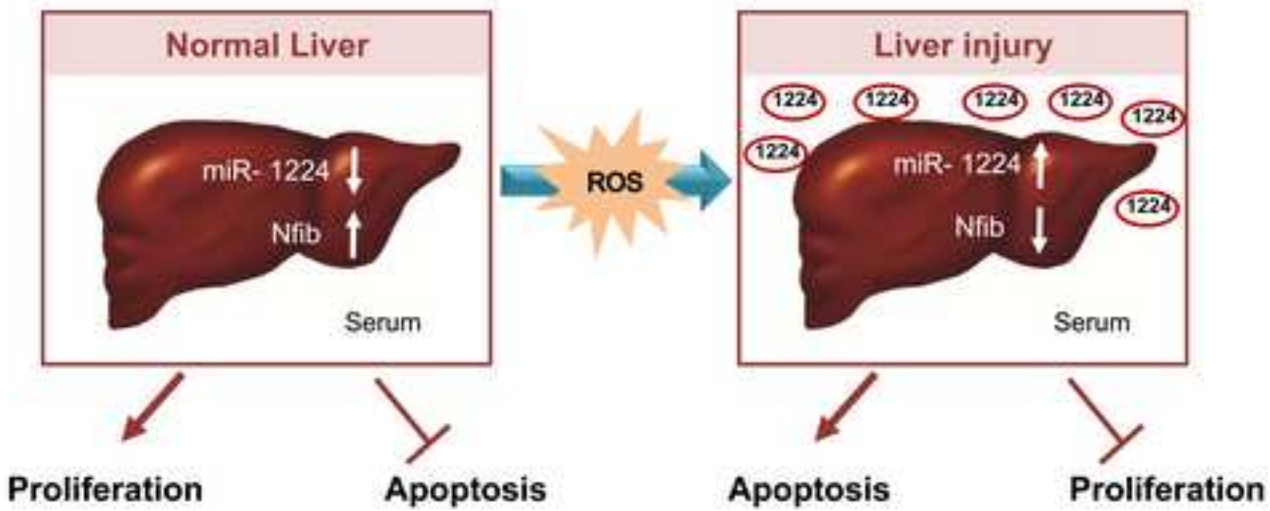


*Graphical Abstract



***Highlights**

Highlights

- miR-1224 is up-regulated in the livers and serum of mice after induction of liver injury
- miR-1224 is up-regulated in the livers and serum of patients with ALF and indicates a poor prognosis
- Upregulated miR-1224 represses proliferation and induces apoptosis in hepatocytes during ALF by targeting Nfib