



HAL
open science

miR-1224 inhibits cell proliferation in acute liver failure by targeting the antiapoptotic gene Nfib

Sanchari Roy, Heike Bantel, Franziska Wandrer, Anne Theres Schneider,
Jérémié Gautheron, Mihael Vucur, Frank Tacke, Christian Trautwein, Tom
Luedde, Christoph Roderburg

► To cite this version:

Sanchari Roy, Heike Bantel, Franziska Wandrer, Anne Theres Schneider, Jérémié Gautheron, et al..
miR-1224 inhibits cell proliferation in acute liver failure by targeting the antiapoptotic gene Nfib.
Journal of Hepatology, 2017, 67, pp.966 - 978. 10.1016/j.jhep.2017.06.007 . hal-03976518

HAL Id: hal-03976518

<https://hal.sorbonne-universite.fr/hal-03976518v1>

Submitted on 21 Feb 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1
2
3
4 **miR-1224 inhibits cell proliferation in acute liver failure by targeting *Nfib***
5
6
7

8
9 Sanchari Roy¹, Heike Bantel², Franziska Wandrer², Anne Theres Schneider¹, Jeremie
10 Gautheron¹, Mihael Vucur¹, Frank Tacke¹, Christian Trautwein¹, Tom Luedde^{1,*},
11 Christoph Roderburg^{1,*}.
12
13
14
15
16
17

18
19 ¹ Department of Medicine III, University of Aachen (RWTH), Pauwelsstraße 30, 52074
20 Aachen, Germany
21
22

23 ² Department of Gastroenterology, Hepatology and Endocrinology, Medical School
24 Hannover, Germany
25
26
27
28
29
30

31 Running title: The role of miR-1224 in acute liver failure
32

33 Keywords: miR-1224, acute liver failure, cell death, proliferation, biomarker, *Nfib*,
34 oxidative stress
35
36
37
38
39

40 Correspondence to: Prof. Dr. Tom Luedde or PD Dr. C. Roderburg; Department of
41 Medicine III, University Hospital RWTH Aachen, Pauwelsstraße 30, 52074 Aachen,
42 Germany; Phone: 0049-241-80-80329; Fax: 0049-241-80-82455; E-mail:
43 tluedde@ukaachen.de.
44
45
46
47
48
49
50
51
52

53 **Abbreviations**
54

55 ALF, Acute liver failure; I/R, Ischemia-reperfusion; miRNA, microRNA; APAP,
56 acetaminophen; H₂O₂, Hydrogen peroxide; CCl₄, Carbon tetrachloride; BrdU, 5-bromo-
57 2'-deoxyuridine; AUC, Area under curve; SR, spontaneous recovery; NSR, non-
58
59
60
61
62
63
64
65

1
2
3
4 spontaneous recovery; siRNA, small interfering RNA; mRNA, messenger RNA; qRT-
5
6 PCR, quantitative real-time polymerase chain reaction; AU, arbitrary units; H/R,
7
8 hypoxia/regeneration; TNF, tumor necrosis factor; MTT, methyl thiazol tetrazolium; cl.
9
10 CASP-3, cleaved Caspase-3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase;
11
12 UTR, untranslated region; TUNEL, terminal deoxynucleotide transferase nick end
13
14 labeling; ROS, reactive oxygen species
15
16
17
18
19
20

21 **Financial Support:**

22
23 This work was supported by project grants from the German Research Foundation (DFG
24
25 RO 4317/4-1) and a START grant from the medical faculty RWTH Aachen to C.R.
26
27 Moreover research in the lab of T.Lu. was supported by a Mildred-Scheel Endowed
28
29 Professorship from the German Cancer Aid (Deutsche Krebshilfe), the German-
30
31 Research-Foundation (DFG) (LU 1360/3-1 and SFB-TRR57 / P06), the Interdisciplinary-
32
33 Centre-for-Clinical-Research (IZKF) Aachen-Germany and the Ernst-Jung-Foundation
34
35 Hamburg. M.V. and J.G. were supported by the Start-Program and the Faculty of
36
37 Medicine of RWTH Aachen university.
38
39
40
41
42
43
44

45 **Authors Contributions**

46
47 S.R., C.R. and T.L. conceived and designed the experiments; S.R. performed and
48
49 analyzed most of the experiments; H.B. and F.W. analysed ALF patient data and
50
51 performed immunohistochemistry experiments; AT.S., J.G. and M.V. contributed to the
52
53 performance and analyses of experiments; S.R., C.R. and T.L. wrote the manuscript;
54
55 C.R., T.L., H.B., F.T., and C.T.. provided intellectual input; H.B. organized patient
56
57 recruitment and collected human serum and tissue samples.
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 H₂O₂ 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

1
2
3
4 ALF. Thus, miR-1224 may represent a target for novel therapeutic and diagnostic
5
6 strategies in the context of ALF and warrants further testing as a biomarker in
7
8 prospective trials.
9

10 Lay summary

11
12 In acute liver failure, miR-1224 expression is modulated by oxidative stress that leads to
13
14 a decrease in hepatocyte cell proliferation and increase in apoptosis. Increase in miR-
15
16 1224 in serum of ALF patients offers a promising candidate for diagnostic purposes.
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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, CCl₄, APAP) and *in vitro* (H₂O₂, serum starvation, TNF stimulation) models of liver

1
2
3
4 failure. In humans, up-regulation of miR-1224 was specific for patients with an
5 unfavourable clinical course and correlated with elevated serum concentrations of miR-
6
7 1224 in patients that succumbed to death from ALF. Functionally, we show that miR-
8
9 1224 inhibited expression of *Nfib* in hepatocytes leading to an impaired cell proliferation
10 and increased cell death. Thus, our data suggest that miR-1224 might not only be a
11 candidate for novel treatment strategies in ALF but also might have potential as a
12 biomarker for diagnosis of ALF and guiding therapeutic decisions in the treatment of
13 patients with liver failure.
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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.

CCl₄-induced damage and APAP –induced toxicity

Acute liver damage in mice by CCl₄ and APAP was induced as recently described [14, 15].

Microarray analysis

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

1
2
3
4 subsequently analyzed with a Geniom Real-Time Analyzer (GRTA) (Febit GmbH,
5 Heidelberg, Germany) [16] using the Geniom Biochip miRNA *Mus Musculus* . The total
6
7 RNA quality was done with the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa
8
9 Clara, CA, United States) using RNA 6000 Nano kit (Agilent Technologies, Santa Clara,
10
11 CA, United States). The 260/280 nm absorbance values were consistently above 1.9.
12
13 The array contained ten replicates of each mouse miRNA and miRNA star (*) sequences
14
15 as annotated in the Sanger miRBase v.14.0. Sample labelling with biotin was carried out
16
17 by microfluidic-based enzymatic chip labeling of miRNAs (MPEA) [17]. Following
18
19 hybridization for 16 h at 42 °C, the biochip was washed as indicated by the supplier and
20
21 signal enhancement was processed with the GRTA. For each array, signal intensities
22
23 were calculated using the Geniom Wizard Software (Febit GmbH, Heidelberg,
24
25 Germany).

32 33 34 35 **Cell culture**

36
37
38 Mouse AML12 hepatocyte cell line was kindly provided by Prof. Dr. Steven Dooley
39
40 (Medizinische Fakultät Mannheim, Mannheim, Germany). AML12 cells were cultured in
41
42 DMEM/F-12 medium supplemented with 10 % FBS (Invitrogen, CA, USA), 1x insulin-
43
44 transferrin-selenite (Pan Biotech, Aidenbach, Germany), dexamethasone (Pan Biotech,
45
46 Aidenbach, Germany), penicillin/streptomycin (Pan Biotech, Aidenbach, Germany). Cells
47
48 were grown at 37 °C in a humidified atmosphere with 5 % CO₂. For serum starvation,
49
50 cells were incubated in serum-free medium for the indicated time points. Cells were
51
52 stimulated with 100 ng/ml TNF α for indicated times and treated with 1 mM H₂O₂ either
53
54 for indicated times or for 3 h.
55
56
57
58
59
60
61
62
63
64
65

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 % CO₂.

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

1
2
3
4 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative
5
6 expression values were normalized to the internal control by using $2^{-\Delta\Delta C_t}$. Primer
7
8 sequences were listed in Supplementary CTAT.
9

10 11 12 13 14 **miRNA isolation from serum**

15
16 Isolation of miRNA from serum samples of mice was performed and normalized against
17
18 SV40 as described [14].
19
20
21
22

23 24 **Patients**

25
26 We investigated sera from 39 ALF patients (mean age 44.7 ± 2.9 years; 30.8 % male).
27
28 The 39 ALF patients included 18 with spontaneous recovery (SR), and 21 with non-
29
30 spontaneous recovery (NSR) that died for miRNA-1224 expression (Table 1A). Causes
31
32 of ALF included drug-induced toxicity (NSR n=6, SR n=9), Budd-Chiari Syndrome (NSR
33
34 n=3, SR n=0), viral hepatitis (NSR n=2, SR n=5), autoimmune hepatitis (NSR n=1, SR
35
36 n=2), and unknown etiology (NSR n=9, SR n=2). Sera were collected within days 1 to 2
37
38 of hospital admission and stored at $-20\text{ }^{\circ}\text{C}$. In addition, liver tissues from 10 ALF patients
39
40 (mean age 42.0 ± 5.4 years; 0% male) with SR (n=4) or NSR who revealed liver
41
42 transplantation (n=3) or died (n=3) were analyzed for miRNA-1224 expression (Table
43
44 1B).
45
46
47
48
49
50
51
52

53 54 **Cell transfection**

55
56 miR-1224 mimic (miR-1224) (MSY0005460), miR-1224 antagomiR (1224ag)
57
58 (MIN0005460) and AllStars negative control siRNA (1027280) or miScript inhibitor
59
60 negative control (1027271) (Neg Ctrl) were purchased from Qiagen (Hilden, Germany).
61
62
63
64
65

1
2
3
4 Nfib plasmid was obtained from Biocat (BC014290). AML12 cells or primary hepatocytes
5
6 were cultured for 24 h and transfected with 50 nM mimics or 100 nM antagomiR using
7
8 Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions
9
10 for 24 h. To knockdown the expression of *Nfib*, small interfering RNA (siRNA) from
11
12 Qiagen (Hilden, Germany) was transfected in AML12 cells for 24 h according to the
13
14 manufacturer's instructions.
15
16
17
18
19
20

21 **Luciferase assay**

22
23 AML12 cells were seeded in 24-well plate for 24 h and transiently transfected with
24
25 luciferase constructs (psiCheck-2TM, Promega, Madison, USA) containing 3'UTR of *Nfib*
26
27 or *Insr* along with miR-mimics using Lipofectamine 2000 (Invitrogen, CA, USA). After 24
28
29 h of transfection, cells were harvested and lysed with 100 μ l of passive lysis buffer
30
31 (Promega, Madison, WI). Lysates were analyzed using dual luciferase assay kit
32
33 (Promega, Madison, WI) according to the manufacturer's instructions. The luciferase
34
35 activities were measured and ratios of Firefly luciferase luminescence to renilla
36
37 luciferase luminescence were calculated. IKK β /E and I κ B α R/R plasmids were gifts
38
39 from Gilles Courtois, INSERM U1038, iRTSV, CEA, Grenoble, France). Cells were
40
41 transfected with NF- κ B-dependent reporter plasmid (I κ B-luc) as described [19].
42
43
44
45
46
47
48
49

50 **Western Blot**

51
52 Cells were homogenized with NP-40 lysis buffer to obtain protein lysates. Proteins were
53
54 separated on SDS-polyacrylamide gel electrophoresis and transferred to PVDF
55
56 membrane. The membranes were probed with the following antibodies: Cyclin A2 ,
57
58 Cyclin E1, Cyclin D1 , anti-GAPDH.
59
60
61
62
63
64
65

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.

1
2
3
4 Image acquisition was performed at a magnification of 20x with a Zeiss Axio Imager.Z1
5
6 microscope, AxioCam MRm and HRc cameras using Axiovision 4.8 software (Carl Zeiss,
7
8 Inc., Oberkochen, Germany).
9

10 11 12 13 14 **Detection of intracellular ROS**

15
16 2',7'-Dichlorodihydrofluorescein diacetate (DCHF-DA) dye (D6883, Sigma-Aldrich, St.
17
18 Louis, MO, USA) diluted with DMEM medium was added to AML12 cells or primary
19
20 hepatocytes and cells were incubated in 5 % CO₂ at 37 °C for 50 min. Cells were fixed
21
22 with 3 % PFA and washed with PBS and analyzed using fluorescence microscopy
23
24 (Leica, Germany). ROS fluorescence was quantified with a microplate cell imaging
25
26 imaging multi-mode reader (Cytation3, Biotek Instruments, Germany) at wavelengths of
27
28 488 nm for excitation and 525 nm for emission. Cells were pre-treated with 100 µM BHA
29
30 for 30 min followed by addition of 1 mM H₂O₂ for 3 h.
31
32
33
34
35
36
37

38 **Immunohistochemical analysis**

39
40 Paraffin sections were obtained from the Department of Pathology at Hannover Medical
41
42 School. Sections were deparaffinized in xylene, followed by rehydration in an ethanol
43
44 gradient. Endogenous peroxidase was blocked with 3 % hydrogen peroxide in distilled
45
46 water. For antigen retrieval, slides were boiled for 5 min in antigen-unmasking solution
47
48 (Vector Laboratories). After washing with 0.05 % Tween-20 in Tris-buffered saline
49
50 (TBST) nonspecific binding sites were blocked with blocking solution (1 % BSA with
51
52 horse serum in TBST; VECTASTAIN ABC kit, Vector Laboratories) for 1 h at room
53
54 temperature. Afterwards, sections were incubated overnight at 4 °C with primary
55
56 antibodies against NFIB (1:300, sab1402289, Sigma-Aldrich, St. Louis, MO, USA). After
57
58
59
60
61
62
63
64
65

1
2
3
4 repeated washings with TBST, sections were incubated with biotinylated secondary
5 antibody Ab solution (VECTASTAIN ABC kit, Vector Laboratories) for 30 min and
6
7 covered with avidin-biotin complex reagent (VECTASTAIN ABC Kit, Vector Laboratories)
8
9 for 1 h at room temperature. Sections were stained in aminoethylcarbazole substrate
10
11 solution (AEC chromogen kit, Sigma-Aldrich, St. Louis, MO, USA) and counterstained
12
13 with hematoxylin. Finally, sections were covered with mounting medium (Aquamount,
14
15 VWR International GmbH, Germany). Ki-67 (1:500, Vector Laboratories, #VP-K451) was
16
17 stained as previously described [2, 11].
18
19
20
21
22
23
24
25

26 **Bioinformatic analysis**

27
28 Predicted targets of miR-1224 are obtained from TargetScan (www.targetscan.org).
29
30 Gene ontology was obtained from DAVID database (<https://david.ncifcrf.gov>). The
31
32 microarray data were obtained from National Center for Biotechnology Information Gene
33
34 Expression Omnibus database (accession number GSE38941).
35
36
37
38
39

40 **Statistical analysis**

41
42 Differences between groups were assessed by an unpaired two-sample t-test or Mann-
43
44 Whitney test and multiple comparisons between more than two groups have been
45
46 conducted by one-way ANOVA with Kruskal-Wallis test for post hoc analysis. Statistical
47
48 analysis was performed by using Graph Pad Prism version 5.0. Data represent means \pm
49
50 standard error of the mean (SEM). A p-value < 0.05 was considered significant.
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

1
2
3
4 for further investigation in our study. Furthermore, we also tested miR-1224 expression
5 in two different acute liver damage models : CCl₄ and APAP intoxication. In both
6 models, miR-1224 expression was time dependently up-regulated (Fig. 1D and
7 Supplementary Fig. 1D), indicating the specificity of miR-1224 in ALF.
8
9

10
11
12
13
14 Based on the striking regulation of miR-1224 in murine liver failure, we next attempted to
15
16 correlate these findings with data from patients. We therefore used samples from
17
18 explanted livers of patients with ALF from different disease entities as well as samples
19
20 from healthy patients [11, 14, 22]. Quantitative polymerase chain reaction (qPCR)
21
22 analysis of miRNA extracts showed a significant up-regulation of miR-1224 in injured
23
24 livers compared to controls (Fig. 1E). To investigate a potential role of miR-1224 in
25
26 determining ALF outcome, we used a second cohort of ALF patients that were
27
28 separated into patients that recovered spontaneously (SR, n= 4) and patients without
29
30 spontaneous recovery (NSR, n=6) who underwent liver transplantation (n=3) or died
31
32 (n=3). Compared to SR patients, those with NSR showed a significantly higher
33
34 expression of miR-1224 (Fig. 1F), suggesting a specific function of miR-1224 in the
35
36 pathophysiology of ALF in mice and human.
37
38
39
40
41
42
43
44

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

46
47
48 There is an ample evidence that circulating miRNAs can serve as biomarkers for organ
49
50 damage such as liver failure [23]. We therefore hypothesized that the up-regulation of
51
52 miR-1224 in injured livers might be associated with the corresponding alterations in
53
54 miR-1224 serum levels. To test this hypothesis, we measured miR-1224 concentrations
55
56 in sera from mice after I/R- or sham-surgery. As shown in Fig. 2A, serum levels of
57
58 miR-1224 were significantly higher in mouse serum upon induction of I/R when
59
60
61
62
63
64
65

1
2
3
4 compared to sham-operated mice. Elevated levels of miR-1224 strongly correlated with
5
6 the established markers of liver failure such as aspartate or alanine transaminase
7
8 (AST/ ALT) (Fig. 2B), suggesting that miR-1224 might represent a previously
9
10 unrecognized serum marker for liver cell death. To confirm the hypothesis that injury of
11
12 hepatocytes is associated with a release of miR-1224 into the extracellular space, we
13
14 performed *in vitro* hypoxia and reoxygenation experiments (H/R) with primary
15
16 hepatocytes and analysed miR-1224 concentrations in the supernatants. As seen in
17
18 Fig. 2C, miR-1224 expression levels in supernatant were significantly higher in cells
19
20 after induction of H/R compared to controls.
21
22
23
24
25

26 We next isolated miRNA from sera of patients with ALF and analyzed miR-1224 levels of
27
28 patients that spontaneously recovered (SR, n=18) and patients without SR who died
29
30 (NSR, n=21). Compared to SR patients, patients who succumbed to death showed
31
32 significantly higher levels of miR-1224 (Fig. 2D), highlighting that elevated miR-1224
33
34 serum levels are associated with an unfavourable disease course in patients with ALF.
35
36 Furthermore, we compared miR-1224 levels in serum of ALF patients with those of
37
38 previously published miRNAs ([11]; miR-21, miR-122 and miR-221) and those of
39
40 differentially regulated miRNAs in the array analysis (Fig. 1A; miR-451, miR-135a* and
41
42 miR-150). As shown in Supplementary Fig. 2A, there was no significant change in the
43
44 expression of miR-451, miR-135a* and miR-150 among SR and NSR patients, while (in
45
46 line to previous results [11]) levels of miR-21, miR-122 and miR-221 were lower in
47
48 patients that did not recover spontaneously. Of note, no correlation between miR-1224
49
50 and any other miRNAs became apparent (Supplementary Fig. 2B), highlighting the
51
52 specificity of alterations in miR-1224 serum levels in this context. Finally, we performed
53
54 receiver operating characteristic (ROC) curve analyses to determine the predictive value
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 of miR-1224 serum concentrations in patients with ALF and calculated an area under
5
6 curve (AUC) value of 0.861 (Fig. 2E), suggesting that miR-1224 represent a new
7
8 biomarker to predict the outcome of patients with ALF.
9

10 11 12 13 14 **Induction of miR-1224 expression in ALF is dependent on increased oxidative** 15 16 **stress**

17
18 To explore the molecular mechanism involved in the regulation of miR-1224 in ALF, we
19
20 characterized the expression of this miRNA in different mouse tissues. Low expression
21
22 of miR-1224 was detected in kidney, lung and spleen, whereas in heart, brain and liver,
23
24 miR-1224 was expressed at higher levels (Supplementary Fig. 3A). To further dissect
25
26 the cell-specific regulation of miR-1224 during ALF, we isolated primary hepatocytes and
27
28 CD45⁺ immune cells from livers of mice subjected to I/R or sham. Upon I/R, miR-1224
29
30 was significantly up-regulated in hepatocytes, whereas no regulation was found in
31
32 CD45⁺ immune cells (Fig. 3A), suggesting a specific role of miR-1224 in hepatocytes
33
34 during ALF.
35
36
37
38

39
40 I/R is associated with a dramatic increase in the release of reactive oxygen species
41
42 (ROS) [3]. As a common ROS inducer, H₂O₂ is used to mimic I/R injury *in vitro*. In both
43
44 primary hepatocytes and immortalized murine hepatocytes (AML12), H₂O₂ stimulation
45
46 resulted in a strong increase of DCF fluorescence as a surrogate for intracellular
47
48 oxidative stress (Fig. 3B and Supplementary Fig. 3B), accompanied by an increase in
49
50 miR-1224 expression (Fig. 3C). Similar results were obtained when these cells were
51
52 serum-starved, representing another *in vitro* model mimicking ROS induction in ALF
53
54 (Fig. 3D). To confirm that ROS represent the main inducer of miR-1224 expression in
55
56 hepatocytes, we treated both primary hepatocytes and AML12 cells with the ROS
57
58
59
60
61
62
63
64
65

1
2
3
4 scavenger BHA (Butylated hydroxyanisole). Indeed, the ROS-dependent increase in
5 miR-1224 expression after H₂O₂ treatment could be completely abolished by pre-
6 treatment with BHA, compared to H₂O₂-treated cells (Fig. 3E and Supplementary Fig.
7 3C). Using another antioxidant N-acetylcysteine (NAC), APAP-induced ROS and miR-
8 1224 expression were inhibited in primary murine hepatocytes when compared with
9 APAP-stimulated hepatocytes (Supplementary Fig. 3D).
10
11
12
13
14
15
16
17
18
19
20

21 Tumour necrosis factor (TNF) is one of the well-known pro-inflammatory mediators
22 released during I/R [24] that is confirmed by strong up-regulation of TNF expression in
23 murine livers upon I/R (Supplementary Fig. 4A). TNF is also known to induce ROS
24 production in liver cells [25]. In order to investigate that ROS induce the expression of
25 miR-1224 up-regulation, we stimulated AML12 cells with TNF for different time points.
26 Indeed, the increase in ROS in TNF-treated AML12 hepatocytes is correlated with the
27 up-regulation of miR-1224 at 6 h after TNF stimulation (Supplementary Fig. 4B). This
28 regulation could be prevented by pre-treatment with BHA (Supplementary Fig. 4C),
29 which is in line to our previous result [14] demonstrating that BHA pre-treatment
30 abolished TNF induced ROS formation in hepatocytes. . Since TNF is known to activate
31 the ROS-dependent transcription factor NF- κ B [25], we finally investigated whether miR-
32 1224 expression is dependent on NF- κ B activation. Therefore, we transfected AML12
33 cells with dominant kinase mutant IKK β E/E (constitutively activation of the NF- κ B
34 pathway) and the super-repressor I κ B α R/R (repression of the NF- κ B pathway)
35 (Supplementary Fig. 4D) and found that miR-1224 expression was up-regulated and
36 down-regulated respectively (Supplementary Fig. 4E), suggesting the dependence of
37 miR-1224 expression on NF- κ B activity. Collectively, these data suggest that ROS
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 produced by both H₂O₂ and TNF in injured livers play a driving factor in the activation of
5
6 miR-1224 expression.
7
8
9

10 11 **miR-1224 up-regulation decreases cell proliferation in ALF**

12
13 To investigate the effect of miR-1224 in regulating cell survival, primary hepatocytes and
14 AML12 cells were transfected with miR-1224 mimic for 24 h and treated with H₂O₂ for
15
16 3 h (Supplementary Fig. 5A). MTT assay on these cells revealed that overexpression of
17
18 miR-1224 alone did not alter cell viability, whereas cells overexpressing miR-1224
19
20 showed an impaired viability upon H₂O₂ treatment compared to All stars negative control
21
22 (Neg ctrl) transfected cells (Fig. 4A). In line with the previous result, knockdown of
23
24 miR-1224 expression (Supplementary Fig. 5B) was associated with a increased cell
25
26 viability (Supplementary Fig. 5C). It is known that ALF-induced compensatory cell
27
28 proliferation is correlated with an impaired clinical outcome. Immunohistological analysis
29
30 of liver biopsies from patients with ALF showed significantly lower numbers of Ki67⁺ cells
31
32 in those patients that did not recover compared to patients with a more favourable
33
34 clinical course (Fig. 4B). Similarly, lower numbers of Ki67⁺ cells became apparent upon
35
36 miR-1224 transfection in H₂O₂-treated cells compared to control (Neg Ctrl) cells (Fig.
37
38 4C), providing a mechanistic link between elevated miR-1224 expression and impaired
39
40 patients prognosis in the context of ALF. To determine how miR-1224 affects cell cycle
41
42 progression in this context, we performed pulse labelling studies using BrdU in H₂O₂-
43
44 treated AML12 cells. The percentage of BrdU⁺ cells was dramatically decreased in miR-
45
46 1224 transfected H₂O₂-treated cells, supporting the involvement of miR-1224 in
47
48 regulating the cell cycle transition from G1 to S phase (Fig. 4D). Moreover, expressions
49
50 of Cyclin D1, Cyclin A2 and Cyclin E1 were reduced in miR-1224 overexpressing in
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 combination with H₂O₂-treated AML12 cells (Fig. 4E and Supplementary Fig. 5D). The
5
6 expression of cyclins was increased and rescued by treating cells with miR-1224
7
8 antagomiR only or in combination with H₂O₂, respectively when compared to All stars
9
10 negative control (Neg ctrl) transfected cells (Supplementary Fig. 5D). Collectively, these
11
12 data demonstrate a role of miR-1224 in the regulation of hepatocyte proliferation during
13
14 ALF.
15
16

17
18 To reinforce the role of miR-1224 in regulating liver cell injury during ALF, we next
19
20 investigated whether these above results could be reproduced in a second model for
21
22 induction of in vitro liver cell injury. We therefore treated both AML12 cells and primary
23
24 hepatocytes with acetaminophen (APAP), leading to a decrease in cell viability
25
26 (Supplementary Fig. 6A), associated with an increase in miR-1224 expression
27
28 (Supplementary Fig. 6B). In line to our previous data on H₂O₂, inhibition of miR-1224
29
30 prevented from APAP induced cell death, while up-regulation of miR-1224, in turn,
31
32 caused a further decrease in cell viability, both in AML12 cells and primary hepatocytes
33
34 (Supplementary Fig. 6C). Thus, similar to H₂O₂, miR-1224 antagonism protects against
35
36 APAP-induced hepatocyte toxicity, as a clinically relevant model of induction of ALF.
37
38
39
40
41
42
43
44

45 **miR-1224 up-regulation promotes apoptosis in ALF**

46
47
48 We hypothesized that miR-1224 might also be involved in the regulation of apoptosis
49
50 during ALF. Therefore, we transfected AML-12 hepatocytes with miR-1224 or control
51
52 vector and stimulated them with H₂O₂. TUNEL stainings were performed to measure the
53
54 amount of cell death. In these analysis, the percentage of TUNEL⁺ cells was significantly
55
56 increased in miR-1224 transfected, H₂O₂-treated cells in comparison to H₂O₂-treated
57
58 control-transfected (Neg Ctrl) cells (Fig. 5A), suggesting a role of miR-1224 in the
59
60
61
62
63
64
65

1
2
3
4 regulation of cell death during ALF. We next analysed the cleavage of Caspase-8 and its
5
6 downstream effector Caspase-3 to deduce the influence of miR-1224 in this process.
7
8 Notably we found significantly higher numbers of cl. Caspase-3⁺ cells in miR-1224
9
10 transfected-H₂O₂-treated cells compared to control-transfected (Neg Ctrl) cells (Fig. 5B).
11
12 Similarly, activities of both Caspase-3 and Caspase-8 were increased upon transfection
13
14 with miR-1224 in combination with H₂O₂ treatment (Fig. 5C), suggesting a yet unknown
15
16 function of miR-1224 in the regulation of cell death during ALF. Recently, an
17
18 up-regulation of miR-1224 in livers of mice after treatment with a Fas antibody (anti-
19
20 CD95, clone Jo2) as an inducer of pure apoptosis was reported [26]. In line, we found
21
22 higher levels of miR-1224 in Jo2- treated AML12 hepatocytes (Fig. 5D). Strikingly,
23
24 miR-1224 promoted Fas-induced apoptosis in these cells, suggesting a specific role of
25
26 miR-1224 in the pathophysiology of apoptosis during ALF (Fig. 5E), which is in line to
27
28 our previous results that indicate an impaired prognosis of patients with a strong
29
30 up-regulation of miR-1224 during ALF (Fig. 1E).
31
32
33
34
35
36
37
38
39

40 ***Nfib* is a direct target of miR-1224**

41
42
43 Next we tested whether miR-1224 is able to modulate the expression of genes involved
44
45 in the regulation of cell proliferation and death. Possible miR-1224 targets
46
47 (Supplementary Table 1) were identified by using the publicly available algorithm
48
49 TargetScan that resulted in 43 unique targets common to both human and mice. In order
50
51 to find proliferation-related target of miR-1224, gene ontology terms were searched on
52
53 these 43 targets. Furthermore, proliferation-related 13 targets were examined for their
54
55 expression in ALF patients (GSE38941) (Supplementary Fig. 7A). Out of 13 targets, 5
56
57 genes were downregulated in ALF patients that were further tested in RNA extracts from
58
59
60
61
62
63
64
65

1
2
3
4 livers of mice after I/R or sham surgery. Out of these, only *Nfib* and *Insr* were found to
5
6 be significantly down-regulated in injured livers, while all other putative target genes
7
8 were not regulated (Fig. 6A).
9

10
11 To verify that *Nfib* and *Insr* are direct targets of miR-1224, we generated a
12
13 luciferase reporter plasmid containing the 3'UTR of *Nfib* or *Insr* flanking the putative
14
15 miR-1224 binding sites. This dual luciferase reporter assay revealed that miR-1224
16
17 significantly reduced the luciferase activity of *Nfib* construct when compared to miR-neg-
18
19 transfected cells (Fig. 6B and Supplementary Fig. 7B). Moreover, mRNA and protein
20
21 level of *Nfib* were dramatically down-regulated in cells transfected with miR-1224
22
23 compared to control-transfected cells (Fig. 6C). Consistent with the data from mice after
24
25 I/R surgery, treatment with serum starvation (Supplementary Fig. 8A) as well as H₂O₂
26
27 (Supplementary Fig. 8B) significantly down-regulated *Nfib* level in AML12-hepatocytes
28
29 and a further reduction in *Nfib* expression was observed when cells were transfected
30
31 with miR-1224 in combination with H₂O₂ (Fig. 6D). To translate these data to the human
32
33 situation, we analysed expression of *NFIB* in liver samples from patients with ALF or
34
35 healthy controls. Both genes were down-regulated in ALF patients, suggesting that miR-
36
37 1224 negatively regulates *NFIB* in humans (Fig. 6E). We hypothesized that, similar to
38
39 miR-1224, *Nfib* might also be predictive for the clinical course in patients with ALF.
40
41 Therefore, we evaluated the protein level of NFIB in liver tissues of ALF patients with SR
42
43 or NSR. In agreement with increased miR-1224 levels (Fig. 1E), we found decreased
44
45 levels of NFIB (Fig. 6F) in liver tissues of patients with an unfavourable outcome (NSR)
46
47 when compared to patients that recovered (SR). Taken together, *Nfib* and *Sp1* are new
48
49 targets of miR-1224 and determine the prognosis of patients with ALF.
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

***Nfib* regulates cell proliferation and cell death in H₂O₂-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 H₂O₂-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 H₂O₂. 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 H₂O₂-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 H₂O₂, TNF and APAP has been provided by DCF fluorescence and co-treatment with antioxidants. In spite 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

1
2
3
4 cell death, namely apoptosis and necrosis. By using an array-based approach, we
5
6 identified a panel of 30 miRNAs deregulated in the context of I/R. This set of miRNAs
7
8 demonstrated a striking overlap with a recently published panel of miRNAs deregulated
9
10 in hepatocytes from mice that were subjected to Fas Ligand-stimulation as an apoptotic
11
12 model of cell death in the liver ([26]; Supplementary Fig. 10). Notably both arrays were
13
14 performed using the same platform leading to a high level of comparability between the
15
16 both studies. Within this subset of miRNA with a likely role in cell death and more
17
18 specifically apoptosis, miR-1224 was also up-regulated after treatment with H₂O₂ in
19
20 hepatocytes. miR-1224 expression directly correlates with AST/ALT serum levels in
21
22 mice and the amounts of TUNEL⁺ and cl.Casp-3⁺ cells in hepatocytes as surrogate for
23
24 apoptotic cell death. Increased amounts of apoptotic cells were recently described in
25
26 patients with an unfavourable clinical outcome compared to other patients [32]. In line,
27
28 extensive TUNEL reactivity was observed in liver samples of NSR patients [33] as well
29
30 as in a model of APAP-induced liver damage [34], further arguing for a role of miR-1224
31
32 in promoting cell death. In our study, NSR patients with higher miR-1224 expression
33
34 demonstrated a significant impaired prognosis compared to other patients.
35
36
37
38
39
40
41
42

43 To further explore the functional relevance of our findings, we investigated
44
45 whether differences in miRNA expression are reflected by a different expression of
46
47 selected target genes in liver tissue of ALF patients. We identified *Nfib* as a direct target
48
49 of miR-1224 in ALF. NFIB is involved in the regulation of cell death, especially apoptosis
50
51 in various cancers [35-37]. In line with increased miR-1224 expression, we found
52
53 significantly decreased *Nfib* expression in liver tissues of spontaneously recovered (SR),
54
55 compared to nonspontaneously recovered (NSR) patients. Since miRNAs can regulate
56
57 hundreds of target genes, and each gene can be regulated by several miRNAs we
58
59
60
61
62
63
64
65

1
2
3
4 cannot exclude that additional miRNAs or other mechanisms might be involved
5
6 regulating liver cell death and regeneration. Nevertheless, our data strongly argue for a
7
8 role of miR-1224 in signalling pathways integrating liver cell death and regeneration in
9
10 ALF.
11
12

13
14 Recently, we demonstrated that serum miRNA levels might reflect
15
16 pathophysiological processes with a direct influence on the prognosis of patients with
17
18 liver diseases. In this present study, we detected significantly elevated serum levels of
19
20 miR-1224 in patients with ALF compared to healthy controls. Notably, within the group of
21
22 ALF patients, elevated miR-1224 levels were restricted to those patients with an
23
24 unfavourable clinical course, while patients that recovered from ALF demonstrated
25
26 almost normal miR-1224 levels. While concordant changes of miR-1224 in serum and
27
28 tissue were previously shown in a mouse model of kidney injury [29], plasma miR-1224
29
30 was down-regulated in contrast to elevated levels in tissue subjected to APAP
31
32 intoxication [27]. These divergent studies suggest that the plasma/ tissue equilibrium of
33
34 miRNAs might vary with distinct pathogeneses of liver diseases. Despite the currently
35
36 unknown mechanism of miRNA regulation in the serum, the striking regulation of
37
38 miR-1224 in the serum of ALF patients might have implications for clinical aspects of
39
40 ALF. Of note, a prognostic function of elevated miR-122, miR-21, and miR-221
41
42 concentrations in patients with ALF was recently demonstrated [11]. In our study,
43
44 miR-1224 showed an outstanding performance in predicting patients prognosis (AUC,
45
46 0.861) (Fig. 2E), which was even superior compared to these recently published
47
48 markers (AUC, 0.6-0.7). Nevertheless, since serum miR-1224 concentrations were
49
50 significantly different but still showed some overlap SR and NSR patients, it is likely that
51
52 not one miRNA but detection of a whole panel might provide the necessary sensitivity
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 and specificity for discriminating between both groups. Thus, when considering miR-
5
6 1224 as a new prognostic marker in ALF, analysis on larger patient cohorts with distinct
7
8 hepatic disease-causes and differential clinical states will have to be analyzed to further
9
10 test the potential of miR-1224 levels in the serum as biomarkers for detection or
11
12 monitoring of ALF. Moreover, *in vivo* analysis using miR-1224 knock-out mice applying
13
14 transient knock-down of miR-1224 would further reinforce the role of miR-1224 in the
15
16 pathophysiology of liver injury.
17
18
19
20

21 Together, our data show that intra- and extracellular miR-1224 is up-regulated in
22
23 human patients with ALF and in mice after I/R mimicking ALF. This translational impact
24
25 might help to make a rational selection of the suitable candidates for liver
26
27 transplantation.
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Acknowledgements

The authors would like to thank Dr. Ioannis Amarantos (Comprehensive Biomarker Center, Heidelberg) for giving assistance to microarray analysis. The authors would like to thank Michaela Roderburg-Goor, Jane Beger-Lüdde, Prof. Christian Liedtke, Dr. Manjeet Mukherjee and members of the Luedde Lab for helpful discussions.

References

- [1] Wang K. Molecular mechanisms of hepatic apoptosis. *Cell death & disease* 2014;5:e996.
- [2] John K, Wielgosz S, Schulze-Osthoff K, Bantel H, Hass R. Increased plasma levels of CK-18 as potential cell death biomarker in patients with HELLP syndrome. *Cell death & disease* 2013;4:e886.
- [3] Bantel H, Schulze-Osthoff K. Mechanisms of cell death in acute liver failure. *Frontiers in physiology* 2012;3:79.
- [4] Sun K, Xie X, Xie J, Jiao S, Chen X, Zhao X, et al. Cell-based therapy for acute and chronic liver failures: distinct diseases, different choices. *Scientific reports* 2014;4:6494.
- [5] Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;136:215-233.
- [6] Valinezhad Orang A, Safaralizadeh R, Kazemzadeh-Bavili M. Mechanisms of miRNA-Mediated Gene Regulation from Common Downregulation to mRNA-Specific Upregulation. *International journal of genomics* 2014;2014:970607.
- [7] Bajan S, Hutvagner G. Regulation of miRNA processing and miRNA mediated gene repression in cancer. *MicroRNA* 2014;3:10-17.
- [8] Ha TY. MicroRNAs in Human Diseases: From Cancer to Cardiovascular Disease. *Immune network* 2011;11:135-154.
- [9] Roderburg C, Luedde T. Circulating microRNAs as markers of liver inflammation, fibrosis and cancer. *Journal of hepatology* 2014;61:1434-1437.
- [10] Szabo G, Bala S. MicroRNAs in liver disease. *Nature reviews Gastroenterology & hepatology* 2013;10:542-552.
- [11] John K, Hadem J, Krech T, Wahl K, Manns MP, Dooley S, et al. MicroRNAs play a role in spontaneous recovery from acute liver failure. *Hepatology* 2014;60:1346-1355.
- [12] Chen X, Zhao Y, Wang F, Bei Y, Xiao J, Yang C. MicroRNAs in Liver Regeneration. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2015;37:615-628.
- [13] Luedde T, Assmus U, Wustefeld T, Meyer zu Vilsendorf A, Roskams T, Schmidt-Supprian M, et al. Deletion of IKK2 in hepatocytes does not sensitize these cells to TNF-induced apoptosis but protects from ischemia/reperfusion injury. *The Journal of clinical investigation* 2005;115:849-859.

- 1
2
3
4 [14] Roy S, Benz F, Alder J, Bantel H, Janssen J, Vucur M, et al. Down-regulation of
5 miR-192-5p protects from oxidative stress-induced acute liver injury. *Clinical science*
6 2016;130:1197-1207.
7
8
9 [15] Mossanen JC, Tacke F. Acetaminophen-induced acute liver injury in mice.
10 *Laboratory animals* 2015;49:30-36.
11
12 [16] Keller A, Leidinger P, Gislefoss R, Haugen A, Langseth H, Staehler P, et al.
13 Stable serum miRNA profiles as potential tool for non-invasive lung cancer diagnosis.
14 *RNA biology* 2011;8:506-516.
15
16
17 [17] Vorwerk S, Ganter K, Cheng Y, Hoheisel J, Stahler PF, Beier M. Microfluidic-
18 based enzymatic on-chip labeling of miRNAs. *New biotechnology* 2008;25:142-149.
19
20
21 [18] Roderburg C, Urban GW, Bettermann K, Vucur M, Zimmermann H, Schmidt S, et
22 al. Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis.
23 *Hepatology* 2011;53:209-218.
24
25
26 [19] Gautheron J, Pescatore A, Fusco F, Esposito E, Yamaoka S, Agou F, et al.
27 Identification of a new NEMO/TRAF6 interface affected in incontinentia pigmenti
28 pathology. *Human molecular genetics* 2010;19:3138-3149.
29
30
31 [20] Liedtke C, Lambertz D, Schnepel N, Trautwein C. Molecular mechanism of
32 Mitomycin C-dependent caspase-8 regulation: implications for apoptosis and synergism
33 with interferon-alpha signalling. *Apoptosis : an international journal on programmed cell*
34 *death* 2007;12:2259-2270.
35
36
37 [21] Sonntag R, Gassler N, Bangen JM, Trautwein C, Liedtke C. Pro-apoptotic
38 Sorafenib signaling in murine hepatocytes depends on malignancy and is associated
39 with PUMA expression in vitro and in vivo. *Cell death & disease* 2014;5:e1030.
40
41
42 [22] Roderburg C, Benz F, Vargas Cardenas D, Koch A, Janssen J, Vucur M, et al.
43 Elevated miR-122 serum levels are an independent marker of liver injury in inflammatory
44 diseases. *Liver international : official journal of the International Association for the*
45 *Study of the Liver* 2014.
46
47
48 [23] Ward J, Kanchagar C, Veksler-Lublinsky I, Lee RC, McGill MR, Jaeschke H, et al.
49 Circulating microRNA profiles in human patients with acetaminophen hepatotoxicity or
50 ischemic hepatitis. *Proceedings of the National Academy of Sciences of the United*
51 *States of America* 2014;111:12169-12174.
52
53
54 [24] Perry BC, Soltys D, Toledo AH, Toledo-Pereyra LH. Tumor necrosis factor-alpha
55 in liver ischemia/reperfusion injury. *Journal of investigative surgery : the official journal of*
56 *the Academy of Surgical Research* 2011;24:178-188.
57
58
59 [25] Kastl L, Sauer SW, Ruppert T, Beissbarth T, Becker MS, Suss D, et al. TNF-
60 alpha mediates mitochondrial uncoupling and enhances ROS-dependent cell migration
61 via NF-kappaB activation in liver cells. *FEBS letters* 2014;588:175-183.
62
63
64
65

- 1
2
3
4
5 [26] Sharma AD, Narain N, Handel EM, Iken M, Singhal N, Cathomen T, et al.
6 MicroRNA-221 regulates FAS-induced fulminant liver failure. *Hepatology* 2011;53:1651-
7 1661.
8
9
- 10 [27] Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, et al. Circulating
11 microRNAs, potential biomarkers for drug-induced liver injury. *Proceedings of the*
12 *National Academy of Sciences of the United States of America* 2009;106:4402-4407.
13
14
- 15 [28] Dolganiuc A, Petrasek J, Kodys K, Catalano D, Mandrekar P, Velayudham A, et
16 al. MicroRNA expression profile in Lieber-DeCarli diet-induced alcoholic and methionine
17 choline deficient diet-induced nonalcoholic steatohepatitis models in mice. *Alcoholism,*
18 *clinical and experimental research* 2009;33:1704-1710.
19
20
- 21 [29] Bellinger MA, Bean JS, Rader MA, Heinz-Taheny KM, Nunes JS, Haas JV, et al.
22 Concordant changes of plasma and kidney microRNA in the early stages of acute kidney
23 injury: time course in a mouse model of bilateral renal ischemia-reperfusion. *PLoS one*
24 2014;9:e93297.
25
26
- 27 [30] Hunsberger JG, Fessler EB, Wang Z, Elkahlon AG, Chuang DM. Post-insult
28 valproic acid-regulated microRNAs: potential targets for cerebral ischemia. *American*
29 *journal of translational research* 2012;4:316-332.
30
31
- 32 [31] Niu Y, Mo D, Qin L, Wang C, Li A, Zhao X, et al. Lipopolysaccharide-induced
33 miR-1224 negatively regulates tumour necrosis factor- α gene expression by
34 modulating Sp1. *Immunology* 2011;133:8-20.
35
36
- 37 [32] Rutherford A, King LY, Hynan LS, Vedvyas C, Lin W, Lee WM, et al.
38 Development of an accurate index for predicting outcomes of patients with acute liver
39 failure. *Gastroenterology* 2012;143:1237-1243.
40
41
- 42 [33] Volkmann X, Anstaett M, Hadem J, Stiefel P, Bahr MJ, Lehner F, et al. Caspase
43 activation is associated with spontaneous recovery from acute liver failure. *Hepatology*
44 2008;47:1624-1633.
45
46
- 47 [34] Barman PK, Mukherjee R, Prusty BK, Suklabaidya S, Senapati S, Ravindran B.
48 Chitohexaose protects against acetaminophen-induced hepatotoxicity in mice. *Cell*
49 *death & disease* 2016;7:e2224.
50
51
- 52 [35] Semenova EA, Kwon MC, Monkhorst K, Song JY, Bhaskaran R, Krijgsman O, et
53 al. Transcription Factor NFIB Is a Driver of Small Cell Lung Cancer Progression in Mice
54 and Marks Metastatic Disease in Patients. *Cell reports* 2016;16:631-643.
55
56
- 57 [36] Jin M, Ande A, Kumar A, Kumar S. Regulation of cytochrome P450 2e1
58 expression by ethanol: role of oxidative stress-mediated pkc/jnk/sp1 pathway. *Cell death*
59 *& disease* 2013;4:e554.
60
61
62
63
64
65

1
2
3
4 [37] Zhang Q, Cao LY, Cheng SJ, Zhang AM, Jin XS, Li Y. p53-induced microRNA-
5 1246 inhibits the cell growth of human hepatocellular carcinoma cells by targeting NFIB.
6 Oncology reports 2015;33:1335-1341.
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 hCCl₄ 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 \pm 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

1
2
3
4 (H/R) and control cells (n = 6 per group). (D) miR-1224 expression in serum was
5
6 determined by qRT-PCR in patients with ALF and are depicted with respect to patients'
7
8 fate [spontaneous recovery (SR) (n = 18), death (NSR) (n = 21)]. (E) Receiver operating
9
10 curve analysis displaying the diagnostic power of miR-1224 serum concentrations in
11
12 predicting patients fate (area under the curve/AUC 0.861). Results are shown as mean \pm
13
14 SEM, * $p < 0.05$; ** $p < 0.01$, Student's *t* test.
15
16
17
18
19
20

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

22
23 (A) Relative expression of miR-1224 was determined by qRT-PCR in hepatocytes and
24
25 CD45⁺ cells isolated from livers of I/R or sham-treated C57Bl/6 mice (n = 4 per group).
26
27 (B) Intracellular ROS production was determined in primary hepatocytes and AML12
28
29 cells after 3 h H₂O₂ treatment by DCF fluorescence (n = 3 per group). (C) miR-1224
30
31 expression was analysed by qRT-PCR in primary hepatocytes and AML12 cells treated
32
33 with H₂O₂ for different time points (n = 3 per group). (D) miR-1224 expression was
34
35 measured by qRT-PCR in serum-starved primary hepatocytes and Aml12 cells for
36
37 different time points (n = 3 per group). (E) miR-1224 was measured by qRT-PCR in
38
39 primary hepatocytes and AML12 cells pre-treated with BHA for 30 min and incubated
40
41 with or without H₂O₂ for 3 h (n = 3 per group). Results are shown as mean \pm SEM,
42
43 * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, ns = not significant, ANOVA with Kruskal-Wallis test.
44
45
46
47
48
49
50
51
52

53 **Fig. 4. Elevated expression of miR-1224 is associated with impaired cell survival**
54
55 **and proliferation in ALF.**

56
57 (A) Primary hepatocytes and AML12 cells were transfected with miR-1224 or negative
58
59 control (Neg Ctrl) for 24 h and treated with or without H₂O₂ for 3 h. Cell viability was
60
61
62
63
64
65

1
2
3
4 measured by MTT assay. (B) Immunohistochemical (IHC) analysis of Ki67⁺ cells in liver
5 tissues of ALF patients with SR or NSR. (n = 3 per group). (C,D) IHC analysis and
6
7 quantification of Ki67⁺ cells (pink) (C) and BrdU (green) (D) in AML12 cells transfected
8
9 with or without miR-1224 and treated with H₂O₂ compared to negative control (Neg Ctrl)
10
11 cells. (E) Western blot analysis on extracts from AML12 cells transfected with or without
12
13 miR-1224 followed by treatment with H₂O₂ compared to negative control (Neg Ctrl) cells.
14
15 All experiments are performed in triplicates. Results are shown as mean ± SEM,
16
17 * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, ns = not significant, ANOVA with Kruskal-Wallis test.
18
19
20
21
22
23
24
25

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

27
28 (A,B) IHC analysis and quantification of TUNEL⁺ (green) (A) and cl. Casp-3⁺ (red) in
29
30 AML12 cells transfected with or without miR-1224 and treated with H₂O₂ compared to
31
32 negative control (Neg Ctrl) cells. (C) Caspase activity in AML12 cell lysates was
33
34 determined using the Caspase-3 substrate DEVD-AMC and the Caspase-8 substrate
35
36 DEVD-AFC. (D) AML12 cells were treated with Fas Agonist for 12 h and relative
37
38 expression of miR-1224 was measured by qRT-PCR (n = 3 per group). (E) AML12 cells
39
40 were transfected with control (Neg Ctrl), miR-1224 or miR-1224ag and treated with Fas
41
42 Agonist. Cell viability was measured with MTT assay. All experiments are performed in
43
44 triplicates. Results are shown as mean ± SEM, * $p < 0.05$; ** $p < 0.01$, ns = not significant,
45
46 ANOVA with Kruskal-Wallis test.
47
48
49
50
51
52
53
54

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

56
57 (A) Schematic diagram illustrating the selection of putative targets of miR-1224 whose
58
59 expression was analysed by qRT-PCR RNA extracts from livers of mice after I/R or
60
61
62
63
64
65

1
2
3
4 sham surgery (n = 6 per group). (B) Luciferase assay performed on AML12 cells
5
6 transfected with *Nfib*-3'UTR reporter plasmid together with miR-1224 or negative control
7
8 (Neg Ctrl) (n = 3 per group). (C) Expression of *Nfib* was analysed by qRT-PCR and
9
10 Western Blot from AML12 cells and Huh7 cells, respectively transfected with miR-1224
11
12 or negative control (Neg Ctrl). (D) Expression of *Nfib* was analysed by qRT-PCR in
13
14 AML12 cells transfected with or without miR-1224 and treated with H₂O₂ compared to
15
16 negative control (Neg Ctrl) cells (n = 3 per group). (E) Expression of *NFIB* was
17
18 measured by qRT-PCR from liver samples of patients with ALF (n = 6) as well as in
19
20 healthy controls (n = 4). (F) IHC analysis and quantification of *NFIB* in liver tissues of
21
22 ALF-patients with SR or NSR (n = 3 per group). Results are shown as mean ± SEM, **p*
23
24 < 0.05; ***p* < 0.01, ****p* < 0.001, ns = not significant, Student's *t* test.
25
26
27
28
29
30
31
32

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

34
35 (A) Expression of *Nfib* was measured by qRT-PCR and Western Blot in AML12 cells and
36
37 Huh7 cells, respectively after transfection with the respective siRNAs for 24 h. (B)
38
39 AML12 cells were transfected with *Nfib* siRNA alone or treated with H₂O₂ for 3 h. Cell
40
41 viability was assessed by MTT assay after treatment. (C) AML12 cells were transfected
42
43 with miR-1224ag in combination with *Nfib* siRNA. Cell viability was measured by MTT
44
45 assay. (D) Cell viability was measured in cells transfected with *Nfib* plasmid along with
46
47 miR-1224. (E) IHC analysis and quantification of Ki67⁺ (pink), BrdU⁺ (green), TUNEL⁺
48
49 (green) and cl. Casp-3⁺ (red) in AML12 cells transfected with siRNAs against *Nfib* and
50
51 treated with H₂O₂ compared to negative control (Neg Ctrl) cells. All experiments are
52
53 performed in triplicates. (F) IHC analysis and quantification of TUNEL⁺ (green) and cl.
54
55 Casp-3⁺ (red) in AML12 cells transfected with siRNAs against *Nfib* and treated with H₂O₂
56
57
58
59
60
61
62
63
64
65

1
2
3
4 compared to negative control (Neg Ctrl) cells. All experiments are performed in
5
6 triplicates. Results are shown as mean \pm SEM, * p < 0.05; ** p < 0.01, *** p < 0.001, ns =
7
8 not significant , ANOVA with Kruskal-Wallis test.
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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
[Click here to download high resolution image](#)

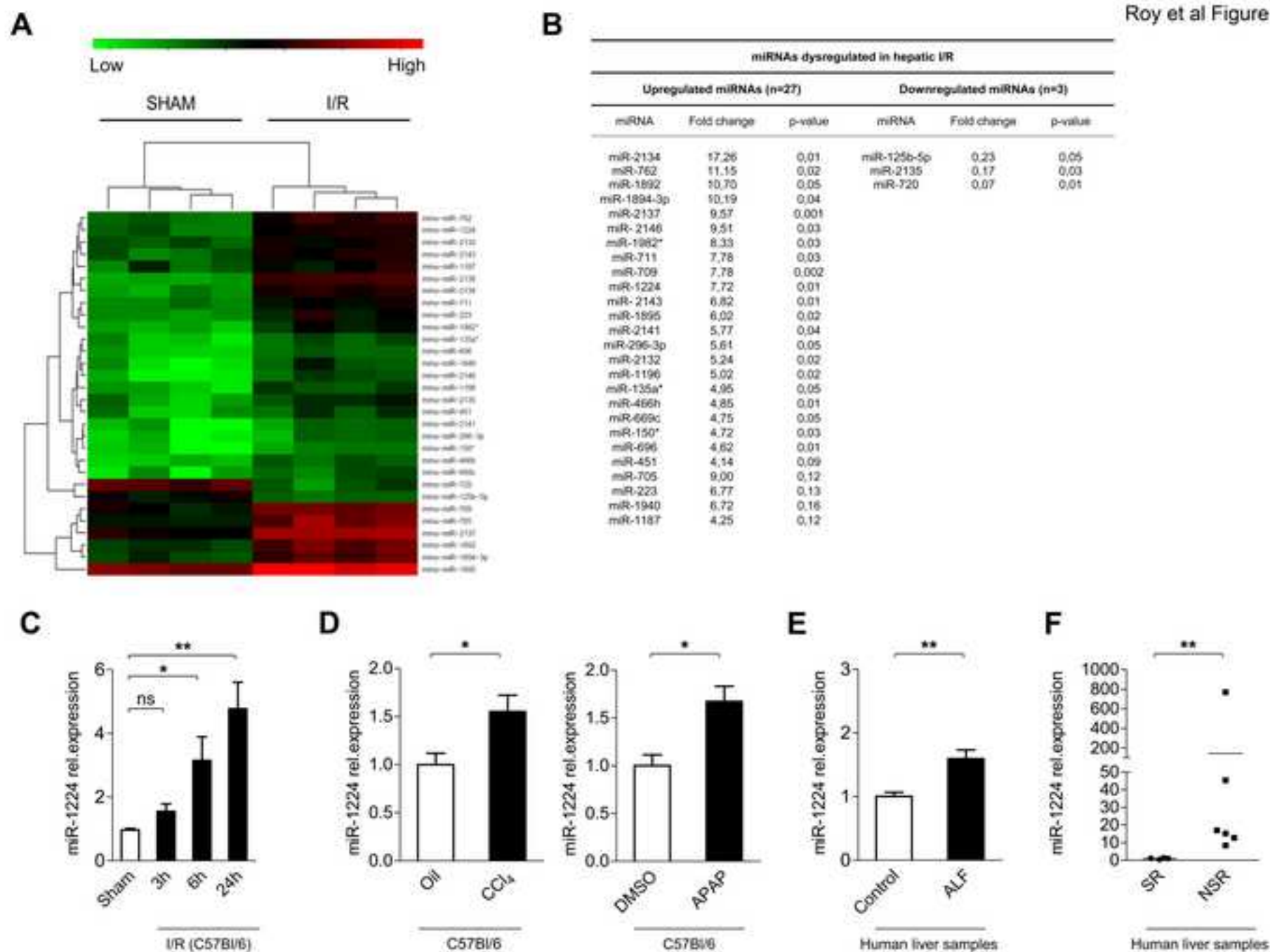


Figure 2
[Click here to download high resolution image](#)

Roy et al Figure 2

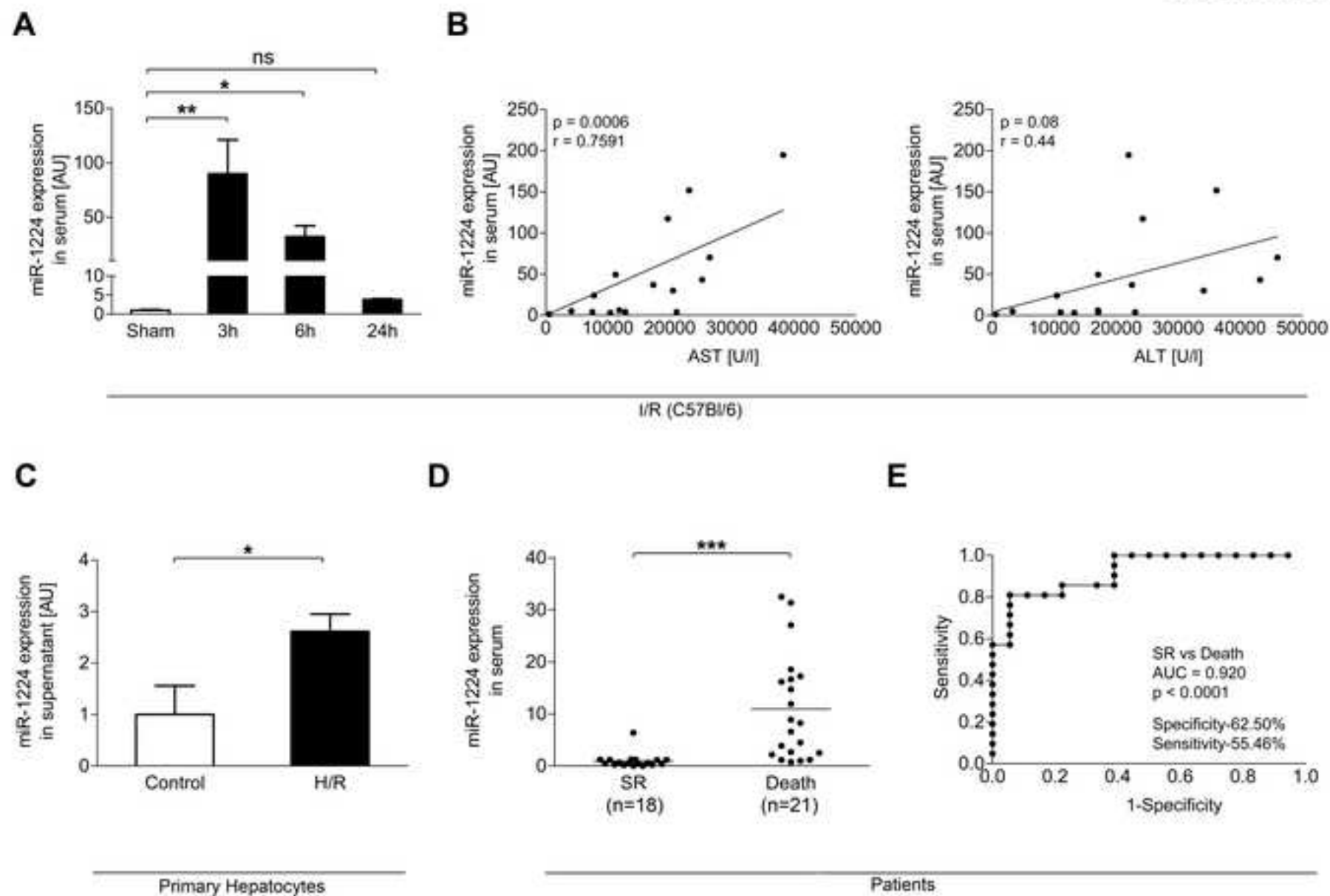


Figure 3
[Click here to download high resolution image](#)

Roy et al Figure 3

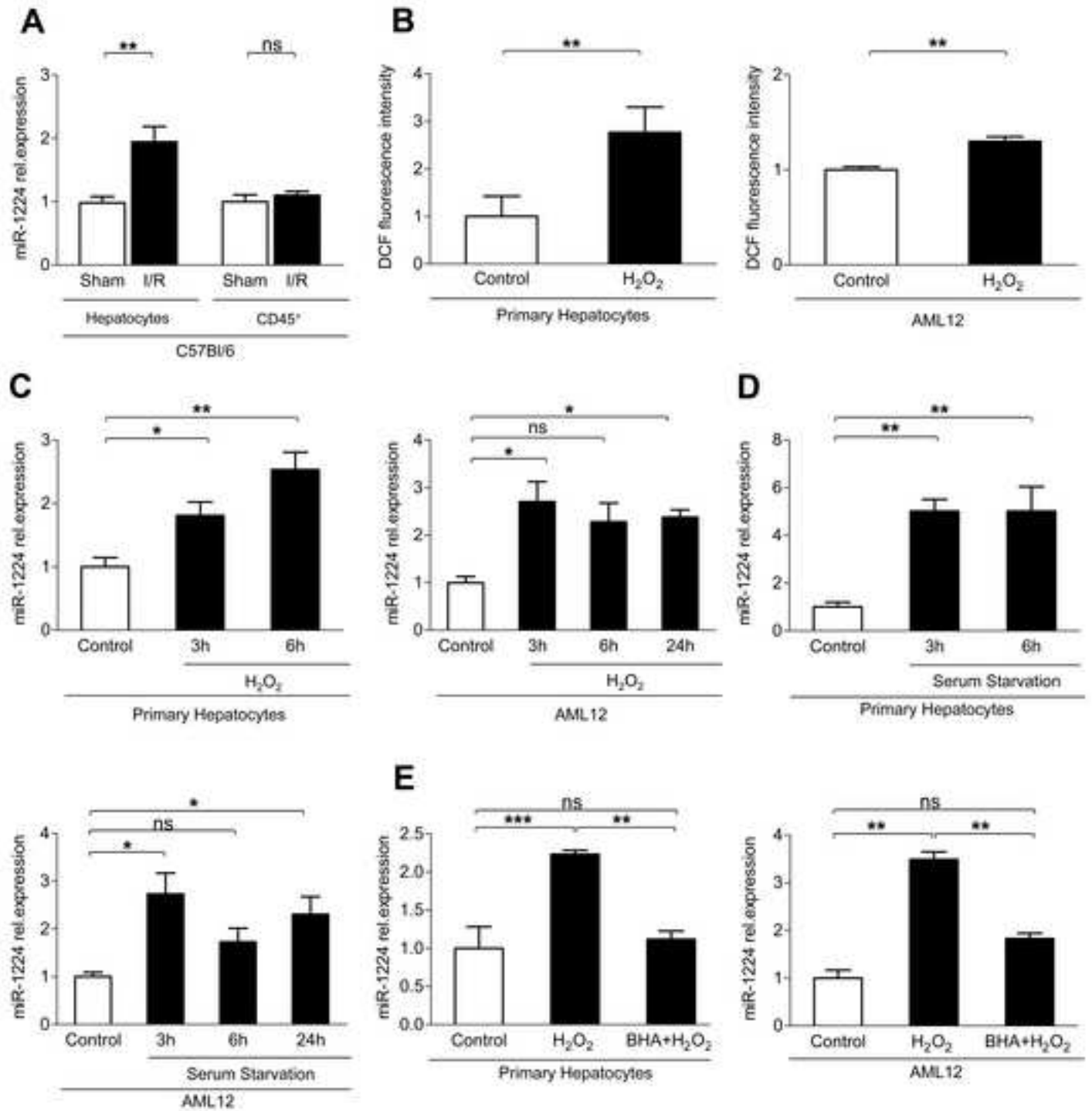


Figure 4
[Click here to download high resolution image](#)

Roy et al Figure 4

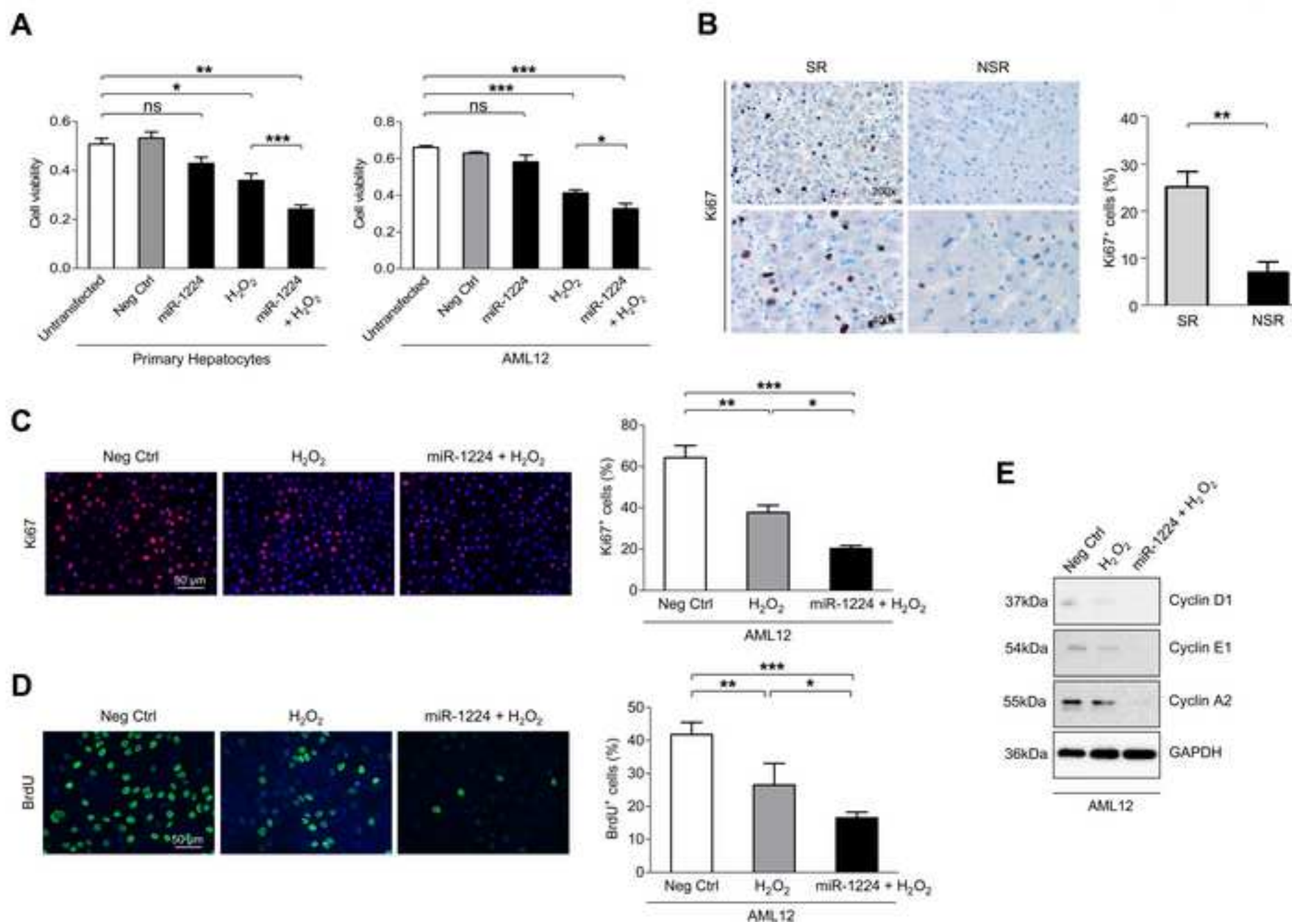


Figure 5
[Click here to download high resolution image](#)

Roy et al Figure 5

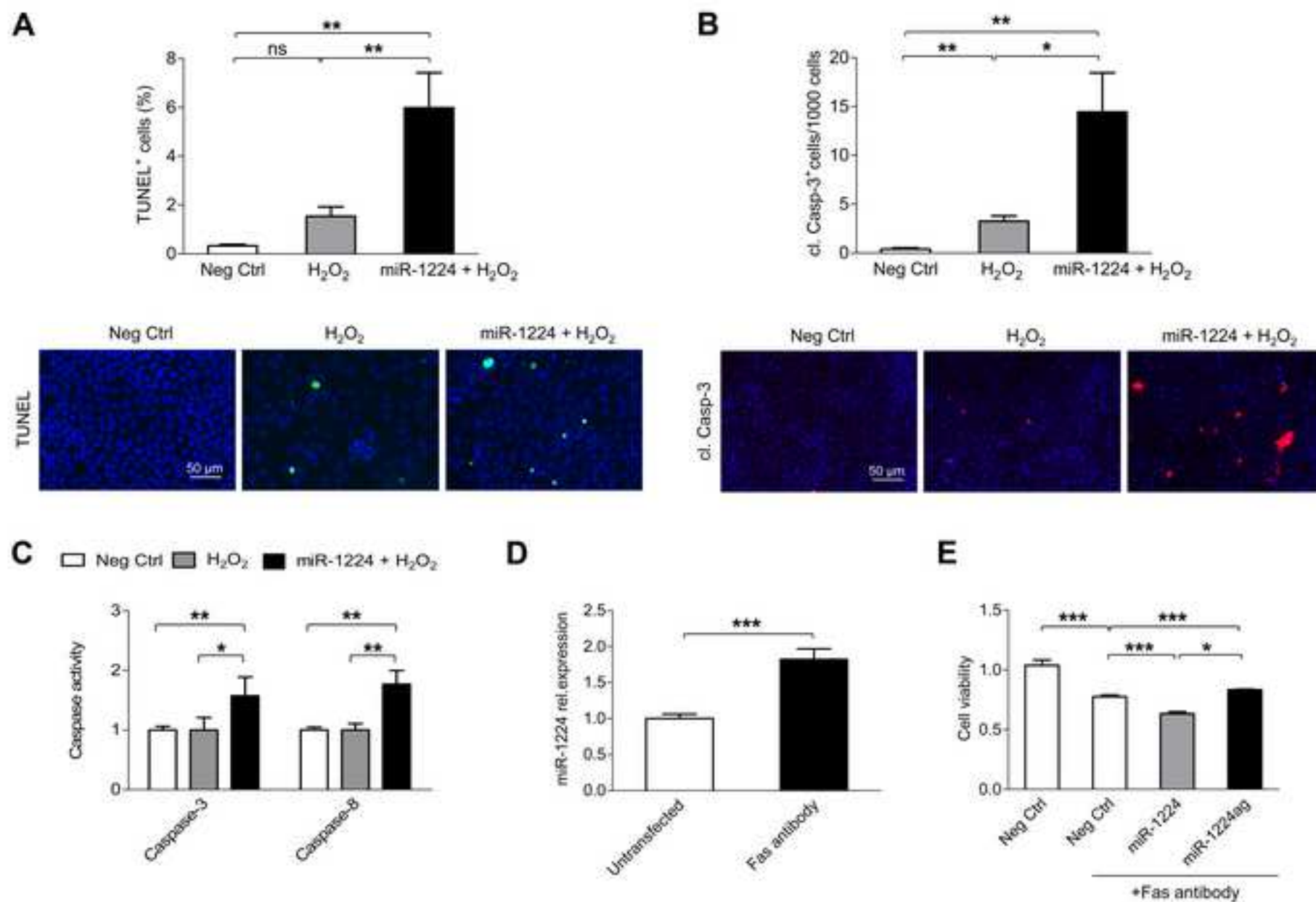
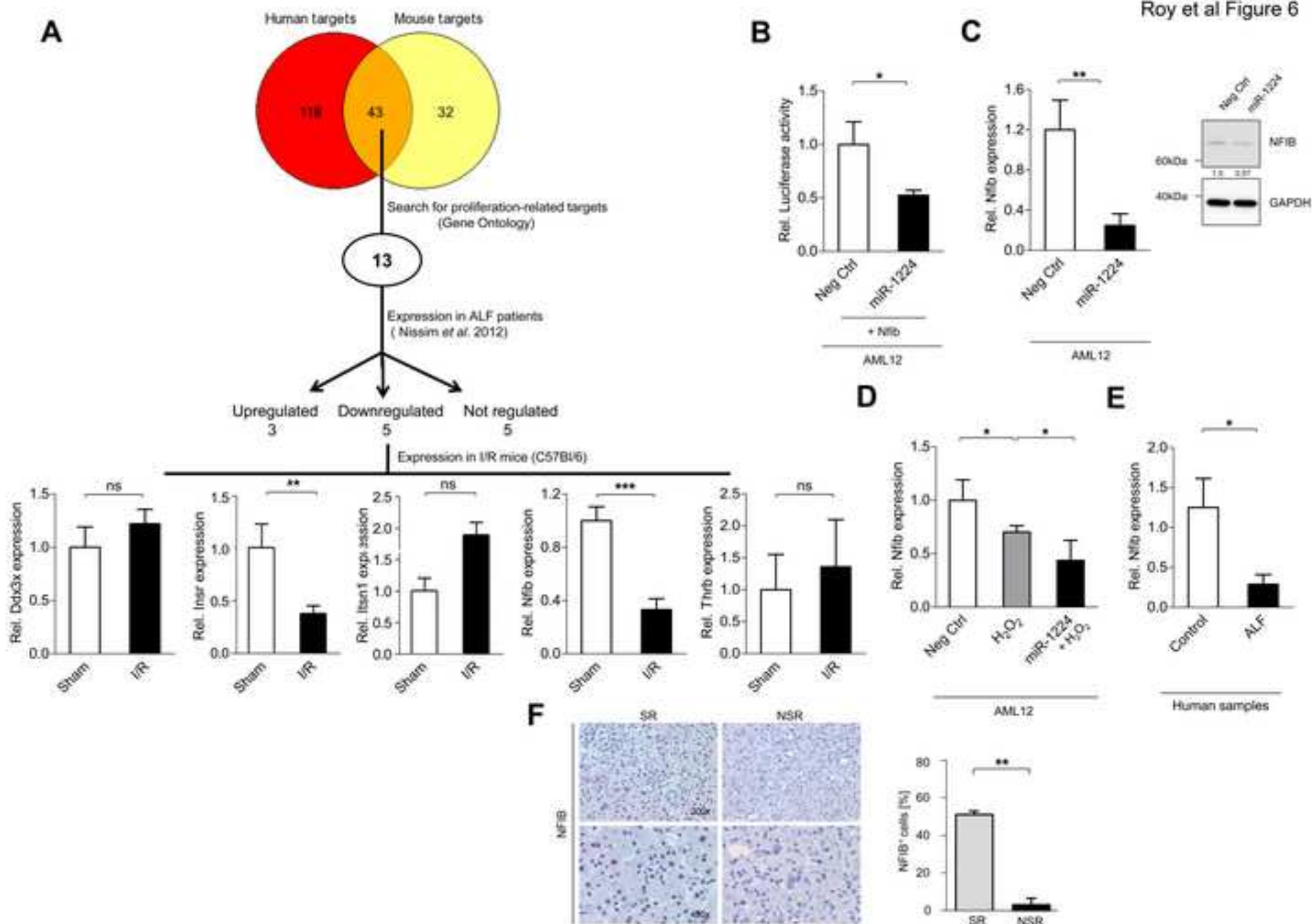
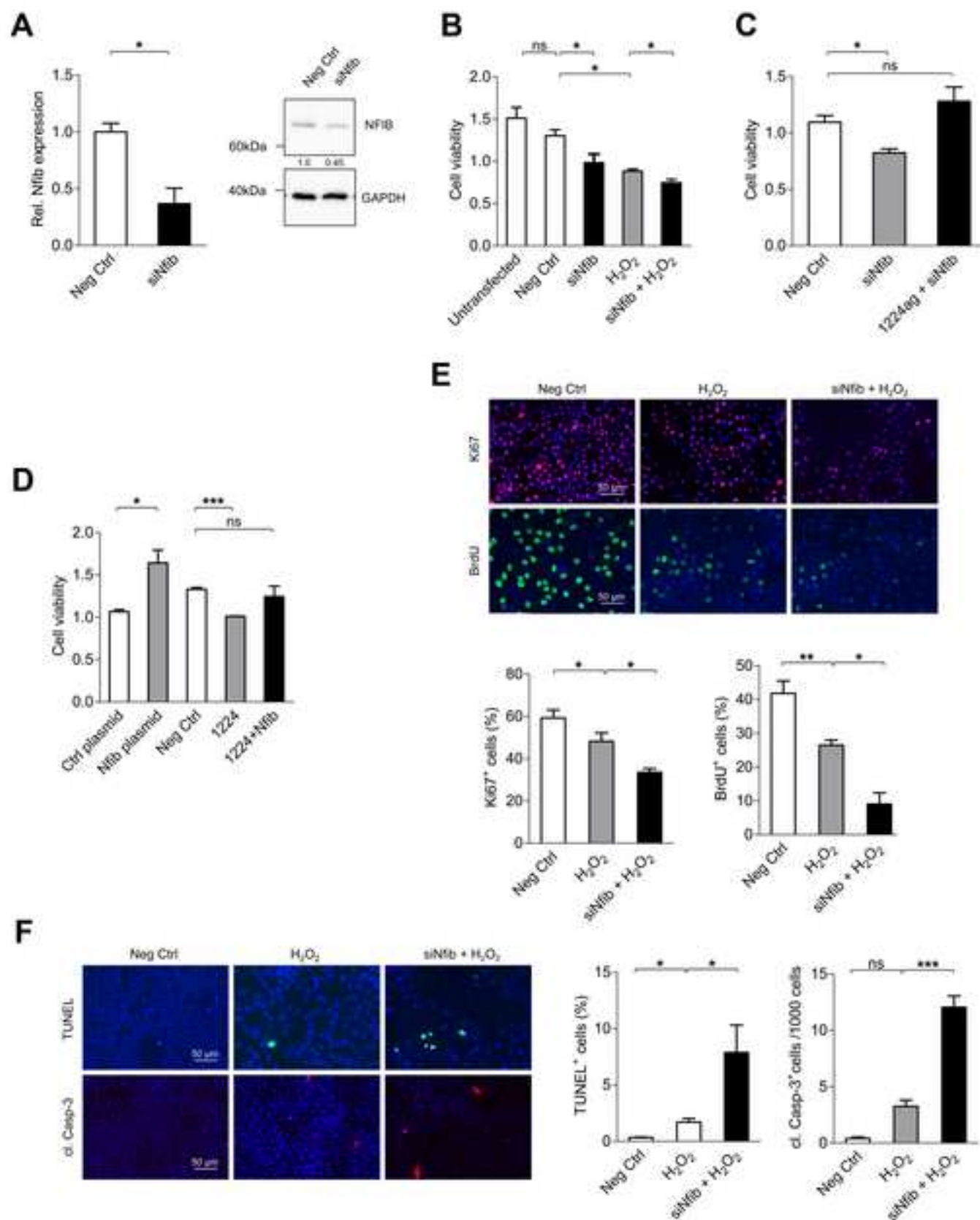
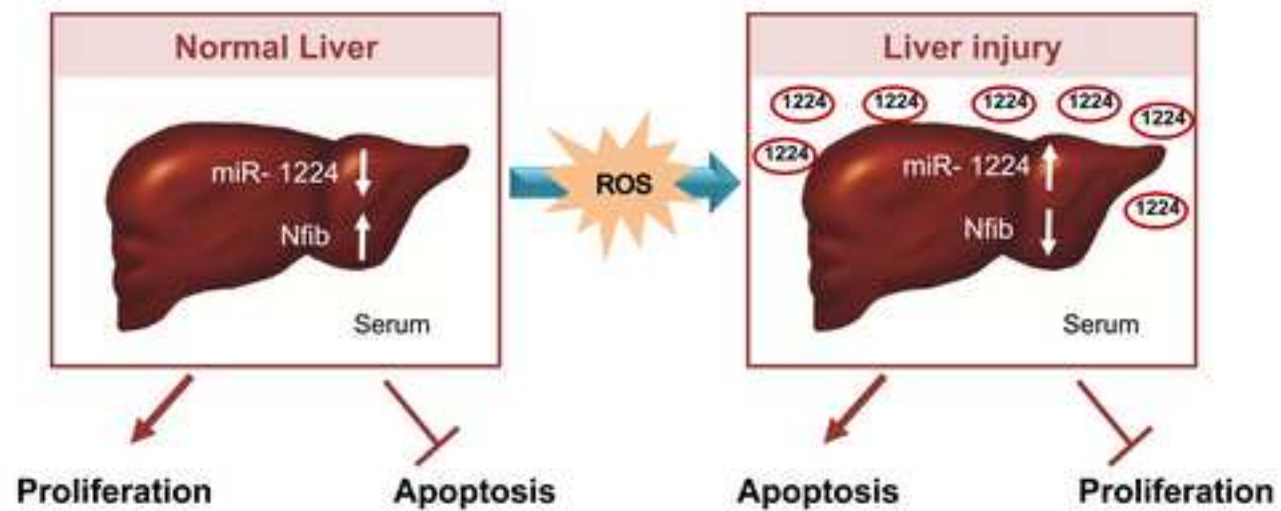


Figure 6
[Click here to download high resolution image](#)







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