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Permalink

https://escholarship.org/uc/item/65q5d6t0

Journal

Liver international : official journal of the International Association for the Study of the Liver, 30(3)

ISSN

1478-3231

Authors

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Publication Date

2010-03-01

DOI

DOI: 10.1111/j.1478-3231.2009.02181.x

Peer reviewed

BASIC STUDIES

Acute liver injury upregulates microRNA-491_5p in mice, and its overexpression sensitizes Hep G2 cells for tumour necrosis factor-α-induced apoptosis

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Abstract
Background: MicroRNAs (miRNAs) have emerged as novel genetic regulators of cell functions such as proliferation, apoptosis and cancer. *Aims:* The aim of this study was to evaluate the role of a specific miRNA in modulating hepatic cell functions. *Methods:* C57Bl/6 mice were administered anti-fas receptor antibodies to induce liver cell apoptosis. miRNAs were purified from the liver tissue and evaluated using an miRNA microarray. The role of miRNA-491_5p, which was overexpressed in the model, in modulating hepatic cell functions was evaluated. miRNA-491_5p was overexpressed in Hep G2 cells, followed by the addition of tumour necrosis factor (TNF)-α, and induction of apoptosis as well as genes involved in apoptosis pathways were evaluated. The effect of miRNA-491_5p target genes on apoptosis was also analysed by inhibiting their expression by siRNA-induced gene silencing. *Results:* Upregulation of miRNA-491_5p was found in a high-dose anti-fas receptor antibody group. Overexpression of microRNA-491_5p sensitized Hep G2 cells for TNF-α-induced apoptosis, and also caused an inhibition of α-fetoprotein, (AFP), heat shock protein-90 (hsp-90) and nuclear factor-κB (NF-κB). Overexpression of miRNA-491_5p or inhibition of AFP and hsp-90 resulted in an increased apoptosis in TNF-α-treated Hep G2 cells. *Conclusions:* One of the miRNAs that is associated with the acute liver injury mouse model, miRNA-491_5p, sensitizes Hep G2 cells for TNF-α-induced apoptosis, at least in part, by inhibiting AFP, hsp-90 and NF-κB.

The liver is a multifunctional organ performing metabolism, biosynthesis, excretion, secretion and detoxification. These processes make the liver susceptible to noxious insults, and create a demand for cell replacement after tissue loss. Enhanced liver cell death and impaired regeneration are features of most liver disorders (1). However, unlike almost any other organ, the liver can regenerate by cell proliferation following either partial hepatectomy or hepatic injury. Depending on the extent of injury, the balance shifts from regeneration to cellular loss and hepatic failure. It has been shown that the injection of anti-fas receptor antibodies at low concen-trations in mice caused minor amounts of hepatocyte apoptosis, which was followed by liver regeneration as evidenced by increased bromodeoxy uridine (BrdU) incorporation (2). Higher concentrations of anti-fas receptor antibodies caused severe apoptosis and liver failure when liver regeneration could not keep up with the pace of hepatocyte apoptosis (3, 4). Administration of D-galactosamine yielded increased DNA synthesis and the expression of several transcription factors that are involved in cell proliferation (5, 6), and another study has shown that acute oxidative stress induced the proliferation and regeneration of hepatocytes (7). Thus, the

available evidence indicates that a low level of insult causes low levels of injury insufficient to cause organ failure; therefore, regeneration occurs to restore the organ structure. High levels of insult cause severe tissue injury, which cannot be repaired in due time by regeneration, leading to organ failure (8).

MicroRNAs (miRNAs) are a family of small non-coding 21–23 nucleotide RNAs that regulate gene expression by targetting mRNAs in a sequence-specific manner, inducing translational repression or mRNA degradation, depending on the degree of complementarity between miRNAs and their targets (9). They appear to function via several mechanisms in repressing gene expression and regulating cellular activities, such as development (10), cell proliferation (11), differentiation (12), apoptosis (13), glucose metabolism (14), stress resistance (15) and cancer (16). An example of an miRNA that is abundant in the liver, and appears to affect hepatic function, is microRNA-122a (miR-122a) (17). When miR-122a was silenced using antagomiR-122a, a cholesterol-conjugated inhibitory molecule of miR-122a, there was a 44% decrease in cholesterol synthesis in hepatocytes (17). The mechanism of this effect appears to be that inhibition of miR-122a caused the activation of a transcriptional repressor protein involved in cholesterol biosynthesis (17). Another study reported that inhibition of miR-122a in the liver caused a marked loss of hepatitis C viral RNAs, and that miR-122a may represent a target for antiviral intervention (18). Several miRNAs have been implicated in the process of apoptosis and cell proliferation in non-liver systems (13, 19). For example, miR-21 has been shown to inhibit caspase activation, thereby promoting anti-apoptosis (20). miR-15 and miR-16 were shown to promote apoptosis by interacting with Bcl-2 (13) and downregulation of miR-27b caused apoptosis (21). Another study shows that miR-126 increased the growth and proliferation of megakaryocytes (22), and that miR-451 enhanced the differentiation of erythroid cells (23).

In the present study, we explored the differential expression of miRNAs during post-injury regeneration and apoptosis using an anti-fas receptor antibody-induced liver injury model system. One of the miRNAs that was highly expressed was microRNA-491_5p (miR-491_5p). We have characterized the role of this miRNA in modulating Hep G2 function, and our results suggest that downregulation of some of its target genes appears to enhance tumour necrosis factor (TNF)-α-induced hepatic cell apoptosis.

Materials and methods

Animals and anti-fas receptor antibody treatments

Male C57Bl/6 mice (five mice per group; 8 weeks old; Charles River Labs, MN, USA) were maintained on a 12-h light/12-h dark cycle and fed with commercial diet and water. All animal experiments were performed according to the National Institutes of Health guidelines for the ethical care and use of laboratory animals, and the experimental protocol was approved by the Institutional

Animal Care and Use Committee of the University of California, Davis. The mice were injected with different concentrations of anti-fas receptor antibodies (BD Biosciences, San Jose, CA, USA; 0, 0.125, 0.25 and 0.5 μg/g mouse, i.p.). After 48 h, the serum was separated and serum alanine transaminase (ALT) was measured using an ALT measurement kit (Catachem, Bridgeport, CT, USA), and the liver tissue was collected for apoptosis assays and miRNA purification.

MicroRNA isolation, labelling, purification and microarray

Total RNA enriched with miRNA was isolated from 50 mg liver tissue using the mirVana miRNA isolation kit (Applied Biosystems San Diego, CA, USA) according to the manufacturer's instructions. MicroRNAs were purified using the FlashPAGE gel electrophoresis system (Applied Biosystems) and labelled with Alexa fluor 555 (Invitrogen, Carlsbed, CA, USA), followed by hybridization with mirVana miRNA bioarray slides according to the manufacturer's instructions. The slides were washed and then scanned with a Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). Raw data were analysed using GENEPIX PRO 6 software (Molecular Devices, Sunnyvale, CA, USA). Normalization was performed by expressing each miRNA replicate relative to a control miRNA (provided in the Bioarray essentials kit, Applied Biosystems) added to each sample, thus allowing for comparisons among chips. Average values of the mean intensity of each replicate in the three groups were generated.

Stem-loop real-time quantitative reverse transcriptase-polymerase chain reaction

To confirm miR-491_5p expression, stem-loop real-time quantitative reverse transcriptase-polymerase chain reaction (SLqPCR) was performed. Total RNA (10 ng) was used for first-strand cDNA synthesis using an miR-491_5p-specific stem-loop primer or Rnu-43, a control endogenous RNA (Applied Biosystems), followed by real-time PCR amplification with a gene-specific forward primer and a reverse primer along with a probe, in an ABI Prism 7500 PCR machine (Applied Biosystems). The relative expression of miR-491_5p was calculated from miR-491_5p and Rnu-43 levels.

Transfection of Hep G2 cells

To study the functional role of miR-491_5p, mature miR-491_5p precursor molecules and non-specific miRNA (used as negative control) were obtained from Applied Biosystems. We cultured a hepatocellular carcinoma cell line, Hep G2, in MEM media containing 10% fetal bovine serum, antibiotics and sodium pyruvate. The cells were transfected with either miR-491_5p or non-specific miRNA (NS-miRNA; 50 nM each). Oligos (12.5 μl of 10 μM diluted in 87.5 μl of Opti-MEM medium) were mixed with SiPORT neoFX transfection reagent (5 μl diluted in 95 μl of Opti-MEM) and dispensed on six-well

plates. The cells (2.3×10^5 cells in 2.3 ml) were overlaid on the transfection mix and allowed to grow for 24 h, and the medium was replenished. Some of the cells were analysed for transfection efficiency using SLqPCR. For siRNA transfection experiments, the cells were plated 24 h before transfection. The cells were washed and siRNA oligos (50 nM) were mixed with siPORT transfection reagent and added to the cells in a low serum growth medium. After overnight incubation, the cells were washed and the growth medium was replenished. After 48 h of transfection with miRNAs or siRNAs, all the experiments were performed.

Labelling of microRNA with fluorescent probes

The miR-491_5p oligos (Applied Biosystems; 10 nmol in 200 μl) were mixed with 50 μl of 0.25 M sodium acetate buffer, 20 μl of double-distilled water and 10 μl of 5 mM sodium periodate. The reaction mixture was incubated at 25 °C in the dark for 90 min for the oxidation of the 3′ terminus of miRNA. The oxidized miRNA was purified and biotinylated using biotin hydrazide (25 nmol). Biotin–miRNA 200 μl, (0.8 μg/μl; 8 nmol) and streptavidin (SA) 180 μl (2 μg/μl; 8 nmol) were mixed and incubated at room temperature for 1 h. The reaction mixture was purified by an HPLC SEC 3000 column (Waters Corp., Milford, MA, USA) using a UV_{254} detector at a flow rate of 1 ml/min PBS buffer and stored at $-70\,°C$ until transfection experiments.

Apoptosis and survival assays

The transfected cells with or without TNF-α treatments were washed and analysed for apoptosis. The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was performed using the Apoptag kit from Serologicals Corporation (Billerica, MA, USA) as we have carried out previously (24). In separate wells, 4′, 6-diamidino-2-phenylindole (DAPI) staining was performed using a mounting medium containing DAPI. The caspase-3 activity assay kit was obtained from EMD Biosciences (San Diego, CA, USA), and the assay was performed according to the manufacturer's instructions. For cell survival assay, WST-1 reagent (Roche Diagnostics, Indianapolis, IN, USA) was used according to the manufacturer's instructions.

Electrophoretic mobility shift assay

For determining the activation of nuclear factor-κB (NF-κB), electrophoretic mobility shift assays (EMSA) were performed using a non-radioactive detection method. All the reagents and kits needed for this assay were obtained from Pierce Biotechnology (Rockford, IL, USA). After different treatments, the cells were washed and nuclear protein was isolated. NF-κB oligos (Promega Corp., San Luis Obispo, CA, USA) were labelled with biotin according to the manufacturer's instructions. The labelled oligos were purified using spin columns and binding reactions between the labelled oligos and the nuclear protein were conducted. The bound DNA–protein complex was separated by polyacrylamide gel electrophoresis, followed by the detection of the shifted bands, as we have carried out previously (25, 26).

Reverse transcriptase-polymerase chain reaction and real-time polymerase chain reaction array

The cells were collected, total RNA was isolated using Qiagen's RNA isolation kit (Valencia, CA, USA) and cDNA was synthesized. Human-specific heat shock protein (hsp)-90, α-fetoprotein (AFP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Hsp-90 and AFP were amplified for 25 cycles and GAPDH was amplified for 20 cycles. The PCR conditions were one cycle of 94 °C for 5 min, 20–25 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, followed by one cycle of 72 °C for 10 min. The amplified bands were resolved in 1.5% agarose gels. For real-time PCR array, the total RNA isolated from Hep G2 cells was isolated and first-strand cDNA synthesis was performed using the first-strand cDNA synthesis kit (Invitrogen, San Diego, CA, USA). The cDNA was amplified using a real-time PCR machine according to the manufacturer's instructions (Superarray Biosciences, Frederik, MD, USA).

Western blots

Cell lysates from different incubations were collected in a mammalian cell extraction solution (Pierce Biotechnology) and the protein content was quantified using bicinconinic acid reagent (Pierce Biotechnology). The samples (30–60 μg/lane) were separated using 10% polyacrylamide gels and transferred to polyvinylidene fluoride membranes and detected using antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) against hsp-90, AFP, bax, bcl-2, mcl-1, caspase-3 or β-actin (Sigma Aldrich, St Louis, MO, USA) as described previously (27).

Statistical analysis

All the experiments were performed in triplicate and at least three times. The statistical significance was calculated using analysis of variance, followed by paired *t*-tests. The data are expressed as means ± standard error of the mean, and $P < 0.05$ was considered as statistically significant.

Results

We have utilized an *in vivo* mouse model of anti-fas receptor antibody-induced injury to identify the differential expression of miRNAs in the liver. *In vivo* administration of low-dose anti-fas receptor antibodies (0.125 μg/g mouse) did not cause any significant injury, whereas high-dose anti-fas receptor antibodies (0.5 μg/g mouse) caused significant liver injury as measured by the serum ALT levels (Fig. 1A). High-dose anti-fas receptor
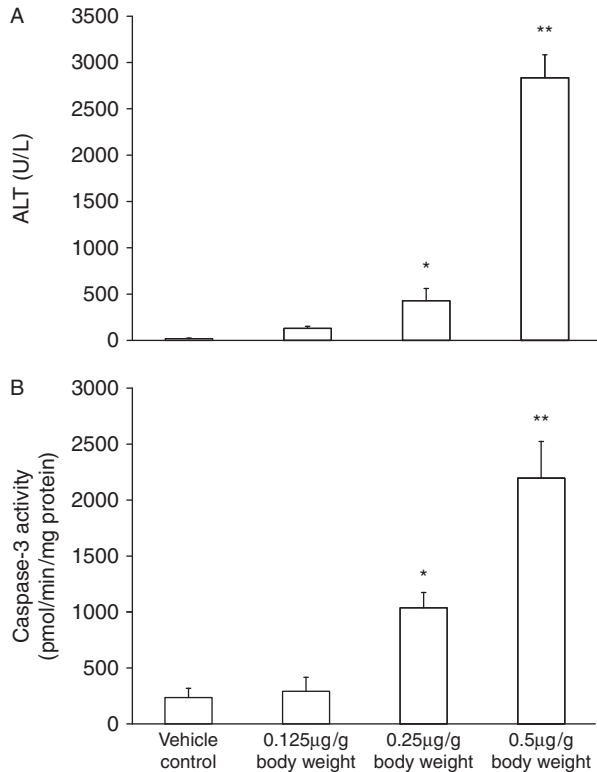
Fig. 1. Anti-fas receptor antibodies induce a liver injury in C57Bl/6 mice. (A) Serum alanine transaminase (ALT) levels in mice injected with anti-fas receptor antibodies (0, 0.125, 0.25 and 0.5 µg/g body weight, i.p.). (B) Caspase-3 activity in the liver tissue was analysed using a caspase-3 activity assay kit. The data are presented as means ± standard error of the mean. $n = 5$/group. *$P < 0.01$, **$P < 0.001$.

Table 1. Expression of microRNAs in mice liver tissue

| miRNA list	Anti-fas receptor antibodies (fold change over vehicle control)	
	Low dose (0.125 µg/g mouse)	High dose (0.5 µg/g mouse)
miR-491_5p	Not expressed	186.3 ($P < 0.001$)
miR-451	1.3 ± 0.07	5.1 ± 0.3 ($P < 0.01$)
miR-22	3.6 ± 0.23 ($P < 0.05$)	0.5 ± 0.02 ($P < 0.05$)
miR-148a	2.7 ± 0.12 ($P < 0.05$)	0.5 ± 0.03 ($P < 0.05$)
miR-30a_5p	2.1 ± 0.1 ($P < 0.05$)	0.6 ± 0.07
miR-30c	2.5 ± 0.22 ($P < 0.05$)	0.7 ± 0.04
miR-21	5.0 ± 0.37 ($P < 0.01$)	1.3 ± 0.06
miR-126	2.5 ± 0.19 ($P < 0.05$)	1.2 ± 0.03

C57Bl/6 mice were administered anti-fas receptor antibodies with either vehicle control, low dose (0.125 µg/g) or high dose (0.5 µg/g). After 48 h, the liver tissue was homogenized and miRNA microarray analysis was performed. The expression of miRNAs in vehicle control mice is calculated as 1 and the results are expressed as fold change over vehicle control. $n = 5$/group.
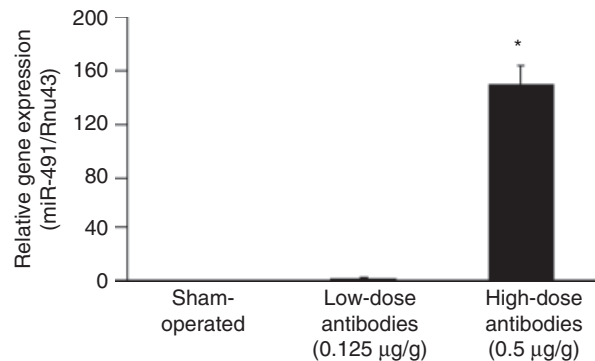
Fig. 2. Expression of miR-491_5p by stem-loop real-time quantitative reverse transcriptase-polymerase chain reaction (SLqPCR). The microarray results were confirmed by performing SLqPCR in the mice liver tissue with different treatments. Rnu43 was used as an internal control. The data are presented as means ± standard error of the mean. $n = 5$/group. *$P < 0.01$.

antibody administration also caused increased apoptosis as measured by the caspase-3 activity assay (Fig. 1B). Liver tissue was collected from the vehicle control mice and the animals in the low-dose anti-fas receptor antibody group (0.125 µg/g mouse) and the high-dose anti-fas receptor antibody group (0.5 µg/g mouse). Total RNA was isolated from the livers and miRNA microarray was performed. The results are presented in Table 1. We found that miR-22, miR-148a, miR-30a_5p, miR-30c, miR-21 and miR-126 were upregulated in the low-dose group compared with the control animals. miR-451 was unchanged in both the untreated and the low-dose group but was highly upregulated in high-dose group. Another miRNA, miR-491_5p, was not expressed either in the control animals or in the low-dose group, but was highly expressed in the high-dose injury group. Hence, miR-491_5p was selected for further studies. The microarray results were confirmed by performing the SLqPCR technique. As an internal control, Rnu43, a small nucleolar RNA molecule that is expressed constitutively, was used for all SLqPCR reactions. The ratio of miR-491_5p expression to Rnu43 was calculated between the vehicle control and the high-dose antibody group. The results

showed that there was a significant increase in miR-491_5p expression in the high-dose antibody group compared with the vehicle control group (Fig. 2).

Next, the kinetics of miR-491_5p uptake in the cells was determined. For this experiment, the precursor miR-491_5p molecules were labelled with Alexa fluor® 488-streptavidin fluorescent molecules. The schematic diagram is presented in Figure 3A. The labelled miRNA was purified using an HPLC system. The final product of Alexa fluor® 488-SA-miR491 showed > 90% purity by SEC3000-HPLC (Fig. 3B). Following purification, the labelled miR-491_5p was transfected into Hep G2 cells, and the transfected cells were examined using fluorescent microscopy at different time points. We found that all the cells were transfected with labelled miR-491_5p after 12 h of incubation (Fig. 3C). The expression of miRNA was sustained up to 5 days after transfection.

A

B

C
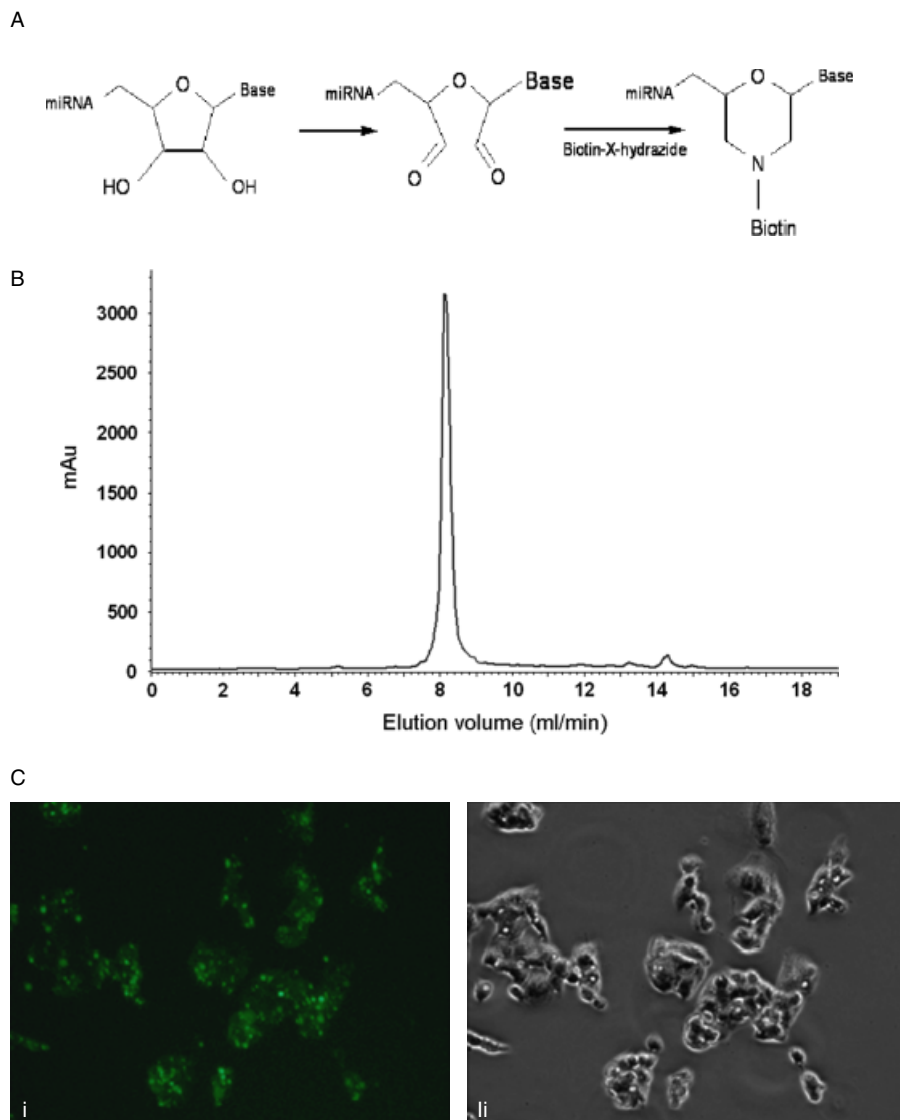
Fig. 3. Maximum transfection efficiency was achieved by 12 h after transfection. (A) Schematic representation of the process of labelling of miR-491_5p. (B) miR-491_5p was labelled with biotin and was conjugated with SA-Alexa fluor® 488 and purified using high-performance liquid chromatography (HPLC). The graph shows SEC-3000 HPLC trace of purified Alexa fluor® 488-SA-miR491. (C) Hep G2 cells were transfected with labelled miR-491_5p, and were observed at different time points. The photomicrograph shows a 12 h time point at which all the cells were transfected, as shown by the cells under (i) the fluorescent microscope and (ii) the corresponding phase micrograph.

The effect of overexpression of miR-491_5p on TNF-α-induced apoptosis in Hep G2 cells was studied. Both precursor miR-491_5p molecules and a negative control NS-miRNA molecule, an miRNA that has been shown not to inhibit any known human mRNA, were transfected into the Hep G2 cells. In parallel experiments, FITC-conjugated siRNAs were transfected to evaluate the efficiency of transfection and 100% of the cells were found to be transfected (data not shown). After 24 h, the cells were washed and replenished with fresh media. The next day, the cells were treated with TNF-α for 4 h. Before TNF-α treatment, some of the cells were collected for SLqPCR to assess the miRNA presence inside the cells.

We found that there was more than a 1500-fold increase in the miR-491_5p levels in the cells that were transfected with the miR-491_5p (Fig. 4A). The remaining cells were collected for either DAPI or TUNEL staining, or caspase-3 activity assay. The results indicated that miR-491_5p sensitized the cells for TNF-α-induced apoptosis as determined by DAPI (Fig. 4B) and TUNEL staining (Fig. 4C). The apoptotic nuclei were counted and the total cells were counted in at least 10 different high-power fields and the percentage of apoptosis was calculated. There was a significant increase of apoptosis in miR-491 transfected cells treated with TNF-α (Fig. 4D). The caspase-3 activity was also significantly increased in
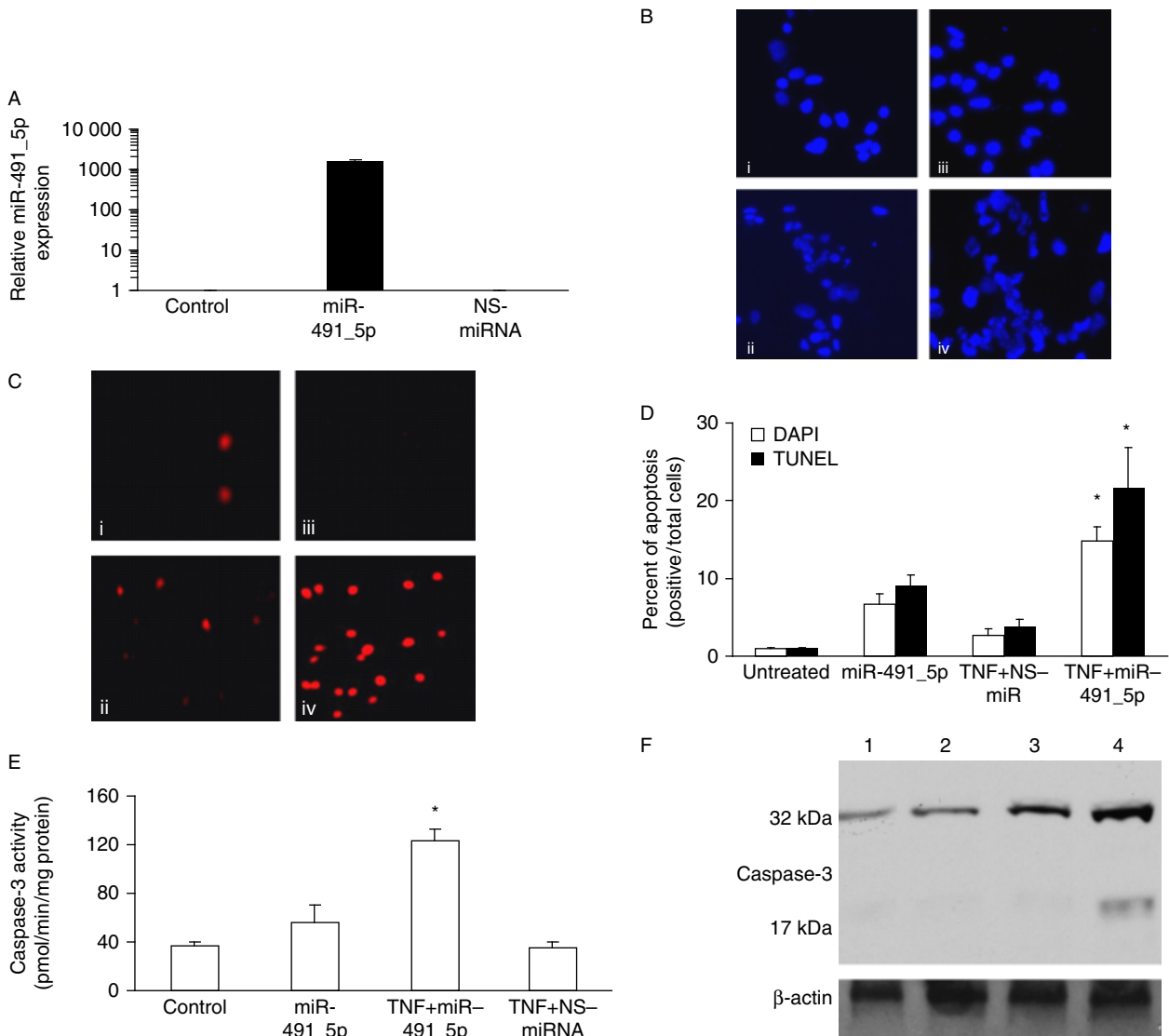
Fig. 4. Overexpression of miR-491_5p induces apoptosis in Hep G2 cells. (A) Hep G2 cells overexpressing miR-491_5p were collected, miRNA was isolated, and the presence of miR-491_5p was analysed using real-time RT-PCR. (B) The transfected cells were treated with TNF-α for 4 h, followed by fixation and permeabilization. The cells were stained using a mounting medium containing DAPI or (C) the terminal deoxynucleotidyl transferase dUTP nick end-labelling (TUNEL) assay was performed according to the manufacturer's instructions. (i) Untreated cells, (ii) miR-491_5p alone, (iii) tumour necrosis factor-α (TNF-α)+NS-miRNA, and (iv) TNF-α+miR-491_5p. (D) The results from 4′,6-diamidino-2-phenylindole (DAPI) and TUNEL experiments were quantified and the data are presented. *$P < 0.05$ compared with control. (E) The transduced cells were sensitized with TNF-α and the protein was analysed for caspase-3 activity. *$P < 0.05$ compared with control. (F) Hep G2 cells transfected with miR-491_5p alone or with TNF-α. Cleaved caspase-3 was detected using Western blots. β-actin was used as an internal control. Lane 1, untreated cells; lane 2, siPORT+TNF-α; lane 3, miR-491_5p alone; lane 4, TNF-α+miR-491_5p. The photograph is representative of three experiments.

miR-491_5p-transfected cells treated with TNF-α (Fig. 4E). The caspase-3 activation was confirmed by performing Western blots for caspase-3 cleavage and similar results were found (Fig. 4F).

Each miRNA may have hundreds of targets; hence, a bioinformatic approach using miRBase, Targetscan 4.1 and miRanda was used to find the predicted protein targets. Among the potential targets, α-fetoprotein (AFP), hsp-90 and the rel B subunit of NF-κB were considered as key targets that could affect Hep G2 cell survival. We therefore analysed these proteins in cells overexpressing miR-491_5p and treated with TNF-α. The Western blot results indicated that TNF-α induced the expression of both AFP and hsp-90 in Hep G2 cells, compared with the control cells; however, this increase was significantly decreased in miR-491_5p
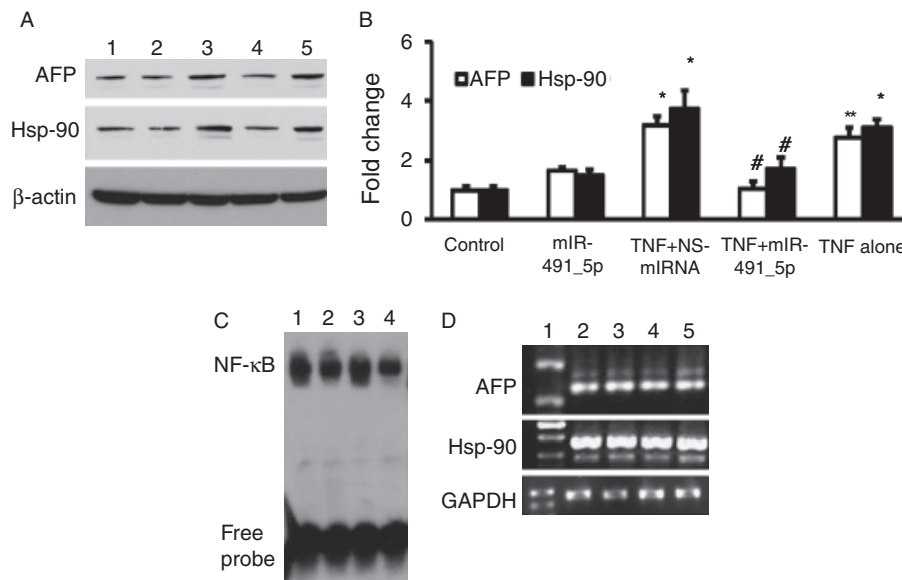
Fig. 5. miR-491_5p degrades its predicted key target proteins in Hep G2 cells. (A) The miR-491_5p overexpressing cells were sensitized with TNF-α and the total cellular protein was isolated. Western blots for AFP, Hsp-90 and β-actin were performed. This is a representative photograph from three experiments. Lane 1, untreated cells; lane 2, miR-491_5p alone; lane 3, TNF-α+NS-miRNA; lane 4, TNF-α+miR-491_5p; and lane 5, TNF-α alone. (B) The results from the above 3 experiments were quantified and presented as fold change over control. $*P < 0.05$ compared with control; $^{\#}P < 0.05$, compared with TNF-α+NS-miRNA. (C) The cells were collected and the nuclear protein fraction was isolated and EMSA for NF-κB was performed. The picture shows a representative experiment. Lane 1, untreated cells; lane 2, miR-491_5p alone; lane 3, TNF-α+NS miRNA and lane 4, TNF-α+miR-491_5p. (D) The total RNA was isolated from miR-491_overexpressing cells and RT-PCR was performed for AFP, hsp-90 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and a representative experiment is shown. Lane 1, molecular weight markers; lane 2, untreated cells; lane 3, miR-491_5p alone; lane 4, TNF-α+NS miRNA; lane 5, TNF-α+miR-491_5p.

overexpressing cells (Fig. 5A and B). The nuclear translocation of NF-κB was also significantly inhibited in miR-491_5p-transfected cells treated with TNF-α (Fig. 5C). Furthermore, we analysed whether the decrease in the levels of these proteins was because of mRNA degradation or translational repression. Therefore, we analysed AFP and hsp-90 gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR) and confirmed that there was no change in mRNA levels (Fig. 5D).

Involvement of both AFP and hsp-90 in Hep G2 cell survival was analysed using siRNA-induced gene silencing for AFP and hsp-90. First, intracellular gene expression of AFP and hsp-90 was estimated in siRNA-transfected cells. More than 80% of the intracellular levels of AFP and hsp-90 were inhibited in siRNAs to AFP or hsp-90 respectively (Fig. 6A). The cells were then analysed for the induction of apoptosis by TUNEL assay and a significant increase was found in apoptosis of TNF-α-treated Hep G2 cells transfected with either AFP siRNA or hsp-90 siRNA compared with the control cells (Fig. 6B). Next, overexpression of miR-491_5p, or inhibition of AFP and hsp-90 using siRNA oligos on TNF-α-induced Hep G2 cell survival was analysed using the WST-1 reagent. The results showed that TNF-α enhanced the survival, and this increase was significantly inhibited by overexpressing miR-491_5p or by inhibiting AFP or hsp-90 with siRNA oligos (Fig. 6C).

To study the involvement of miR-491_5p, AFP and hsp-90 in modulating apoptosis-related genes, a real-time RT-PCR array was performed using the Superarray RT-PCR array system (Superarray Biosciences, Frederik, MD, USA). Hep G2 cells were transfected with miR-491_5p, AFP siRNA or hsp-90 siRNA and sensitized with TNF-α. The total RNA was isolated, first-strand cDNA was synthesized and real-time RT-PCR array was performed. We found that there was a significant decrease in bcl-2 and mcl-1, whereas bax, bad and caspase-3 genes were upregulated in all the transfected cells compared with TNF-α treatment (Fig. 7A). We then analysed the protein levels of bcl-2, bax and mcl-1 by Western blots. The results confirmed the RT-PCR results (Fig. 7B). β-actin was used as a loading control for all the experiments.

Discussion

In this study, we have utilized the anti-fas receptor antibody-induced liver injury mouse model system to elucidate the differential expression of miRNAs that are modulated between normal, post-injury regeneration and acute liver injury causing cell death. It has been shown that the injection of anti-fas receptor antibodies at a low dose in mice caused minor amounts of hepatocyte apoptosis, which was followed by liver regeneration as evidenced by increased BrdU incorporation (2). Higher
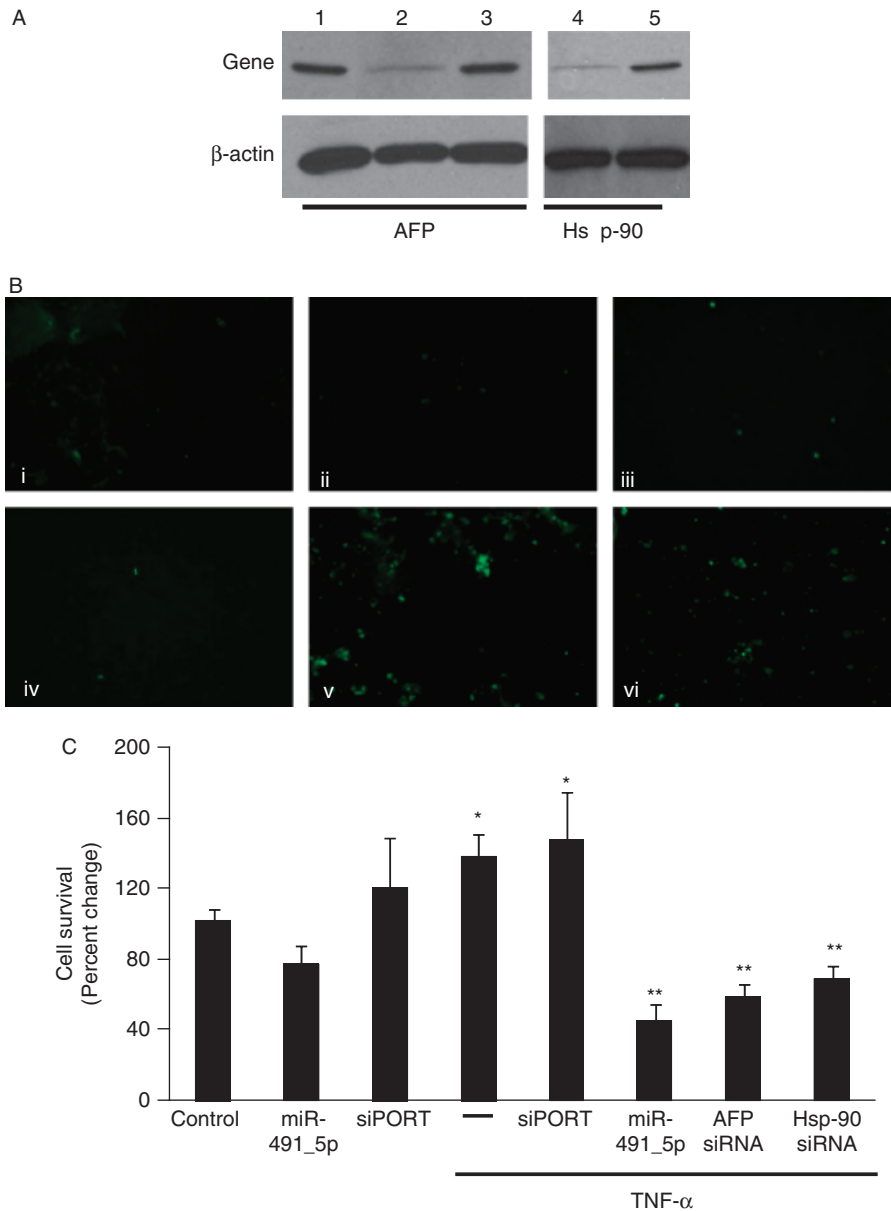
Fig. 6. TNF-α induces Hep G2 cell survival and inhibition of AFP or hsp-90 causes apoptosis. (A) Hep G2 cells were transfected with either siRNAs to AFP, hsp-90 or scrambled siRNA. The intracellular expression of AFP (upper panel, lanes 1–3), hsp-90 (upper panel, lanes 4–5) or β-actin (lower panel) was measured by Western blots. Lane 1, untreated cells; lane 2, siRNA to AFP; lane 3, scrambled siRNA; lane 4, siRNA to hsp-90 and lane 5, scrambled siRNA. (B) TUNEL assay was performed in Hep G2 cells transfected with siRNAs and treated with TNF-α. (i) untreated cells, (ii) siPORT+TNF-α, (iii) miR-491_5p alone, (iv) TNF-α alone, (v) TNF-α+AFP siRNA and (vi) TNF-α+hsp-90 siRNA. This is a representative photograph from three experiments. (C) Hep G2 cells were transfected with miR-491_5p, siRNA oligos to AFP or hsp-90. As controls, TNF-α alone, siPORT alone or siPORT transfection agent was added with TNF-α-treated cells. The cells were treated with TNF-α for 4 h and the cell survival assay was performed using the WST-1 reagent. TNF-α-induced cell survival was inhibited by either overexpressing miR-491_5p or inhibiting AFP or hsp-90 using siRNA-mediated gene silencing ($n = 5$; *$P < 0.05$ compared with control, **$P < 0.01$ compared with TNF-α treated cells).

concentrations of anti-fas receptor antibody caused severe apoptosis and liver failure when liver regeneration could not keep up with the pace of hepatocyte cell death (3, 4). Our results also showed that at low-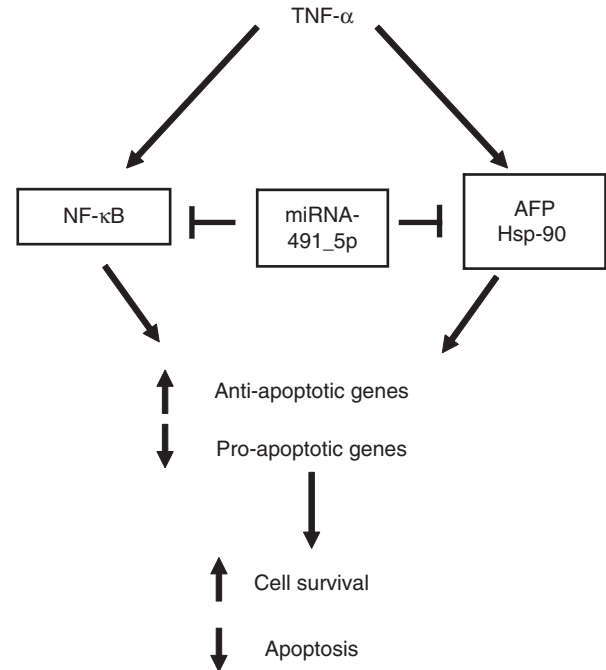dose injury (0.125 μg/g mouse), there was no increase in ALT and caspase-3 activity. However, at a high dose (0.5 μg/g mouse) the liver injury was severe as determined by serum ALT levels and increased caspase-3 activity. Thus, it appears that depending on the degree of injury, liver cells undergo either predominantly proliferation or
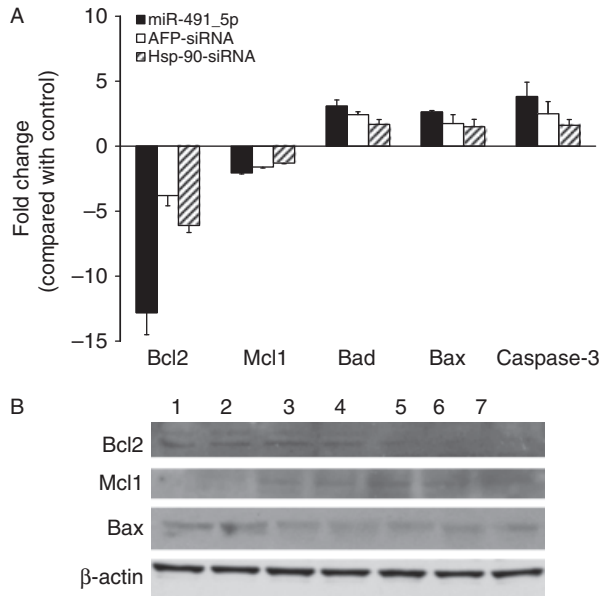
Fig. 7. Overexpression of miR-491_5p or inhibition of AFP or hsp-90 using siRNA oligos yielded increased pro-apoptotic and decreased anti-apoptotic genes. (A) Expression of apoptosis-related genes was estimated using a real-time RT-PCR array in untreated cells, cells overexpressing miR-491_5p, or inhibiting α-fetoprotein (AFP) or heat shock protein (hsp)-90 with siRNAs. The fold change was calculated compared with the tumour necrosis factor (TNF)-α-treated cells. (B) The protein was isolated from the miRNA or siRNA transfected cells and the Western blots were performed for bcl-2, mcl-1, bax and β-actin. Lane 1, untreated cells; lane 2, TNF-α+scrambled siRNA; lane 3, miR-491_5p alone; lane 4, TNF-α alone; lane 5, TNF-α+miR-491; lane 6, TNF-α+AFP siRNA; lane 7, TNF-α+hsp-90 siRNA. The photograph is representative of three experiments.

Fig. 8. Proposed pathway by which miR-491_5p sensitizes Hep G2 cells for tumour necrosis factor (TNF)-α induced apoptotis miR-491_5p inhibits TNF-α-induced cell survival and sensitizes TNF-α-induced apoptosis in Hep G2 cells, at least in part, by inhibiting the protein levels of α-fetoprotein (AFP) and hsp-90, and nuclear factor-κB (NF-κB) nuclear translocation.

apoptosis. Both regeneration and apoptosis are controlled by the differential expression of various genes. The gene expression is controlled in a variety of different ways, and we hypothesized that one such mechanism may be through miRNA expression. We purified the miRNA from the vehicle control mice, a low-dose group and a high-dose group. The miRNA microarray results indicated that there was a differential pattern of miRNA expression among groups. Several miRNAs that were upregulated in the low-dose group were inhibited in the high-dose group, and a number of these miRNAs have been shown to induce proliferation and differentiation in a variety of non-hepatocyte cell types. For example, miR-21 has been shown to promote hepatocellular growth (28), or anti-apoptotic in megakaryocytes (20). miRNA 126 has been shown to inhibit the invasion of lung cancer cell lines (29) and miR-30c has been shown to enhance the proliferation of megakaryocytes (22). The function of the other miRNAs that are upregulated in the low-dose group remains unknown.

Among the differentially expressed miRNAs, two of them (miR-451 and miR-491_5p) were upregulated in the high-dose antibody group. Recently, it has been shown

that miR-451 causes chemotherapeutic drug resistance in the breast cancer cell line (30). We decided to focus our attention on miR-491_5p which was not expressed either in the vehicle control group or in the low-dose antibody group, but was strongly expressed in the high-dose group, and its function is unknown. Hence, we speculated that miR-491_5p could be involved in modulating hepatic cell functions when these cells undergo apoptosis. To investigate this hypothesis, we overexpressed miR-491_5p in Hep G2, cells which are miR-491_5p negative without transfection. Although miR-491_5p did not induce apoptosis, the transfection sensitized these cells for TNF-α-induced apoptosis; otherwise, TNF-α caused increased cell survival. As a control, a non-specific negative control miRNA, an miRNA found not to inhibit any known mRNA, was used in all the transfection experiments. Firstly we studied whether miRNA is transfected into the cells and the time it takes for the transfection. The results suggested that the 100% transfection efficiency was achieved after 12 h of transfection using siPORT NeoFX transfection reagent.

We selected TNF-α for treating the cells for the following reasons: (a) TNF-α does not induce apoptosis in hepatocellular carcinoma cells (31, 32), (b) AFP, one of the predicted target proteins for miR-491_5p, had been shown to protect Hep G2 cells from TNF-α-induced apoptosis (32, 33), (c) elevated levels of TNF-α are

present in both hepatocellular carcinoma cells (34) and inflammation (35) and (d) TNF-α enhances the activation of NF-κB leading to increased cell survival in cancer cells (35). The TNF receptor family has dual signalling pathways, including apoptosis and NF-κB activation leading to cell survival (36), and TNF-α is one of the important cytokines that plays a significant role in inducing inflammation in several cells (37, 38). TNF-α can act either as an apoptosis-inducing agent (39) or an inflammatory agent (37, 38) depending on the cell type and pathological conditions. In hepatocellular carcinoma cells, TNF-α induces NF-κB and subsequently cell survival (40), whereas in hepatocytes TNF-α can induce apoptosis (31, 41). Although high non-physiological concentrations of AFP or AFP-derived peptides have displayed some growth inhibiting properties (42), at physiologically relevant concentrations (10–100 μg/ml), they showed a protective role against TNF-α-induced apoptosis (32, 33).

Using known bioinformatics approaches such as miR-Base, targetscan 4.1 and miRANDA, the key targets of miR-491_5p were identified such as hsp-90, AFP and the rel B subunit of NF-κB. Hsp-90 is highly expressed in cancer cells, and one treatment for cancer is to inhibit hsp-90 expression (43). *In vitro* studies have shown AFP to be involved in pleiotropic activities affecting cell growth and apoptosis (42). These results strongly suggest that AFP stimulates HCC growth in humans (44). NF-κB has been well established as a survival protein in cancer cells by several investigators (45).

Western blot experiments were conducted to determine the modulation of hsp-90 and AFP expression in Hep G2 cells. It was found that TNF-α enhanced the expression of these two proteins in Hep G2 cells compared with the control cells and increased the cell survival. In miR-491_5p overexpressing cells, TNF-α-induced AFP and hsp-90 expression was significantly decreased and apoptosis was increased. EMSA results also suggested that miR-491_5p expression caused a decrease in the nuclear translocation of NF-κB, suggesting that miR-491_5p decreases another important survival protein in Hep G2 cells. Next, to study whether miR-491_5p inhibits the translation or degrades the mRNA of the target genes in Hep G2 cells, RT-PCR experiments were performed and it was found that the mRNA levels were not changed over time. Thus, it appears that miR-491_5p may act by suppressing the translation of these genes.

Various genes involved in the apoptosis pathway were evaluated by overexpressing miR-491_5p in these cells. Real-time RT-PCR results showed that pro-apoptotic genes were upregulated in miR-491_5p-expressing cells and that survival genes were inhibited. It appears that miR-491_5p overexpression enhances apoptosis via inhibiting anti-apoptotic genes such as bcl-2 and mcl-1, while increasing the pro-apoptotic genes such as bax and caspase-3. It was determined whether the inhibition of the miR-491_5p target genes induced apoptosis through

an effect on bcl-2, mcl-1, bax and caspase-3. When AFP or hsp-90 protein expression was neutralized using siRNA-induced gene silencing in TNF-α-sensitized Hep G2 cells, cell survival was decreased and apoptosis was increased in both the siRNA-transfected cells, with a more pronounced effect in the AFP siRNA-transfected cells. Previous data have shown that inhibition of hsp-90 causes the activation of the intrinsic pathway of apoptosis (46). In our experiments, we confirmed the previous findings that inhibition of hsp-90 resulted in an increase in the bax protein. In addition, our data indicate that inhibition of hsp-90 resulted in decreased bcl-2 levels. This could be because of the decreased NF-kB activation, needed for the expression of bcl-2 family members (47). Our data in Hep G2 cells confirm that inhibition of AFP resulted in decreased bcl-2, and increased bax and caspase-3 levels, suggesting that AFP might directly be involved in the growth and survival of hepatocellular carcinoma cells (48). Although inhibition of AFP or hsp-90 and transfection of miR-491_5p in TNF-α-treated cells resulted in an upregulation of pro-apoptotic proteins and inhibition of anti-apoptotic proteins, the exact mechanism is not known. Figure 8 shows a characterization of how miR-491_5p may induce apoptosis in Hep G2 cells. Our data show that miR-491_5p sensitizes Hep G2 cells for TNF-α-induced apoptosis, at least in part, by inhibiting the expression of AFP, hsp-90 and NF-κB.

Acknowledgements

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