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Knockdown of TIGAR by RNA interference induces apoptosis and autophagy in HepG2 hepatocellular carcinoma cells

Ling Ye [a,b,c], Xiaoping Zhao [b,d], Jian Lu [b], Guanxiang Qian [b], Jialin C. Zheng [a,c,*], Shengfang Ge [b,e,*]

[a] Laboratory of Neuroimmunology and Regenerative Therapy, Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198-5930, USA
[b] Department of Biochemistry and Molecular Biology, Shanghai Jiaotong University School of Medicine, Shanghai 200025, People's Republic of China
[c] Center for Translational Neurodegeneration and Regenerative Therapy, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200092, People's Republic of China
[d] Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200127, People's Republic of China
[e] Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200011, People's Republic of China

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ABSTRACT

Apoptosis and autophagy are crucial mechanisms regulating cell death, and the relationship between apoptosis and autophagy in the liver has yet to be thoroughly explored. TIGAR (TP53-induced glycolysis and apoptosis regulator), which is a p53-inducible gene, functions in the suppression of ROS (reactive oxygen species) and protects U2OS cells from undergoing cell death. In this study, silencing TIGAR by RNAi (RNA interference) in HepG2 cells down-regulated both TIGAR mRNA (~75%) and protein levels (~80%) and led to the inhibition of cell growth ($P < 0.01$) by apoptosis ($P < 0.001$) and autophagy. We demonstrated that TIGAR can increase ROS levels in HepG2 cells. The down-regulation of TIGAR led to the induction of LC-3 II (specific autophagic marker), the formation of the autophagosome, and increased Beclin-1 expression. 3-MA (3-Methyladenine), an inhibitor of autophagic sequestration blocker, inhibited TIGAR siRNA-enhanced autophagy, as indicated by the decrease in LC-3 II levels. Consequently, these data provide the first evidence that targeted silencing of TIGAR induces apoptotic and autophagic cell death in HepG2 cells, and our data raise hope for the future successful application of TIGAR siRNA in patients with hepatocellular carcinoma (HCC).

1. Introduction

Currently, HCC ranks among the five most important causes of cancer-related mortality worldwide [1]. The dysregulation of the balance between proliferation and cell death represents a protumorigenic principle in human hepatocarcinogenesis and is mainly due to the overactivation of antiapoptotic and antiautophagic signals. Apoptosis (programmed cell death type I) and autophagic cell death (programmed cell death type II) are crucial physiological mechanisms that control the development, homeostasis, and elimination of unwanted and malignant cells [2,3]. Overactivation of antiapoptotic signals contributes to tumorigenesis and chemotherapy resistance in HCC cells [4]. Recent data demonstrated that autophagy is involved in the major fields of hepatology. In HCC, the autophagy levels are reduced, and autophagy plays an antitumor role and has a beneficial effect in patients with HCC [5,6]. Collectively, these data indicate that the status of apoptosis and autophagy is a key factor that determines therapeutic response to HCC therapies. Therefore, therapeutic strategies to inhibit selectively antiapoptotic or antiautophagic signals in HCC cells have the potential to provide powerful tools to treat HCC. If siRNA becomes therapeutically practical, antiapoptotic and antiautophagic proteins would be potential therapeutic targets [7].

It has been determined that TIGAR is a p53-induced apoptosis and autophagy regulator, and it plays an essential role in apoptosis induced by both nuclear and cytoplasmic p53 [8,9]. Previous studies have demonstrated that TIGAR expression correlates with the ability to protect cells from ROS-associated apoptosis; consequently, knockdown of endogenous TIGAR expression sensitizes

Abbreviations: siRNA, small interference RNA; RNAi, RNA interference; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; TIGAR, TP53-induced glycolysis and apoptosis regulator; siTIGAR, siRNA for TIGAR.

* Corresponding authors. Addresses: No. 227, South Chongqing Road, Shanghai 200025, People's Republic of China. Fax: +86 21 63852916 (S. Ge), Laboratory of Neuroimmunology and Regenerative Therapy, Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198-5930, USA. Fax: +1 402 5593744 (J.C. Zheng).

E-mail addresses: jzheng@unmc.edu (J.C. Zheng), geshengfang@sjtu.edu.cn (S. Ge).

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U2OS cells to ROS-intensive death [9]. Expression of TIGAR may therefore modulate the apoptotic response to p53, allowing cellular survival in the face of mild or transient stress signals that may be reversed or repaired. Previous studies have also demonstrated that the modulation of intracellular ROS levels by TIGAR can function in limiting autophagy in U2OS cells [8].

We hypothesized that the expression of TIGAR can affect the balance between cell growth and cell death and that TIGAR modulation influences apoptosis and autophagy in HepG2 cells. To investigate the role of TIGAR in apoptosis and autophagy in HepG2 cells, we transfected cells with siTIGAR (siRNA targeting TIGAR) to regulate specifically the expression of TIGAR. We determined that the silencing of TIGAR regulated ROS levels and increased specific autophagic protein expression that resulted in the induction of apoptosis and autophagy in HepG2 cells. These data demonstrate a novel mechanism for growth inhibition induced by TIGAR knockdown in HepG2 cells. Better understanding of the mechanisms involved in siTIGAR-induced cell death may hold the promise of better management of HCC in patients.

2. Materials and methods

2.1. Cell lines and reagents

The HepG2 cell line was grown in Dulbeco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum at 37 °C in a 5% CO_2. The cells were treated with 3-MA (Sigma–Aldrich, St. Louis, MO, USA) at a final concentration of 10 mM for 24 h before transfection. For the HBSS treatment, the cells were nutrient starved for 2 h, and these cells were used as a positive control.

2.2. Transfections with siRNAs and RT-PCR analysis

Exponentially growing, untreated HepG2 cells were plated at 2×10^5/well in 2 ml of medium for 24 h before transfection. The plated cells were transfected with either the control siRNA, the double-stranded siRNA targeting the TIGAR mRNA, or the transfection reagent alone. For the siRNA-mediated down-regulation of TIGAR, the following 4 siRNAs oligonucleotides were designed and employed (Genepharm Co., Shanghai, China): siRNA-1 (siTIGAR-80), 5′-GAU GAA CCU CUU UCA GAA AdTdT-3′; siRNA-2 (siTIGAR-225), 5′-GCA AAG AUA UGA CGG UAA AdTdT-3′; siRNA-3 (siTIGAR-496), 5′-GGC AGA GAU AUU UCC UUU AdTdT-3′; siRNA-4 (siTIGAR-772), 5′-CCU ACA GGA UCA UCU AAA UdTdT-3′. The non-silencing control siRNA (siCON) duplexes were synthesized using scrambled sequences. siRNA transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer′s instructions. Briefly, on the day the cells were transfected, the medium was removed and replaced with 1 ml of fresh medium. One microgram of siRNA was mixed with the transfection reagent and then added to each well. For the RT-PCR analysis, cells were collected and total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and then cDNA was obtained using PCR. The real-time PCR amplification was performed using QuantiTect SYBR green (Qiagen, CA, USA) following the manufacturer's instructions. The TIGAR (5′-AGG GAA GAG TGC CCT GTG TT-3′) and GAPDH-specific primers were used in the real-time PCR reactions. The C_T (threshold cycle) value of TIGAR amplification was normalized to that of the GAPDH control.

2.3. Western blot analysis

After treatment with siCON, siTIGAR or HBSS, the cells were harvested. Proteins were separated using 12% (w/v) SDS–polyacrylamide gel electrophoresis (PAGE) gels and transferred to PVDF membranes. After blocking in PBS/Tween (0.01%) with 5% nonfat milk, the membrane was incubated with the primary antibodies at 4 °C overnight. The following antibodies were used: TIGAR (1:1,250; Abcam Inc., Cambridge, UK); β-actin (1:5000; Sigma–Aldrich, St. Louis, MO, USA); LC3 (1:500; Abcam Inc., Cambridge, UK); Beclin-1(1:500; Abcam Inc., Cambridge, UK). The membranes were then incubated with secondary antibody conjugated to horseradish peroxidase, the bands were visualized using enhanced chemiluminescence (Pierce, IL, USA), and the blots were exposed to film (Kodak, USA). The films were scanned using a CanonScan scanner for data quantification, and the acquired images were then analyzed on a computer using the public domain NIH image program (http://rsb.info.nih.gov/nih-image/).

2.4. MTT assay

Cell viability was assessed using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) reduction assay as described previously [10]. Briefly, HepG2 cells were incubated with 10% MTT (Sigma–Aldrich) solution in DMEM medium for 30 min at 37 °C. The extent of MTT conversion to formazan by mitochondrial dehydrogenase, which is indicative of cell viability, was determined by measuring the absorbance at a wavelength of 490 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. In situ TUNEL assay

After siCON or siTIGAR treatment in HepG2 cells, apoptosis was assayed using a commercially available terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit (Roche, Manheim, Germany). The total number of TUNEL positive cells divided by the total number of cells (DAPI staining of nuclei) was used to indicate the percentage of apoptosis. At least ten images were acquired from each immunostained treatment group using an Olympus microscope.

2.6. MDC staining

The autofluorescent agent MDC (monodansyl cadaverine) (Sigma, St. Louis, MO, USA) was recently introduced as a specific autophagolysosome marker to analyze autophagic process [11]. HepG2 cells were plated into 24-well plates at 0.2×10^6 cells/well 24 h before siRNA treatment. The cells were transfected with siTIGAR, control siRNA, or left untreated for 72 h. The control and treated cells were harvested using trypsin–EDTA and washed in 1 ml of phosphate-buffered saline (PBS). Autophagic vacuoles were then labeled with MDC by incubating the cells with 50 μM MDC in DMEM at 37 °C for 10 min. After incubation, the cells were washed twice with PBS and immediately analyzed by FACS analysis.

2.7. Transmission electron microscopy

HepG2 cells were grown on six-well plates and transfected with siTIGAR or control siRNA, fixed for 2 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), post-fixed in 1% OsO_4 in the same buffer, and then subjected to electron microscope analysis as described previously. Representative areas were chosen for ultrathin sectioning and viewed using a Philip electron microscope (Eindhoven, Netherlands).

2.8. Statistical analyses

All experiments were performed in triplicate, and the data were analyzed using one-way analysis of variance (ANOVA) followed by

a Tukey's test for multiple comparisons. The results are expressed as the mean ± S.D., and the criterion for statistical significance was $P < 0.05$.

3. Results

3.1. TIGAR-specific siRNA down-regulates TIGAR expression in HepG2 cells

RNAi was performed by transfecting siRNA to specifically knockdown TIGAR expression in HepG2 cells. We first investigated the transfection efficiency of siRNA by using FAM-labeled negative control siRNA and found that the efficiency was more than 85% (12 h transfection) (data not shown). Next, we evaluated a time-course of GAPDH siRNA (siGAPDH)-induced changes in GAPDH mRNA and protein level in HepG2 cells at 12, 24, 48, 72 h post-transfection (Fig. 1A). We observed that siGAPDH-induced the down-regulation of GAPDH RNA and protein starting at 24 h (RNA) or 48 h (protein) and reaching maximum down-regulation at 48 h (RNA) (Fig. 1A) or 72 h (protein) (data not shown) post-transfection. Therefore, for siTIGAR study we used the 48 h post-transfection time point for RNA studies and 72 h post-transfection for protein studies.

TIGAR expression was then measured using quantitative real-time PCR. We treated HepG2 cells with four different siRNAs targeting TIGAR (siRNA 1, 2, 3 and 4). Based on the real-time PCR data from the 48 h treatment samples, these siRNAs decreased TIGAR RNA by approximately 50%, 30%, 20%, and 75%, respectively, relative to the control cells (Fig. 1B). We next investigated the inhibition of TIGAR protein by performing a western blot on samples from 72 h post-transfection. As shown in Fig. 1C, TIGAR and β-actin expression levels were determined and, siTIGAR down-regulated the TIGAR protein. Similar to the real-time PCR result (Fig. 1B), the western blot (Fig. 1D) also demonstrated that siRNA 4 (siTIGAR 772) had the greatest interference efficiency (nearly 80%) out of the four siRNAs. Therefore, from this point we only used siTIGAR4 for the remaining experiments, and henceforth dubbed "siTIGAR".

3.2. HepG2 cells undergo cell death and apoptosis upon TIGAR knock down

We first investigated whether the inhibition of TIGAR expression by siRNA alone results in enhanced growth inhibition and cell death in HepG2 cells. Treating HepG2 cells using siTIGAR caused cell death, as detected using an MTT assay (Fig. 2A). A time-dependent decrease in HepG2 cell viability was tightly associated with the down-regulation of TIGAR protein following siTIGAR treatment, but this was not observed using the same dose of the negative control (data not shown). After TIGAR was silenced by RNAi for 72 h, cell viability decreased dramatically relative to the untreated control and the non-silencing siRNA control cells (Fig. 2A).

Knockdown of endogenous TIGAR protein has been shown to induce apoptosis in U2OS cells [9], and the expression of TIGAR may modulate the apoptotic response to p53. To understand the cell death mechanism in HepG2 cells, which occurred in TIGAR-down-regulated cells, we next investigated whether knockdown of TIGAR expression by siRNA alone can activate apoptotic signaling in HepG2 cells. Therefore, HepG2 cells were transfected with siRNA targeting TIGAR or non-silencing control siRNA. Twenty-four hours after transfection, the cells were using a TUNEL apoptosis assay. TUNEL staining indicated that the apoptosis rate of the transfected HepG2 cells was approximately 35%, which was triggered by the down-regulation of TIGAR expression by RNAi. Previous studies have shown that TIGAR can inhibit apoptosis by limiting ROS levels and was not effective at regulating non-ROS-dependent apoptosis [9]. Therefore, we detected the intracellular ROS levels in HepG2 cells after TIGAR was silenced and found that TIGAR had the ability to decrease ROS levels (Fig. 2C). Similar to the observations in the previous study, our data indicated that TIGAR can control ROS accumulation and inhibit apoptosis, which is related to ROS level.

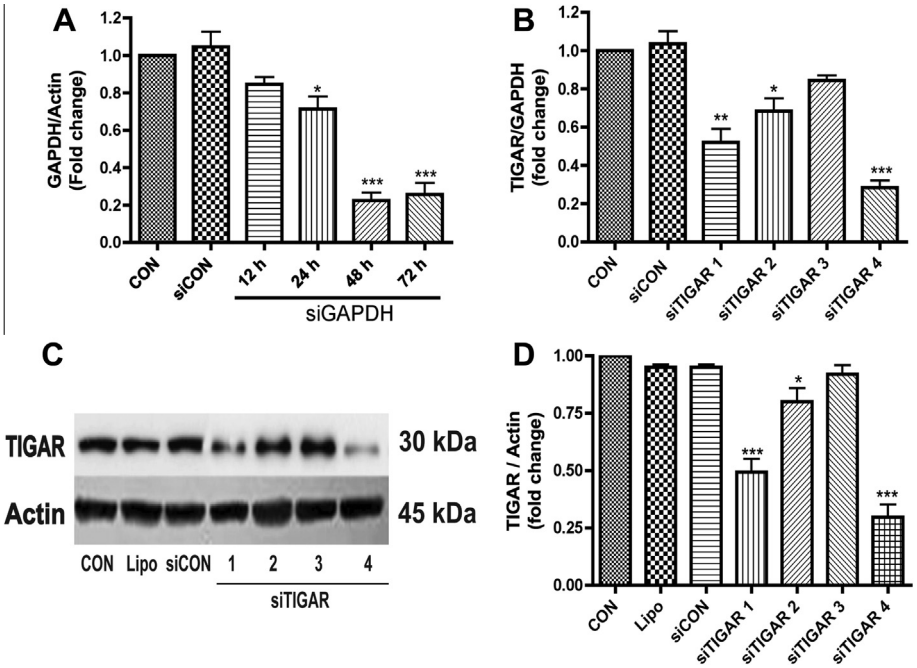

Fig. 1. RNAi of GAPDH and TIGAR. (A) HepG2 cells were transfected with siGAPDH for 12, 24, 48 and 72 h. GAPDH and β-actin mRNA levels were detected by real-time PCR. (B) HepG2 cells were treated with either siCON or siTIGAR (1, 2, 3, and 4) for 48 h. The TIGAR mRNA level was quantified by real-time PCR and normalized to GAPDH mRNA level. (C) After 72 h of siCON and siTIGAR treatments, the protein extracts were subjected to western blot and β-actin was used as a loading control. (D) Levels of TIGAR (C) were normalized as a ratio to actin after densitometrical quantification and presented as fold change relative to control. Results are expressed as the mean ± SD of samples from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ compared with untreated group.

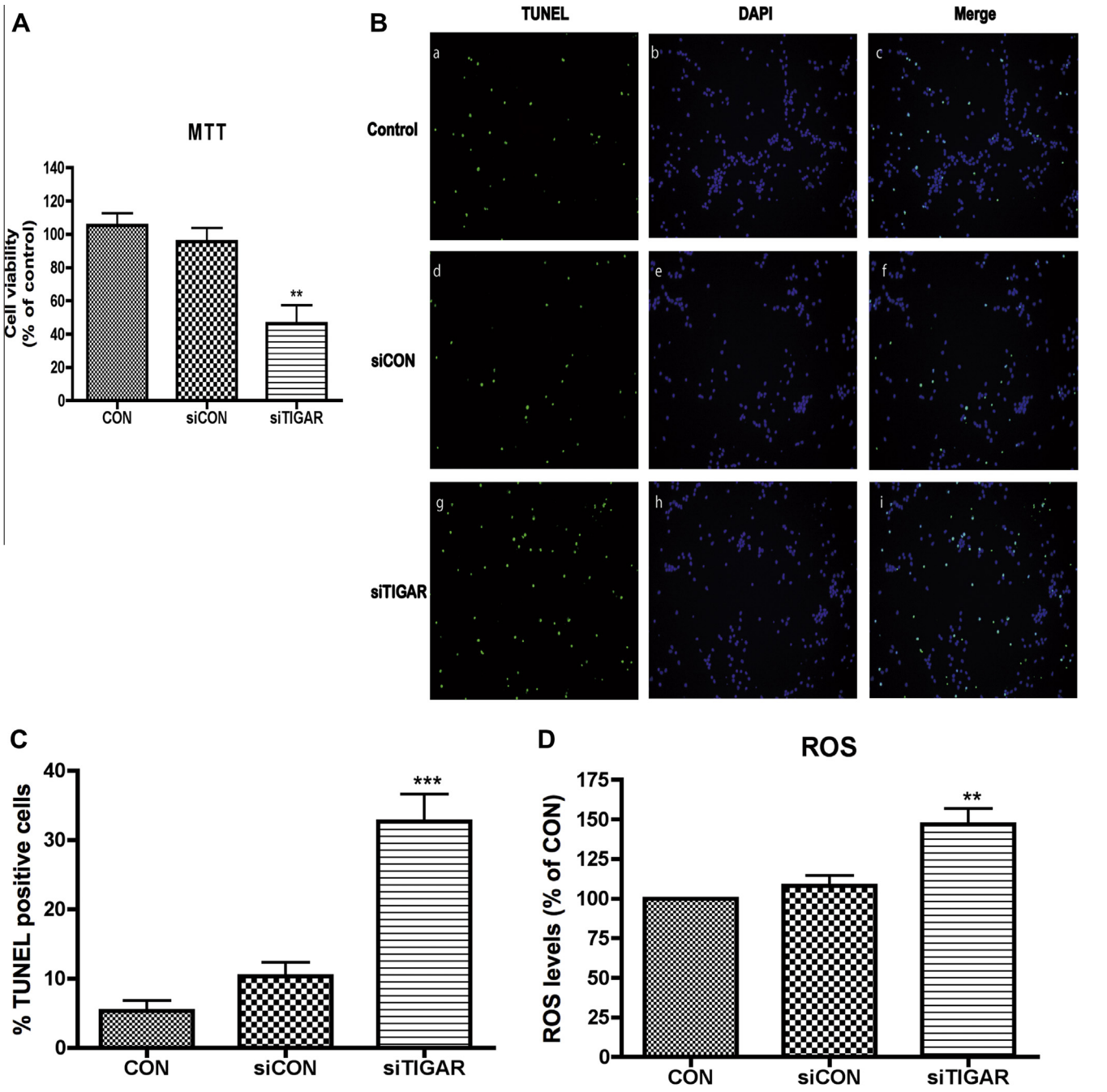

Fig. 2. siTIGAR induced cell death and apoptosis. (A) HepG2 cells were transfected with either siCON or siTIGAR for 72 h. Cell viability of HepG2 cells were assessed by MTT assay. Results are expressed as average ± SD of triplicate sample. (B) Using TUNEL assay to examine the siTIGAR-induced apoptosis at 24 h posttreatment. Photographs have a magnification 20×. (C) Apoptosis is expressed as percentage of TUNEL positive cells (green dots) over the total amount of cells (nuclei staining by DAPI, blue dots). For quantification, a minimum of 10 photos were analyzed. (D) ROS levels in HepG2 cells transfected with either siCON or siTIGAR for 24 h. ROS levels were measured by FACS after transfection. The results are expressed as the mean DCF fluorescence (±SD), from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.0001$ compared with untreated group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. siTIGAR induces autophagic cell death

In mammalian cells, some data have suggested that apoptosis and autophagy could participate in cell death, and the induction of autophagy has been verified to enhance the apoptotic response [12]. In fact, the connection between apoptosis and autophagy is a developing area of research. One previous study demonstrated that tetrandrine induced the production of ROS and autophgay, and the treatment with ROS scavengers significantly abrogated tetrandrine-induced autophagy [13]. Recently, TIGAR has been shown to indirectly modulate ROS and function to inhibit autophagy [9,14]. Because the TIGAR protein has been shown to protect cells from undergoing apoptosis (Fig. 2B and C) by modulating ROS levels (Fig. 2D), and ROS can regulate autophgay, we next investigated the effect of siTIGAR on the induction of autophagy by performing MDC staining. A previous study showed that MDC accumulates in mature AVs (autophagic vacuoles), such as autophagolysosomes, and MDC staining can be used to detect autophagic vacuoles. When cells were analyzed by FACS, the 72-h siTIGAR-treated HepG2 cells resulted in a significant increase in the number of MDC labeled

vesicles (Fig. 3A) relative to the untreated or siCON treated cells. This result indicates that there is an induction of AV formation by siTIGAR transfection.

Previous work showed that during the autophagy process, microtubule-associated protein 1 light chain 3 (LC3) is converted to LC3-I and conjugated to phosphatidylethanolamine to form LC3-II, which is a hallmark of autophagy and located on the autophagosomal membrane [15]. The expression of autophagy-specific LC3-II was detected by western blot analysis. Both siTIGAR transfection and HBSS treatment enhanced LC3-II levels in HepG2 cells (Fig. 3B). To test if increased LC3-II levels were a result of increased autophagosome formation, we further analyzed the induction of autophagy and the ultrastructure of HepG2 cells by transmission electron microscopy (Fig. 3C). As shown in Fig. 3C, siTIGAR cells contained multiple cytoplasmic autophagic vacuoles (arrows in Fig. 3d). We also observed abundant vacuolar elements after HBSS treatment, which were most likely to be of autophagic origin (arrows in Fig. 3f).

Collectively, our data clearly demonstrate that siTIGAR treatment, but not siCON treatment (Fig. 3a and b), induced the formation of double-membraned autophagic vesicles containing cellular organelles with merging autophagic vesicles with lysosomes and lysed cellular content in the autophagosomes, indicating activity of lysosomal function and degradation.

3.4. Inhibition of TIGAR induces expression of Beclin-1 autophagy promoting protein

To evaluate the contribution of an autophagic mechanism to siTIGAR-induced cell death, the effects of the autophagy inhibitor 3-MA on siTIGAR-induced alterations in LC3-II proteins was detected by western blot assay (Fig. 4A). Quantification data (Fig. 4B) indicated that pretreating with 3-MA significantly inhibited siTIGAR- and HBSS-induced autophagy. Recent developments indicate that Beclin-1 is one of the most important mediators in the formation of the autophagosome because it is involved in the initial step of autophagosome formation [16].

To address whether Beclin-1 is involved in siTIGAR-induced autophagy, the effect of siTIGAR on the expression of Beclin-1 was checked by western blot analysis. We determined that

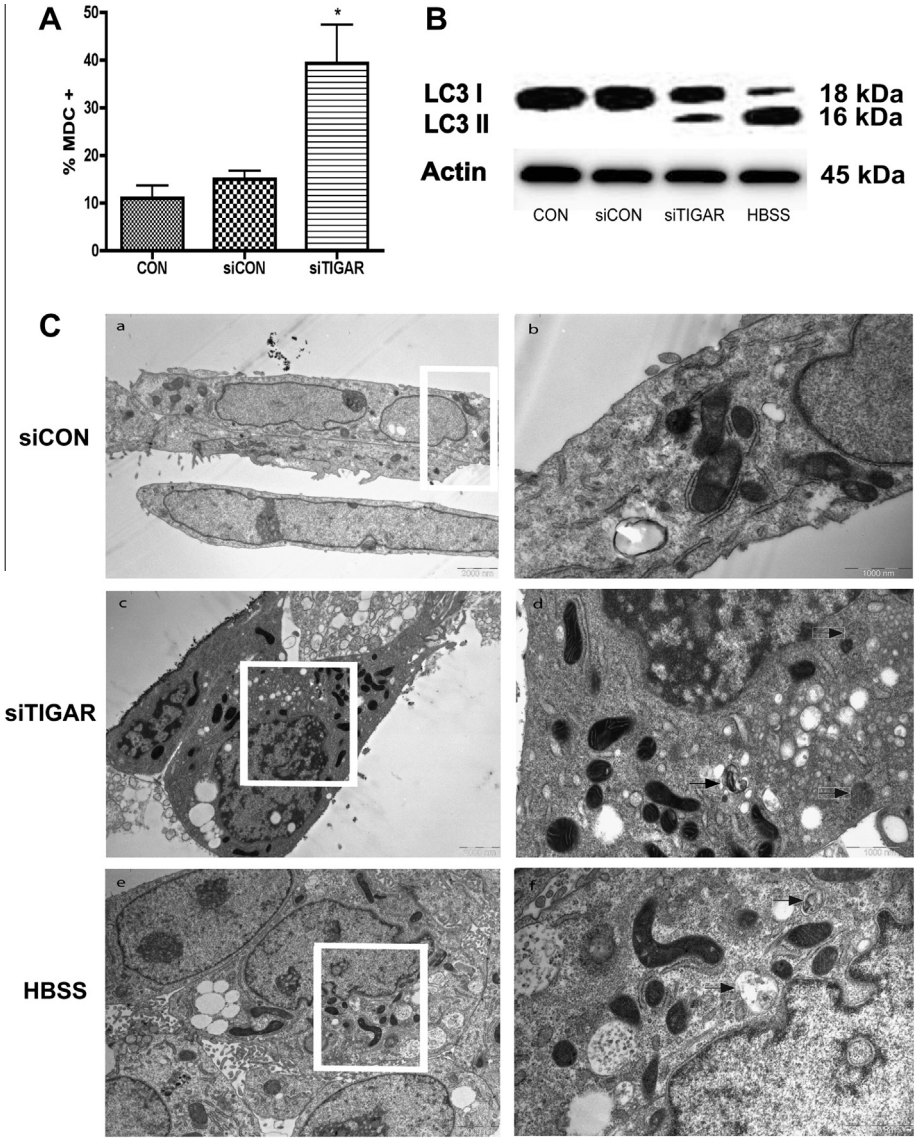

Fig. 3. siTIGAR induced autophagy in HepG2 cells. (A) HepG2 cells treated with siCON or siTIGAR for 72 h. The cells were then stained with MDC for detection by FACS. Bar graph represents the percentage of MDC-positive cells. (B) LC 3-II expression was detected after 72 h transfection or 2 h nutrient starvation by Western blot analysis. (C) Electron micrographs showed the ultrastructure of HepG2 cells treated with siCON (a and b) or siTIGAR (c and d) for 72 h or exposed to HBSS (e and f). Numerous autophagic vacuoles (indicated by arrows) were observed in the siTIGAR-treated and HBSS-treated cells. $^{*}P < 0.05$ compared with untreated group.

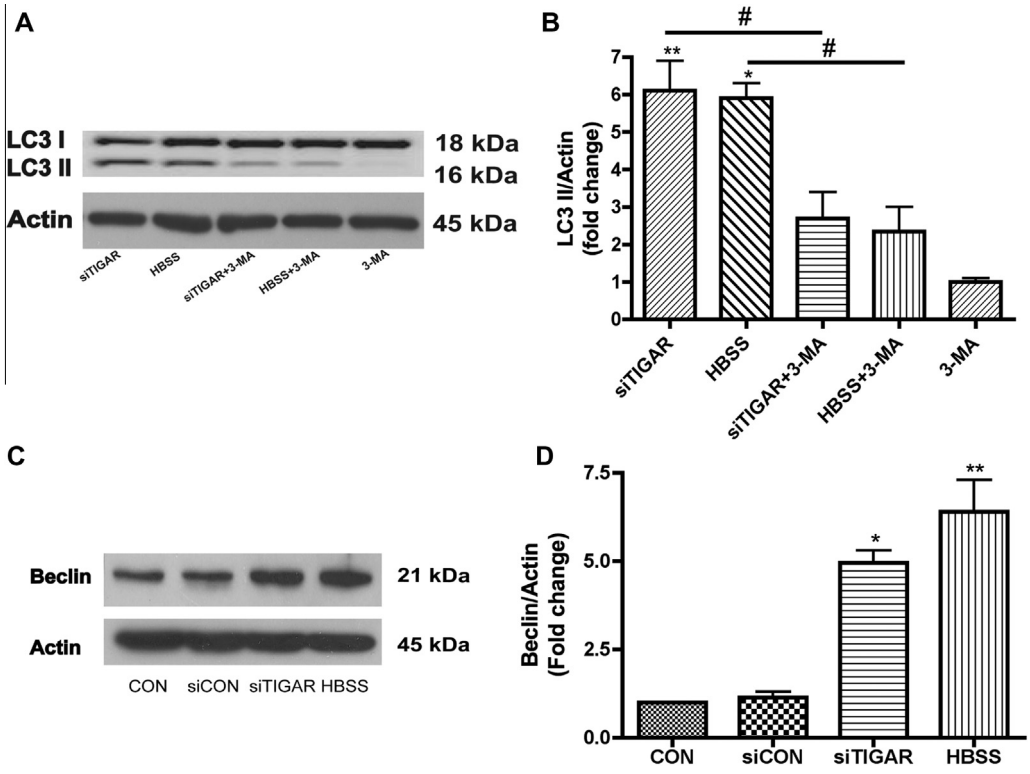

Fig. 4. TIGAR expression modulates autophagy. (A) HepG2 cells were pretreated with 3-MA before siRNA transfection or nutrient starvation and were collected after 72 h transfection. Protein extracts were subjected to western blot and β-actin was used as a loading control. Results are representative of three independent experiments. (B) The films were scanned and the acquired images were analyzed using the public domain NIH image program for data quantification. Levels of LC3-II were normalized as a ratio to β-actin after densimetrical quantification and shown as percentage of control group. (C) HepG2 cells were treated with siRNA for 72 h or nutrient starvation for 2 h. Protein extracts were subjected to western blot and β-actin was used as a loading control. (D) Densities of protein bands were analyzed with the public domain NIH image program. Levels of Beclin-1 were normalized as a ratio to β-actin after densimetrical quantification and shown as percentage of untreated group. $^{*}P < 0.05$, $^{**}P < 0.01$compared with untreated group, $^{\#}P < 0.05$.

Beclin-1 was upregulated during siTIGAR transfection (Fig. 4C). Quantification data (Fig. 4D) showed that siTIGAR dramatically increased Beclin-1 expression relative to siCON treatment. HepG2 cells exposed to HBSS also displayed upregulated Beclin-1 expression.

4. Discussion

Although the relationship between apoptosis and autophagy is still a debated topic, and the functional relationship between apoptosis and autophagy within the carcinogenesis process is very complex, there is mounting evidence that the combined inactivation of apoptosis and autophagy contributes to tumorigenesis [17,18]. Previous studies have indicated that in HCC, antiapoptotic stimuli inhibit apoptosis induced by various stress-inducing conditions such as p53 activation and nutrient starvation [4,19]. Furthermore, the heterozygous disruption of the B autophagy gene has been shown to promote tumorigenesis and accelerate the development of hepatitis B virus-induced premalignant, which results in increased cellular proliferation and reduced autophagy *in vivo*.

Accumulating evidence indicates that autophagy plays dual roles in cell death [20]. Some results suggest that autophagy is harmful [21]; however, there are some reports that autophagy is protective [22]. One recent study determined that the expression of autophagic genes was extremely low in HCC cells, especially in highly malignant HCC cells relative to low-grade malignant cells. These data indicate that the malignant potential or differentiation of HCC is only closely correlated with Beclin-1 expression in a Bcl-xL-positive background [18].

One study showed that TIGAR has the ability to limit the level of fructose-2, 6-bisphosphatase and via the induction of the pentose phosphate pathway TIGAR has an anti-apoptotic effect in U2OS cells [9]. TIGAR also has been shown to translocate to the mitochondria and to interact with hexokinase 2, resulting in the regulation of mitochondrial membrane potential and a reduction in ROS levels [23]. However, recent studies have also highlighted the important contribution of TIGAR to autophagy and determined that the autophagy inhibitory function of TIGAR was tightly correlated with the suppression of ROS. These findings indicate that TIGAR is very important for anti-programmed cell death.

The present study provides the first evidence that the targeted silencing of TIGAR expression results in enhanced apoptosis and autophagy levels in HCC cells. Our findings support the hypothesis that TIGAR plays an important role in protecting HepG2 cells from cell death. Furthermore, our data demonstrate that ROS levels play a critical role in siTIGAR-induced apoptosis and that Beclin-1 expression is responsible for autophagic cell death in HepG2 cells (Fig. 4C and D). The reported structure of TIGAR [24] will further help us to explore the mechanisms of siTIGAR-induced cell death.

In this paper, we used a chemically modified siRNA, which is thought to be more stable and effective than a non-modified siRNA. The introduction of siRNA targeting TIGAR into HepG2 resulted in efficient and specific inhibition of endogenous TIGAR expression, as demonstrated by real-time PCR and western blotting. These results suggest that the prepared siRNA that targeted the TIGAR gene is capable of inducing marked inhibitions of TIGAR RNA transcription and significant enhancement of apoptosis and autophagy in HCC cell lines. Taken together, we demonstrated that the specific down-regulation of TIGAR by RNAi is a promising approach to promote HCC cell apoptosis and autophagy and that this method has potential to be a molecularly targeted therapy, thereby shedding light on a potential new strategy in gene silencing therapy in HCC.

Acknowledgments

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