Biochemical and Biophysical Research Communications 459 (2015) 306–312

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A hairpin within YAP mRNA 3′UTR functions in regulation at post-transcription level

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ARTICLE INFO

Article history:
Received 12 February 2015
Available online 26 February 2015

Keywords:
YAP
Hairpin
3′UTR
esiRNA
Post-transcription

ABSTRACT

The central dogma of gene expression is that DNA is transcribed into messenger RNAs, which in turn serve as the template for protein synthesis. Recently, it has been reported that mRNAs display regulatory roles that rely on their ability to compete for microRNA binding, independent of their protein-coding function. However, the regulatory mechanism of mRNAs remains poorly understood. Here, we report that a hairpin within YAP mRNA 3′untranslated region (3′UTR) functions in regulation at post-transcription level through generating endogenous siRNAs (esiRNAs). Bioinformatics analysis for secondary structure showed that YAP mRNA displayed a hairpin structure (termed standard hairpin, S-hairpin) within its 3′UTR. Surprisingly, we observed that the overexpression of S-hairpin derived from YAP 3′UTR (YAP-sh) increased the luciferase reporter activities of transcriptional factor NF-κB and AP-1 in 293T cells. Moreover, we identified that a fragment from YAP-sh, an esiRNA, was able to target mRNA 3′UTR of NF2 (a member of Hippo-signaling pathway) and YAP mRNA 3′UTR itself in hepatoma cells. Thus, we conclude that the YAP-sh within YAP mRNA 3′UTR may serve as a novel regulatory element, which functions in regulation at post-transcription level. Our finding provides new insights into the mechanism of mRNAs in regulatory function.

1. Introduction

RNA plays a central role in molecular biology and encompasses both informational and catalytic functions as a primordial molecule of life [1]. Global evidence suggests that mRNA is also widely involved in the regulation of genome organization and gene expression besides acting as a messenger between DNA and protein [2]. The regulatory RNA seems to operate in the epigenetic processes that control differentiation and development. These discoveries suggest a central role for RNA in human evolution and ontogeny. Accumulated documents witness that the antisense RNA, circular RNAs, pseudogene and hybrid RNA provide a crucial new perspective on the centrality of RNA in gene regulation [3–6]. Interestingly, the messenger RNAs (mRNAs) also modulate gene expression [7]. It has been reported that the mRNA transcripts possess a biological activity independent of their protein-coding function, but relying upon their ability to compete for miRNA binding [8,9]. The structure of RNA involving regulatory elements is closely associated with the function in regulation. The regulatory mechanisms of many genes occur at the level of mRNA, including the control tasks mediated by structured mRNA elements for sophisticated genes [10]. Basically, RNA folds are assembled by base-pairing elements. The conserved structural motifs and tertiary interactions are stabilized by different forces, including hydrogen bonds, stacking, electrostatic, and van der Waals interactions [11]. Interestingly, hairpin loops are crucial structural elements of RNA, defining the three-dimensional structures of large RNAs and offering potential nucleation sites for RNA folding and interaction with other nucleic acids and proteins [12,13]. The secondary structure of characteristic hairpin within a precursor can be processed by Dicer generating microRNAs [14,15]. The miRNAs are processed from short hairpins termed pre-miRNAs, which are the products of much longer precursors known as pri-miRNAs [16]. The secondary

Abbreviations: 3′UTR, 3′untranslated region; esiRNAs, endogenous siRNAs; S-hairpin, standard hairpin; YAP-sh, S-hairpin derived from YAP 3′UTR; siNC, negative control siRNA; mimics NC, mimics negative control; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diph-enyltetrazolium bromide; EdU, 5-ethynyl-2′-deoxyuridine.

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http://dx.doi.org/10.1016/j.bbrc.2015.02.106
0006-291X/

structure of mRNA predicted by bioinformatics analysis displays the helix, bulge, hairpin loop, internal loop, and multi-loop [17].

The Hippo-signaling pathway, initially discovered in Drosophila, is a well-conserved potent regulator of cell growth and apoptosis in mammals [18]. As a negatively regulated down-stream effector of the Hippo-signaling pathway, Yes-associated protein (YAP) functions as a transcriptional modulator [19]. However, the roles of those secondary structures of mRNA in function remain poorly understood.

In this study, we try to identify the mechanism that YAP mRNA functions in regulation through the secondary structure assessment of mRNA using bioinformatics analysis. Interestingly, we identify a novel secondary structural element within the 3′UTR of YAP mRNA, a hairpin structure, which functions in regulation at post-transcriptional level. Our finding provides new insights into the mechanism by which mRNA functions in regulation.

2. Materials and methods

2.1. Bioinformatics analysis

We examined the secondary structure of YAP mRNA by bioinformatics analysis using RNAstructure and RNAdraw. Interestingly, we demonstrated a secondary structure of YAP mRNA which was determined as a standard hairpin (termed YAP-sh), based on the rules, such as formulation of a stable hairpin with continuous sequences at different free energy levels; 50–130 nucleotides long; stem loop with 6–20 nucleotides; stem with bubbles as small as possible. Additionally, sequence alignment was using the software of CLC sequence viewer 6.3.

2.2. Total RNA isolation and reverse-transcriptional PCR

Total RNA was extracted from the cells using Trizol (Invitrogen) according to the manufacturer's protocol. To test small RNA derived from YAP-sh, total RNA was polyadenylated by poly (A) polymerase (Ambion, Austin, TX, USA) as described previously. Reverse transcription was performed using poly (A)-tailed total RNA and reverse transcription primer (5′GCGAGCACAGAATTAATACGACTCACTATA GGT TTTTTTTTTTTTTTTTVN3′) with ImPro-II Reverse Transcriptase (Promega) according to the manufacturer's protocol. Primers were listed in Supplementary Table S1.

2.3. Plasmid construction

The DNA sequences of YAP-sh (5′CGCGGATCCGCATGTTGTTGGG ATTTTTTTTAA TGTGCAGAAGATCAAAGCTACTTGGAAGGAGTGCCT ATAATTTGCCGGTACCCCG3′), YAP-non-sh (5′CGCGGATCCTTATTAA GAAAACAGCAGAAAGATTAAATCTTGAAT TAAGTCTGGGGGGAAAT GGCCACTGCAGATGGAGGGTACCCCG3′) and YAP-sh-mut (5′CGC GGATCCGCATGTACTTGGGTGATTTGGTATGTGATTTCAATAAGCTACT-TGGCAGAGTGCAAATAGTTAATTTGCCGGTACCCCG3′) were synthesized by Augct (Beijing, China), and then were cloned into pRNAT-U6.1/neo vector *via* KpnI and XhoI sites. The 3′UTRs of YAP and NF2 mRNA were amplified by PCR using the cDNA of HepG2 as templates. Primers were listed in Supplementary Table S1. The PCR products were inserted into the downstream of the pGL3-control vector (Promega, USA) *via* FseI and XbaI sites, respectively. Mutant construct of NF2 3′UTR, carrying a substitution of four nucleotides, was cloned into pGL3-control vector using overlapping extension PCR [20]. YAP-sh-3p20 PCR products derived from 293T cells were inserted into pEASY-T1 vector and sequenced (BGI, Beijing, China).

2.4. Cell culture and transfection

Hepatoma cell line HepG2 and human embryonic kidney cell line 293T was maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) [21]. The cells were cultured in a 6-well or 24-well plate for 12 h and then were transfected with plasmid or siRNA. All transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

2.5. Luciferase reporter gene assays

Luciferase reporter gene assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Cells (3×10^4/per well) were transferred into 24-well plates. After 12 h, the cells were transiently co-transfected with 0.1 μg/well of pRL-TK plasmid (Promega) containing the Renilla luciferase gene used for internal normalization, and various constructs containing pGL3-Ap-1, pGL3-NF-κB, pGL3-YAP 3′UTR and pGL3-NF2 3′UTR, respectively. The luciferase reporter pGL3-Ap-1 (or pGL3-NF-κB) contains transcriptional factor AP-1 (or NF-κB) binding element in the promoter region which can be activated by Ap-1 (or NF-κB) when the plasmid was transfected into the cells [22,23]. The luciferase activities were measured as previously described [24]. All experiments were performed at least three times.

2.6. Western blotting analysis

Western blotting protocol was described previously [24]. Primary antibodies were rabbit anti-YAP (Proteintech, Chicago IL, USA), rabbit anti-NF2 (Proteintech, Chicago IL, USA), rabbit anti-CTGF (Proteintech, Chicago IL, USA), rabbit anti-Dicer (Boster, Wuhan, China), rabbit anti-phosphorylated YAP (Proteintech, Chicago IL, USA) and mouse anti-β-actin (Sigma–Aldrich, St. Louis, MO). Every experiment was repeated three times.

2.7. Oligonucleotides and transfection

Small interfering RNAs (siRNAs) targeting human Dicer mRNA (siDicer-1: 5′-UGCUUGAAGCAGCUCUGGA-3′; siDicer-2: 5′UUUGU UGCGAGGCUGA UUC3′) [25], negative control siRNA (siNC), YAP-sh-3p20 mimics (5′ GCUACUUGGAAGGAGUGCC U3′) and mimics negative control (mimics NC) were synthesized by RiboBio (Guangzhou, China). Transfections were performed in 6- or 24-well plates after seeded cells were cultured for 24 h siRNA reagents or/and different doses of plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.8. Analysis of cell proliferation

HepG2 cells was seeded in 96 well plates for 24 h before transfection and 3-(4,5-dimethylthiazol-2-yl)-2,5-diph-enyltetrazolium bromide (MTT) assays were used to assess the cell proliferation every day from the first day until the third day after transfection. The protocol was described previously [24]. Five-ethynyl-2′-deoxyuridine (EdU) incorporation assay was carried out using the Cell-Light™ EdU imaging detecting kit according to the manufacturer's instructions (RiboBio, Guangzhou, China). Flow cytometry analysis was processed as earlier described. Proliferation index, $PI = (G2 + S)/(G1 + G2 + S) \times 100\%$ [26].

2.9. Statistical analysis

Statistical significance was assessed by comparing mean values (±standard deviation; SD), using a Student's *t*-test for independent groups, and was assumed for $P < 0.05$, $P < 0.01$, and $P < 0.001$. Each experiment was repeated at least three times.

3. Results

3.1. An S-hairpin within YAP mRNA 3′UTR owns regulatory function

It has been reported that the secondary structure of mRNA has a positive influence based on the ligand recognition and gene regulation in structural studies of riboswitches [27]. In this study, we focused on the investigation of YAP mRNA 3′UTR structure using the software of RNAdraw [28] and RNAstructure [29]. Interestingly, we observed that the secondary structure of YAP mRNA 3′UTR was complicated at different free energy levels, and the secondary structure analysis displayed a stable hairpin structure (termed YAP-sh) relative to other hairpin structures, such as YAP non-standard hairpin (YAP-non-sh) (Fig. 1A and Supplementary Fig. 1). The hairpin structure within the 3′UTR of the Pol β mRNA demonstrates an element influencing the expression of a reporter gene [30], but its regulatory function is unclear. Then, we try to investigate the function of the hairpin. Next, we constructed a vector with U6 promoter for the overexpression of YAP-sh. Since the transcription factors of AP-1 and NF-κB can induce expression of various target genes related to proliferation, lipid metabolism, angiogenesis, apoptosis and metastasis in the cells [22,23], we used the reporter system of AP-1 and NF-κB to evaluate the response of cells to the overexpression of YAP-sh. Strikingly, luciferase reporter gene assays showed that YAP-sh increase the luciferase activities of AP-1 and NF-κB in 293T cells, but the YAP-non-sh failed to work (Fig. 1B). Next, we cloned the mutant of YAP-sh, termed YAP-sh-mut, which was a cripple of YAP-sh (Fig. 1C). Interestingly, YAP-sh-mut failed to activate the reporter of AP-1 and NF-κB in 293T cells (Fig. 1D). To better understand the biological significance, we aligned the sequence of YAP-sh in different species and found that it was highly conserved in higher mammals (Fig. 1E). Thus, we conclude that the S-hairpin within YAP mRNA 3′UTR functions in regulation in the cells.

3.2. YAP-sh functions in regulation of YAP at post-transcription level

Given that protein binding can alter mRNA folds, thereby affecting transcription termination or translation initiation [31], we supposed that YAP-sh might regulate the gene expression at the

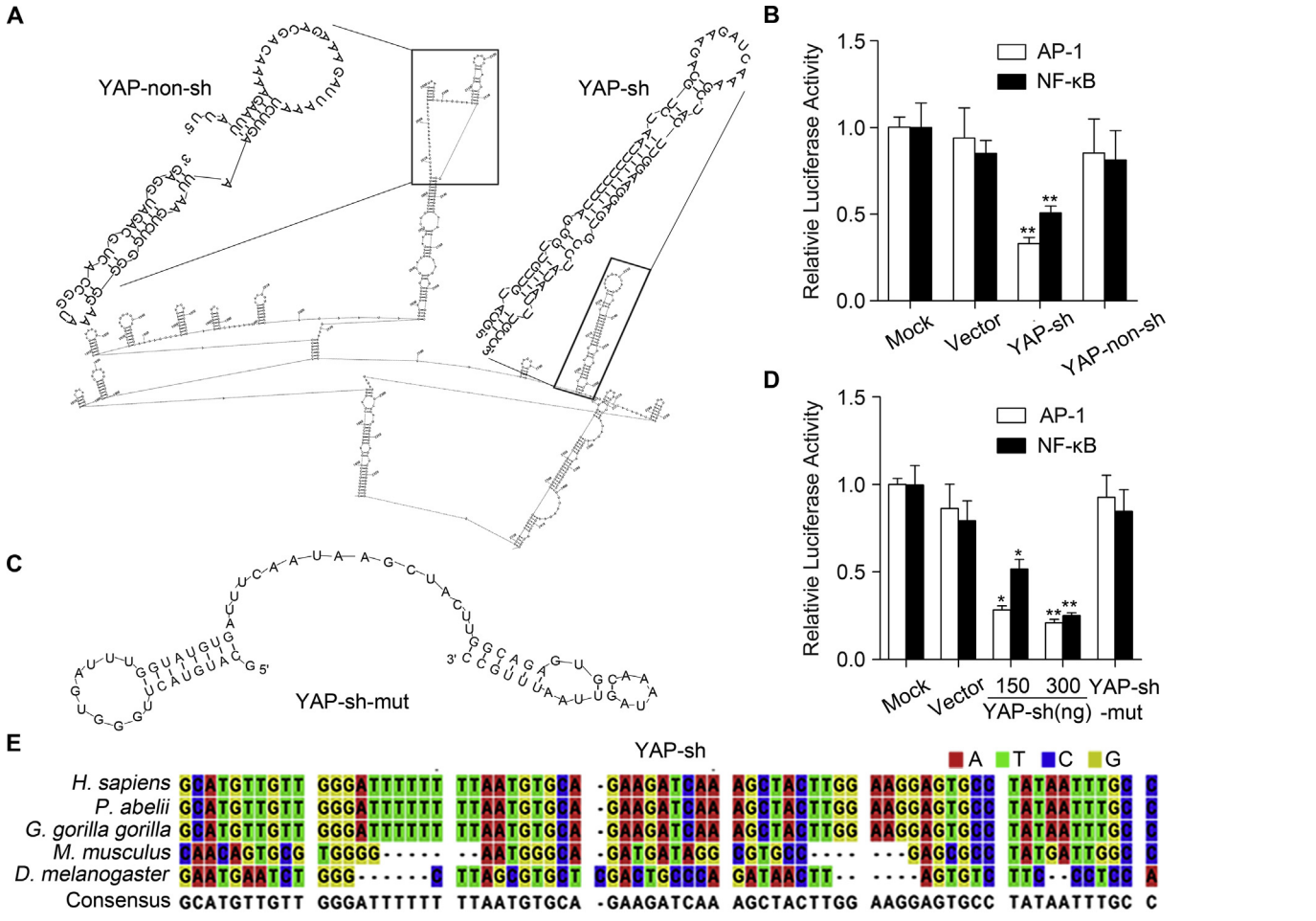

Fig. 1. An S-hairpin within YAP mRNA 3′UTR owns regulatory function. (A) The diagram of the secondary structure of YAP mRNA 3′UTR, including YAP-sh and YAP-non-sh, was analyzed by using RNAstructure and RNAdraw. (B) The effect of YAP-sh and YAP-non-sh on promoter activities of AP-1 and NF-κB was examined by luciferase reporter gene assays. (C) The secondary structure of YAP-sh-mut with cripple of YAP-sh sequence was analyzed using RNAstructure and RNAdraw. (D) The effect of YAP-sh and YAP-sh-mut on promoter activities of AP-1 and NF-κB was examined by luciferase reporter gene assays. Each experiment was repeated three times. Mock, without plasmid DNA; Vector, empty plasmid DNA. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, Student's *t*-test. Error bars represent s.d. ($n = 3$). (E) The sequence alignment of YAP-sh in different species was displayed.

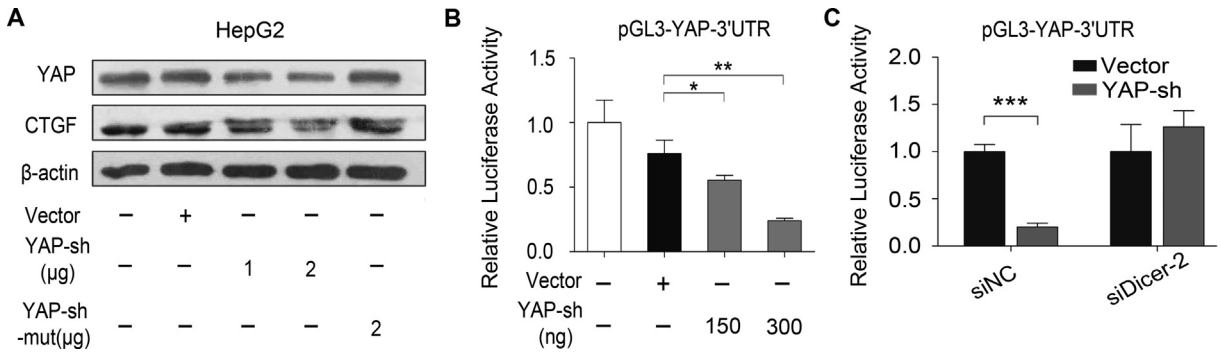

Fig. 2. YAP-sh functions in regulation of YAP at post-transcription level. (A) The effect of YAP-sh and YAP-sh-mut on expression of YAP and CTGF (a down-stream effector of YAP) was measured by Western blot analysis in HepG2 cells. Vector presents empty plasmid DNA. (B) The effect of YAP-sh on luciferase activities of pGL3-YAP-3′UTR was examined by luciferase reporter gene assays in 293T cells. (C) The effect of 100 nM siDicer-2 on luciferase activities of pGL3-YAP-3′UTR induced by YAP-sh was detected in the system. Error bars represent s.d. (n = 3). *p < 0.05; **p < 0.01; ***p < 0.001, student's *t*-test.

post-transcriptional level. Western blot analysis showed that the overexpression of YAP-sh led to the down-regulation of YAP in the cells in a dose-dependent manner, but not by YAP-sh-mut (Fig. 2A). Similarly, it worked on CTGF mRNA 3′UTR, which was a down-stream effector of YAP (Fig. 2A). Accordingly, the luciferase reporter gene assays could be used to reflect the interaction of microRNAs with their target genes [32]. Then, we constructed a vector of luciferase reporter with a fragment of 567 bp YAP 3′UTR containing YAP-sh in pGL3-control vector, termed pGL3-YAP-3′UTR (Supplementary Fig. 2A). Strikingly, we observed that the luciferase

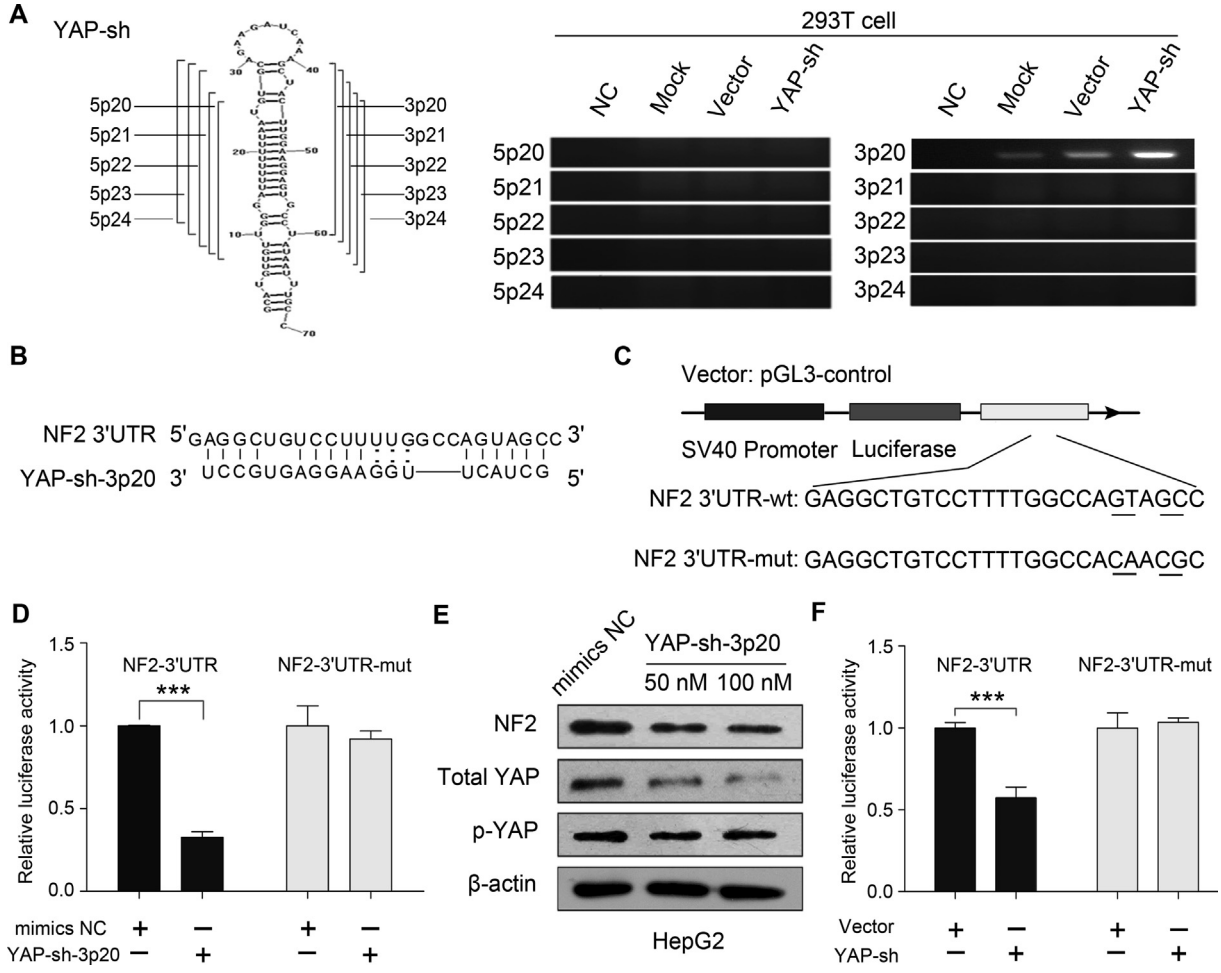

Fig. 3. YAP-sh-3p20 serves as an esiRNA derived from YAP-sh. (A) Diagram of designed walking PCR primers, each containing 20 nt, for esiRNAs derived from YAP-sh. The esiRNA products were examined by RT-PCR after polyadenylation by poly (A) polymerase in 293T cells transfected with YAP-sh. NC, purified water; Mock, without plasmid DNA; Vector presents empty plasmid DNA. (B) A diagram shows the model of YAP-sh-3p20 targeting NF2 locus sites. (C) A diagram shows the luciferase reporter construction of NF2 3′UTR wild type or mutation. (D) Effect of YAP-sh-3p20 on NF2 3′UTR-wt (or NF2 3′UTR-mut) was examined by luciferase reporter gene assays in 293T cells. (E) The effect of YAP-sh-3p20 on NF2, total YAP and phosphorylated-YAP (p-YAP) was determined by Western blot analysis in HepG2 cells. (F) Effect of YAP-sh on NF2 3′UTR-wt (or NF2 3′UTR-mut) was detected by luciferase reporter gene assays in 293T cells. Vector, empty plasmid DNA. Error bars represent s.d. (n = 3). ***p < 0.001, Student's *t*-test.

activities of pGL3-YAP-3′UTR could be attenuated by the treatment with YAP-sh in 293T cells (Fig. 2B), suggesting that an endogenous interference RNA (esiRNA) is generated by YAP-sh cleavage. Since Dicer can recognize stem-loop or double-strand RNA and cleave it into ~22 nucleotide long fragments [33]. Therefore, we supposed that Dicer might be involved in the cleavage of YAP-sh. Interestingly, we found that the YAP-sh mediated down-regulation of pGL3-YAP-3′UTR luciferase activities could be rescued by siDicer-2 in 293T cells (Fig. 2C), in which the efficiency of siDicer-1 and siDicer-2 was validated (Supplementary Fig. 2B), suggesting that Dicer might be responsible for the cleavage of YAP-sh in the cells. Therefore, we conclude that YAP-sh acting as a regulatory element functions in regulation of YAP at post-transcription level.

3.3. YAP-sh-3p20 serves as an esiRNA derived from YAP-sh

According to above data that the cleavage of YAP-sh was associated with Dicer, resulting in the suppression of protein synthesis, we presumed that a fragment derived from YAP-sh might be responsible for the regulatory function in the cells. It has been reported that the transcribed pseudogene functions as gene regulator by generating of esiRNAs that regulate human cell growth [34]. The small RNA fragments can be identified after the total RNA are polyadenylated by poly (A) polymerase [35]. Accordingly, we designed the different PCR primers for YAP-sh (Fig. 3A). Interestingly, primer walking PCR showed that a fragment of 20 nucleotides was detectable in 293T cells transfected with YAP-sh using YAP-sh-3p20 primers (Fig. 3A). PCR products from 293T cells were proved by sequencing. At the same time, the sequences were matched to YAP-sh, suggesting that YAP-sh-3p20 is a kind of esiRNA derived from YAP-sh.

Given that esiRNAs silence their target genes in the cells, we predicted that NF2 might be one of the target genes of YAP-sh-3p20, using the software of RNAhybrid [36] (Fig. 3B). Meanwhile, we cloned the 3′UTR of NF2 mRNA (termed NF2 3′UTR-wt) and mutant of NF2 (termed NF2 3′UTR-mut) into the pGL3-control vector (Fig. 3C). Interestingly, our data revealed that YAP-sh-3p20 could attenuate the luciferase activities of NF2 3′UTR-wt, but not the NF2 3′UTR-mut (Fig. 3D). Furthermore, Western blot analysis showed that YAP-sh-3p20 could down-regulate NF2 and YAP at the levels of protein, but not phosphorylated YAP (p-YAP) (Fig. 3E). Moreover, we observed that YAP-sh containing YAP-sh-3p20 could decrease the luciferase activities of NF2 mRNA 3′UTR-wt, but not NF2 mRNA 3′UTR-mut (Fig. 3F), indicating that YAP-sh-3p20 as an esiRNA is able to silence its target genes in the cells. Therefore, we conclude that the fragments derived from hairpins serving as an esiRNA functions in post-transcription regulation.

3.4. YAP-sh-3p20 down-regulates cell proliferation in vitro

To better understand the significance of YAP-sh-3p20 in function, we tested the effect of YAP-sh-3p20 on cell proliferation. Our results showed that YAP-sh-3p20 could decrease the proliferation of HepG2 cells (Fig. 4A–C), using MTT, EdU and flow cytometry assays, suggesting that YAP-sh-3p20 functions as an esiRNA in the cells. Therefore, we conclude that the YAP-sh-3p20, as an esiRNA, is able to decrease cell proliferation *in vitro*.

4. Discussion

The messenger RNA plays crucial roles in the translation of proteins as templates. However, the regulatory function of mRNAs

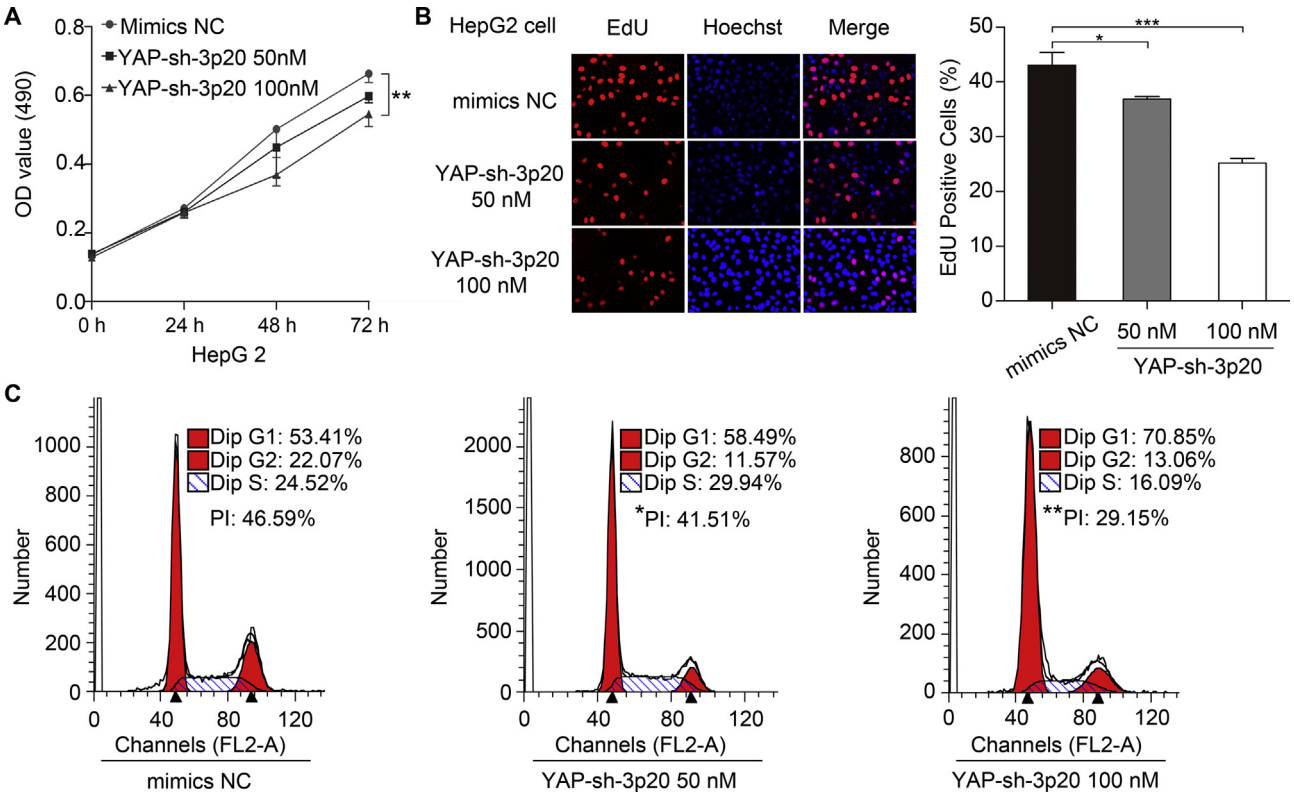

Fig. 4. YAP-sh-3p20 down-regulates cell proliferation *in vitro*. (A, B) The effect of YAP-sh-3p20 on cell proliferation was determined in HepG2 cells by MTT (A) and EdU incorporation assays (B), respectively. Population of EdU positive cells were calculated by software Image J. (C) The effect of YAP-sh-3p20 on HepG2 cells was examined by flow cytometry analyses. Error bars represent s.d. (n = 3). Statistical significant differences are indicated: *p < 0.05; **p < 0.01; ***p < 0.001; Student's *t*-test.

is mysterious. It has been reported that regulatory RNAs [37], both protein-coding and non-coding RNAs, demonstrate significant biological importance [8,9]. In this study, we are interested in the basic functions of YAP mRNA in gene expression regulation.

Generally, the mRNA 3′UTR shows a new motif at the post-transcriptional regulation level [29], and stem loops play an important role in mRNA stability [38] and localization [39]. To better understand the mechanism of YAP mRNA in gene expression regulation, we investigated the regulatory activity of YAP mRNA after its secondary structure of mRNA 3′UTR was analyzed in the cells. Interestingly, we observed a hairpin within the mRNA 3′UTR of YAP. The secondary structures of RNA are abundant, such as helix, bulge, hairpin loop, internal loop, and multi-loop [17]. Hairpin loops could construct the three-dimensional structure of large RNAs and provide potential nucleation sites for RNA folding and interaction with other nucleic acids and proteins [12,13]. Moreover, we found that YAP-sh could function in the cells.

Next, we try to identify the significance of the hairpin within YAP mRNA. The microRNA precursors with a characteristic hairpin secondary structure are processed by Dicer generating microRNAs, small noncoding RNA gene products about 22 nt long [13–15]. Therefore, we assumed that the hairpin within YAP mRNA might be processed by Dicer as well. Strikingly, we validated that the Dicer was involved in the cleavage of hairpin within YAP mRNA using luciferase reporter gene assays. Interestingly, the primer walking PCR [40] and sequencing could identify the fragment of YAP-sh-3p20 containing 20 nt.

Then, we speculated that the endogenous small RNA YAP-sh-3p20 might serve as an esiRNA. It has been reported that miRNAs collectively regulate thousands of endogenous target transcripts *via* as little as 7 nt “seed” pairing with nucleotides 2–8 of the miRNA [41]. Thus, we validated that the YAP-sh-3p20 regulated its target genes according to the above rule. Strikingly, the target gene NF2 of esiRNA YAP-sh-3p20 was a member of Hippo-signaling pathway [42]. It suggests that the esiRNA from YAP mRNA contributes to the precise regulation of Hippo-signaling pathway. Thus, we conclude that a hairpin within YAP mRNA 3′UTR acts as a regulatory element in the gene expression regulation.

In summary, we show the regulatory functions of YAP mRNA 3′UTR in a model (Supplementary Fig. 3). The secondary structure analysis of mRNA 3′UTR displays multiple hairpins. The esiRNA YAP-sh-3p20 derived from YAP-sh functions in the regulation of Hippo-signaling pathway by targeting mRNA 3′UTR of YAP and NF2. Our finding provides new insights into the mechanism by which mRNA functions in the gene expression regulation.

Conflict of interests

Xiaodong Zhang and Lihong Ye conceived the projects, designed the experiments and drafted the manuscript. Yuen Gao designed the experiments, drafted the manuscript and performed the experiments. Yuan Wang, Jinyan Feng, Guoxing Feng, Minying Zheng, Zhe Yang, Zelin Xiao and Zhanping Lu performed the experiments. The authors declare no competing financial interests.

Acknowledgments

We thank Prof. Yunping Luo from Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China for providing the vector of pRNAT-U6.1/neo. This work was supported by grants from the National Basic Research Program of China (973 Program, No. 2015CB553703, No. 2015CB553905), National Natural Science Foundation of China (No. 31470756, No. 81272218) and Project of Prevention and Treatment of Key Infectious Diseases (No. 2014ZX0002002-005).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.106.

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