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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Photoclickable MicroRNA for the Intracellular Target Identification of MicroRNAs

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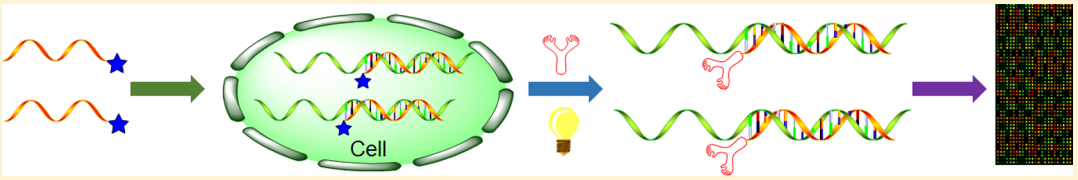
Supporting Information



ABSTRACT: MicroRNAs (miRNAs) are important gene regulators that bind with target genes and repress target gene expression at the post-transcriptional level. The identification of target genes associated with miRNAs inside different cells is a major challenge in miRNA chemical biology due to the lack of functional miRNAs bearing appropriate tags. Here we report photoclickable miRNAs as appropriately pretagged miRNAs that keep the intracellular function of miRNAs and allow the addition of molecular handles through photoclick reaction. The photoclickable miRNAs upon transfection inside cells were able to form functional complexes with target genes and repress target gene expression. Target genes associated with the photoclickable miRNAs in the complexes were then tagged with the molecular handle through photoclick reaction for pull-down and identification. Using photoclickable miR-106a, miR-27, and miR-122, we first verified that their intracellular function was comparable to that of intact miRNAs, which showed obvious advantage over corresponding biotinylated miRNAs. After attaching the biotin handle to the associated complexes containing the photoclickable miRNAs through the tetrazole-ene photoclick reaction, target genes previously bound with these miRNAs inside cells were successfully pulled town and analyzed. The application of this strategy was demonstrated by the identification of several new target genes of miR-122, followed by revealing a novel regulatory pathway in HepG2 cells with regard to the role of PEG10 in miR-122-promoted cell apoptosis.

INTRODUCTION

MicroRNAs (miRNAs) are endogenous gene regulators that form RNA-induced silencing complexes (RISC) with functional proteins inside cells to repress target gene expression through binding with their 3′-untranslated regions (3′-UTR).[1] Over 30% of human genome is estimated to be regulated by miRNAs,[2] and miRNAs are thereby extensively involved in diverse physiological processes, such as proliferation, differentiation, and apoptosis.[3] Meanwhile, abnormal expression or function of miRNAs is found to be closely associated with the occurrence or development of various human diseases, including cancers.[4] In light of their significant roles in physiology and pathology, miRNAs are emerging as novel biomolecular targets for chemical−biological studies, including regulation and detection.[5] However, the identification of intracellular targets of functional miRNAs, which is the basic way to understand miRNA-involved cellular processes, still remain as a major challenge in miRNA chemical−biology due to the lack of practical strategies.

While bioinformatic methods based on theoretical computation represent the most efficient way to predict miRNA targets,[6] experimental methods to identify intracellular targets of miRNAs are in high demand. Two typical biochemical methods were commonly used for this purpose.[7] One relies on the quantification of gene expression changes following miRNA overexpression or inhibition, and the other is based on the classical protein immunoprecipitation method to analyze possible miRNA target genes in RISC. Chemical−biological pull-down protocol has emerged as a straightforward method to identify the biospecies associated with biotinylated miRNAs transfected into live cells through affinity purification.[7b,c,8] Nevertheless, the 3′-biotinylated miRNAs were recently reported to lose their ability to form RISC with target genes, which greatly hampered their function to repress target gene expression inside cells.[9] It is therefore important to find alternative strategies to tag miRNA without changing its intracellular function to repress target gene expression.

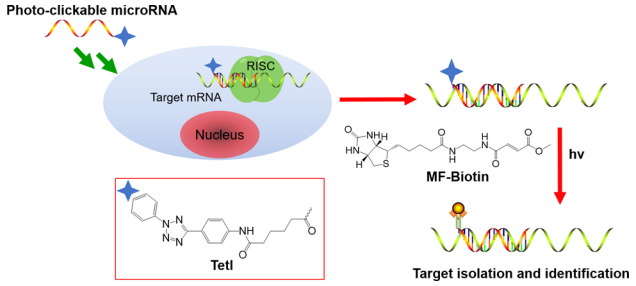
The tetrazole-ene photoclick reaction has been developed as a unique bio-orthogonal reaction that happens rapidly upon

Received: August 15, 2016
Published: November 18, 2016

DOI: 10.1021/jacs.6b08521
J. Am. Chem. Soc. 2016, 138, 15943−15949

mild light irradiation.[10] Biaryl tetrazoles were commonly used as photoclickable tags on proteins or other biomolecules for postlabeling through photoclick reaction.[11] Whether tetrazoles can be used as photoclickable tags on RNAs remains to be explored. We noticed that the presence of aromatic molecules on 3′-miRNA or 3′ short interfering RNA (3′-siRNA) was able to promote the formation of corresponding RISC with target genes.[12] Therefore, we synthesized photoclickable miRNAs through the introduction of biphenyl tetrazoles at 3′-miRNAs. Here we report our investigation on the intracellular biological function of these photoclickable miRNAs and demonstrate their application as novel chemical−biological tools for miRNA target gene identification (Scheme 1).

Scheme 1. Schematic Illustration on the Design of Photoclickable miRNAs for the Intracellular Target Identification Based on Photoclick Chemistry



RESULTS AND DISCUSSION

Structure and Reaction of the TetI Modified miRNAs. Synthetic miRNA mimics usually consist of a guide strand and its complementary passenger strand hybridized together to generate functional miRNA upon delivery inside cells. Through the reaction of commercially available 3′-NH2 miRNAs with NHS esters, it is synthetically convenient to attach bio-orthogonal functionalities to the 3′-end of the guide strand which is responsible for binding with target genes in RISC. **TetI** (Scheme 1) with two phenyl groups attached to the tetrazole core is the most commonly used substrate for the intermolecular photoclick reaction with alkenes.[10] We therefore prepared **TetI** NHS ester and used its reaction with the single-stranded 3′-NH_2 miRNAs to generate **TetI** substituted miRNAs. The cancer-related miR-106a,[9a] miR-27,[9b] and the liver-specific miR-122[13] were modified with **TetI** using this method, and the modified miRNAs were purified with HPLC and characterized with MS (Supporting Information). It is worth mentioning that we also carefully purified commercial 3′-biotinylated miRNAs by HPLC and used the purified sample as control to exclude any false result caused by impurities (Supporting Information). Photoclickable miRNA mimics for transfection were generated by the hybridization of the **TetI** modified miRNA guide strands with their passenger strands prior to cellular assays.

The photoclick reaction of the **TetI** modified miRNA **TetI**-miR122 with monomethyl fumarate-modified biotin (MF-Biotin) was then tested in PBS. With the generation of the biphenyl pyrazoline structure (Figure 1a), obvious fluorescence turn-on was observed upon irradiation with mild light from a hand-held 302 nm UV lamp for 5 min on the mixture containing **TetI**-miR122 and MF-Biotin (Figure 1b). The gel-shift assay also showed successful labeling of miR-122 after the

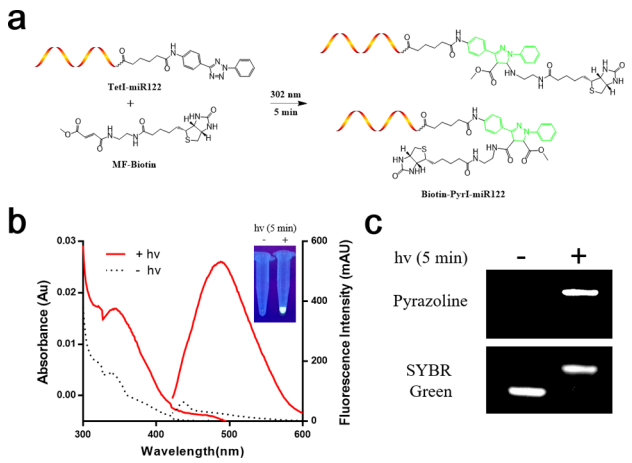


Figure 1. Photoclick reaction between photoclickable **TetI**-miR122 (20 μM) and MF-Biotin (100 μM) in PBS buffer. (a) Schematic illustration for the photoclick reaction, (b) UV/emission spectra, and (c) electrophoresis analysis of the reaction mixtures before and after irradiation by a hand-held 302 nm UV lamp for 5 min. Excitation = 365 nm. Insert figure: 365 nm UV-excited fluorescence images of reaction mixtures. Fluorescence of pyrazoline was acquired using EB channel. SYBR green was used to stain miR-122, and its fluorescence was acquired using SYBR channel.

photoclick reaction (Figure 1c). The rapid speed and high conversion rate of the photoclick reaction (Figure S1) guaranteed the highly efficient introduction of the biotin handle to the pretagged **TetI**-miR122 just by light irradiation. The resultant Biotin-PyrI-miR122 in the reaction mixture was enriched on streptavidin magnetic beads, which was confirmed by quantitative real-time PCR (qRT-PCR) analysis of the eluted sample from the beads (Figure S2).

Confirmation on the Intracellular Function of the Photoclickable miRNAs. We then examined whether the intracellular function of **TetI**-miRNAs was impaired by comparing with both normal miRNA mimics and 3′-biotinylated miRNAs. Using the cellular reporter systems constructed with different luciferase reporter genes,[14] we tested whether the miRNA mimics transfected into cells were able to bind with their target genes in RISC and repress target gene expression through the change of relative luciferase signals.[1b,14] **TetI**-miR106a, **TetI**-miR27, and **TetI**-miR122 were synthesized and purified for subsequent cellular assay. Commercial **Biotin**-miR106a, **Biotin**-miR27, and **Biotin**-miR122 with biotin modified at the 3′-end were also purified (Supporting Information) and used for comparison since miR-106a[9a] and miR-27[9b] upon 3′-biotinylation were reported to lose the ability to form RISC and fail to repress target gene expression.

Figure 2a showed the results of the luciferase assay using cells transfected with corresponding luciferase reporter genes bearing the complementary sequences of tested miRNAs. Compared with normal miRNA mimics, **TetI**-miR106a and **TetI**-miR27 showed similar repression on the reporter gene expression, which indicated that the 3′-modification of miRNAs with **TetI** had minimal impair on the formation of RISC. Several other miRNAs modified by **TetI** also showed effective regulation on corresponding gene regulation (Figure S3). In contrast, **Biotin**-miR106a and **Biotin**-miR27 only showed slight repression on the luciferase reporter gene expression, which was consistent with previous work reported by Hall and Steitz et al.[9] With these control experiments, it seemed that the 3′-

DOI: 10.1021/jacs.6b08521
J. Am. Chem. Soc. 2016, 138, 15943−15949

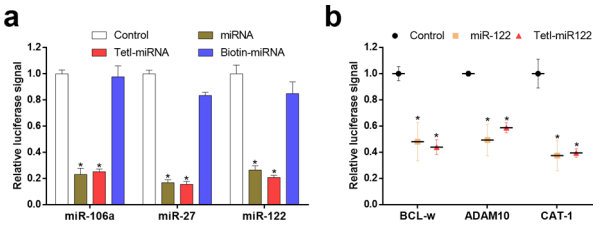


Figure 2. Luciferase assay to test the biological function of tetrazole-modified miRNAs. Relative luciferase signal changes upon introduction of miRNA (30 nM), **TetI**-miRNA (30 nM) or **Biotin**-miRNA (30 nM) into cells transfected with luciferase reporter genes bearing (a) the complementary sequences of miRNAs and (b) 3′-UTR of miR-122 target genes. Data are shown as mean ± SEM (n = 3). *, P < 0.05, relative to control.

modification by **TetI** was much more compatible with miRNA function through the RNA interference (RNAi) pathway than that of the 3′-modification by biotin. Parallel experiment using **TetI**-miR122 and **Biotin**-miR122 also showed that the intracellular function of **TetI**-miR122 to regulate gene expression was much higher than that of **Biotin**-miR122 in HepG2 cells.

The difference of the intracellular function of **TetI**-miRNAs and **Biotin**-miRNAs to regulate gene expression might come from two possibilities. One possibility is that **TetI**-miRNAs could be deprived of the tetrazole moiety upon transfection inside cells to form the normal miRNA mimics. The other possibility is that **TetI**-miRNAs and **Biotin**-miRNAs could have different binding ability with Argonaute 2 (AGO2) to form the functional RISC to further regulate gene expression. We performed Northern blotting analysis, and the results showed that tetrazole-modified miRNAs remained intact inside cells (Figure S4), which excluded the first possibility. The second possibility was confirmed later by the pull-down and Western blotting experiment (Figures S5−S6).

We then tested the regulation of **TetI**-miR122 on the expression of several known target genes of miR-122 inside HepG2 cells. We engineered luciferase reporter genes for BCL2-like 2 (BCL-w),[15] a distintegrin and metallopeptidase family 10 (ADAM10),[16] and cationic amino acid transporter 1 (CAT-1)[17] and obtained reporter cellular assay systems using a similar protocol as we reported before.[14] Then, the repression of these target genes expression upon transfection of **TetI**-miR122 into HepG2 cells was read out by the change on the relative luciferase signal. **TetI**-miR122 showed repression (∼40−50%) on these target gene expressions comparable to that by normal miR-122 (Figure 2b). Therefore, it is possible to use these **TetI**-miRNAs as intracellular probes to perform chemical−biological studies related to miRNA function inside cells.

Practical Pull-Down Protocol Using Photoclickable miRNA as the Pre-Tagged Probe. Pull-down experiment using streptavidin coated beads to enrich biospecies tagged with biotin is a common strategy in chemical−biology for sample enrichment prior to instrumental identification.[18] Since direct biotinylation of miRNA at the 3′-end impairs the intracellular function of miRNA, it is necessary to develop alternative strategies to tag the functional miRNA-bound species in cell lysate with the biotin handle. **TetI**-miRNAs with intact intracellular function may serve well as pretagged probes for the enrichment of miRNA-bound target genes through appropriate pull-down protocol. We then used **TetI**-miR122 to set up a practical protocol for pull-down assay by using the photoclick reaction to add the biotin handle to specific species in complex biological samples (Figure S7).

Validation of the attachment of the biotin handle through photoclick reaction of MF-Biotin with **TetI**-miR122 in cell lysate was based on the following experiment with several control lines. **TetI**-miR122 was transfected into HepG2 cells, and the cell lysate was reacted with an excess amount of MF-Biotin by UV irradiation for 10 min. The dialyzed mixture was then subject to normal pull-down step through streptavidin coated magnetic beads; finally, the abundance of the biotin-tagged miR-122 in the eluted species was analyzed by qRT-PCR. The results shown in Figure 3 validated that this protocol indeed was able to enrich **TetI**-miR122 (∼60-fold) transfected inside cells through the stepwise addition of the biotin-handle.

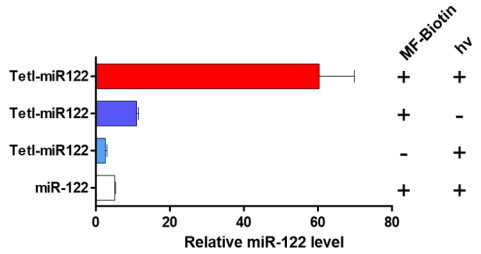


Figure 3. Validation of the pull-down protocol using photoclickable miRNAs as pretagged probes. qRT-PCR analysis of miR-122 in pull-down samples from lysate of HepG2 cells transfected with miR-122 (100 nM) and **TetI**-miR122 (100 nM) followed by different treatments as indicated in the figure. Data are shown as mean ± SEM (n = 3).

As there was concern on the possible side reactions of tetrazole with nucleophiles present in the complex biological systems,[19] three negative control experiments were performed to confirm the importance of the tetrazole-ene photoclick reaction in this pull-down protocol as shown in Figure 3. Cell lysates containing both MF-Biotin and **TetI**-miR122 without photoirradiation gave much lower level of miR-122 present in the pull-down sample. The presence of MF-Biotin and unmodified miR-122 in cell lysate after photoirradiation and the following pull-down procedures did not lead to a high level of miR-122. Photoirradiation on the cell lysate containing **TetI**-miR122 without the addition of MF-Biotin showed minimal enrichment of miR-122 in the pull-down assay. These results indicated that photoclick reaction of **TetI**-miRNA with MF-Biotin in cell lysate containing various nucleophilic species was still efficient enough to transform most of the **TetI**-miRNA into Biotin-PyrI-miRNA for further applications.

Photoclickable miRNAs as Probes for Target Gene Identification. After the confirmation of the intracellular function of photoclickable miRNAs and the validation on the photoclick based pull-down protocol, we then used the photoclickable miRNAs for the identification of intracellular miRNA target genes. The photoclickable miRNAs were transfected into cells and allowed to bind with their target genes to form RISC. Then, the cell lysate containing the **TetI** pretagged species was subjected to photoclick reaction with MF-Biotin to further attach the biotin handle for pull-down and enrichment. **TetI**-NC that is not miRNA mimic was used as the negative control probe. The abundance of different target genes in the pull-down sample was quantified by qRT-PCR analysis.

DOI: 10.1021/jacs.6b08521
J. Am. Chem. Soc. 2016, 138, 15943−15949

Figure 4 showed several target genes pulled down from HeLa cells transfected with **TetI**-miR106a (Figure 4a) and from

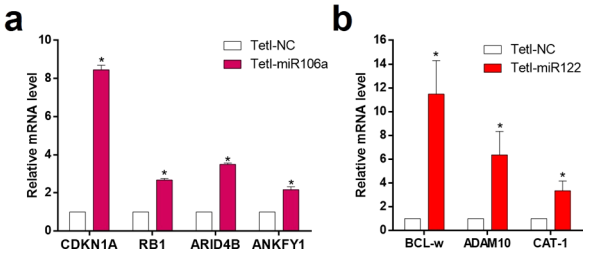


Figure 4. Application of **TetI**-miRNAs as probes to pull down target genes inside cells. qRT-PCR analysis of (a) confirmed miR-106a targets; (b) confirmed miR-122 targets in pull-down samples from lysate of cells transfected with **TetI**-NC (100 nM), **TetI**-miR106a (100 nM) or **TetI**-miR122 (100 nM). Data are shown as mean ± SEM (n = 3). *, $P < 0.05$.

HepG2 cells transfected with **TetI**-miR122 (Figure 4b). Reported target genes of miR-106a,[9a] including cyclin dependent kinase inhibitor 1A (CDKN1A), RB transcriptional corepressor 1 (RB1), AT-rich interaction domain 4B (ARID4B), and ankyrin repeat and FYVE domain containing 1 (ANKFY1), showed around 8.5-, 2.7-, 3.5-, and 2.2-fold enrichment, respectively, in HeLa cells transfected with **TetI**-miR106a compared with those transfected with **TetI**-NC. The confirmed target genes of miR-122 in HepG2 cells, including BCL-w (~11.5-fold),[15] ADAM10 (~6.4-fold),[16] and CAT-1 (~3.3-fold),[17] were also successfully pulled down by using **TetI**-miR122 as the pretagged probe. Additionally, the pull-down efficiency of the two-step biotinylation method through photoclick reaction was also comparable to that of direct biotinylation method (Figure S8). These results demonstrated that photoclickable miRNAs were promising functional pretagged probes to identify miRNA target genes that bind with the miRNAs inside cells.

The association of the photoclickable miRNAs with AGO2 that is the key component of RISC[20] was then confirmed by pull-down experiments using the photoclickable **TetI**-miR122. We performed Western blotting analysis on the abundance of AGO2 in the sample pulled down using **TetI**-miR122 through photoclick reaction with MF-biotin. A parallel test using **Biotin**-miR122 was also performed to compare the pull-down efficiency. We found that the AGO2 band corresponding to **TetI**-miR122 probe with stepwise biotinylation was much more abundant than the band corresponding to the sample using **Biotin**-miR122 probe (Figures S5 and S6). The result was consistent with the fact reported previously that 3′-biotinylation of miRNA to some extent hampered its association with AGO2 inside cells.[9] The reason why **TetI**-miRNA with the biphenyl tetrazole modified at the 3′-end still remained its binding affinity with AGO2 might be ascribed to the enhancement of binding due to the presence of the aromatic rings. Based on the structure of AGO2, the region of AGO2 for recognition and interaction with 3′-end of nucleic acids was composed of hydrophobic aromatic amino acids,[21] and it has also been demonstrated that chemical modifications of miRNA or siRNA with hydrophobic and aromatic molecules at the 3′-end could enhance their binding with AGO2 as well as their silencing activities compared to those of natural molecules.[12] Since the association of miRNA with AGO2 in RISC is essential for miRNA to repress its target gene expression,[20] the difference of the intracellular gene regulation ability of **TetI**-miRNA and **Biotin**-miRNA as shown in the previous part (Figure 2a) can be well-explained.

Discovery of New Target Genes of miR-122 and New miR-122 Involved Regulatory Pathway. After we proved that the use of **TetI**-miR122 in combination with photoclick reaction was able to pull down reported target genes of miR-122 inside HepG2 cell, we further used the probe to find unknown target genes of miR-122 in HepG2 cell. Several predicted miR-122 target genes based on TargetScan[22] were tested on their abundance in the sample pulled down using **TetI**-miR122 from HepG2 cells. As shown in Figure 5a, six of

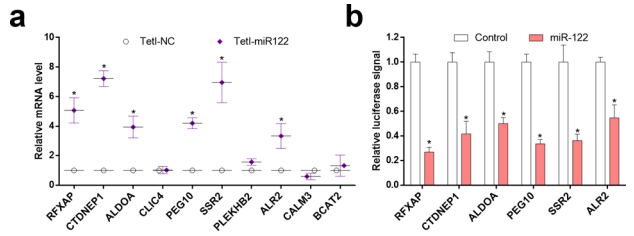


Figure 5. Discovery of new target genes for miR-122. (a) Identification of unknown target genes in pull-down samples from lysate of HepG2 cells transfected with **TetI**-NC (100 nM) or **TetI**-miR122 (100 nM); (b) luciferase assay to validate miR-122 targets identified by photoclickable miRNAs. Relative luciferase signal changes upon introduction of miR-122 (30 nM) into HepG2 cells transfected with luciferase reporter genes bearing the 3′-UTR of identified miR-122 targets. Data are shown as mean ± SEM (n = 3). *, $P < 0.05$.

the predicted target genes, including regulatory factor X-associated protein (RFXAP, ~5.1-fold), CTD nuclear envelope phosphatase 1 (CTDNEP1, ~7.2-fold), aldolase A fructose-bisphosphate (ALDOA, ~3.9-fold), paternally expressed gene 10 (PEG10, ~4.2-fold), signal sequence receptor subunit 2 (SSR2, ~7-fold), and aldose reductase (ALR2, ~3.3-fold), were successfully pulled down. Furthermore, we cloned the 3′-UTR of these genes into the luciferase vectors and coexpressed them in HepG2 cells as previously described to test whether their expression was able to be repressed by miR-122. Strong inhibition of luciferase signals was induced by the increase of miR-122 delivered into the reporter cells (~25−50%, Figure 5b), which suggested that miR-122 was able to target these genes to repress their expression inside HepG2 cells. A very recent biomedical paper[23] published during our preparation of this manuscript simultaneously verified PEG10 as the direct target of miR-122. We also performed Western blotting experiments to check the intracellular protein expression levels of all the six target genes in HepG2 cells upon overexpression of miR-122 (Figure S9). The results indicated that the expression of all these target genes was downregulated inside cells upon overexpression of miR-122, which further confirmed these identified genes as new miR-122 targets.

Among these newly confirmed target genes of miR-122, we noticed that PEG10 was highly expressed in hepatocellular carcinoma cells but absent in normal liver cells.[24] The overexpression of PEG10 was found to promote cell proliferation, while knock-down of PEG10 could inhibit the growth and induce cell apoptosis.[25] Since PEG10 was identified as the direct target of miR-122 in HepG2 cells by our protocol using **TetI**-miR122 (Figure 5), we hypothesized that miR-122 should be able to promote cell apoptosis through the down-regulation of PEG10 in HepG2 cells. We then performed

DOI: 10.1021/jacs.6b08521
J. Am. Chem. Soc. 2016, 138, 15943−15949

experiments using miR-122 overexpressed HepG2 cells to verify our hypothesis which was based on the previous target identification results. As shown in Figure 6a, the PEG10 protein

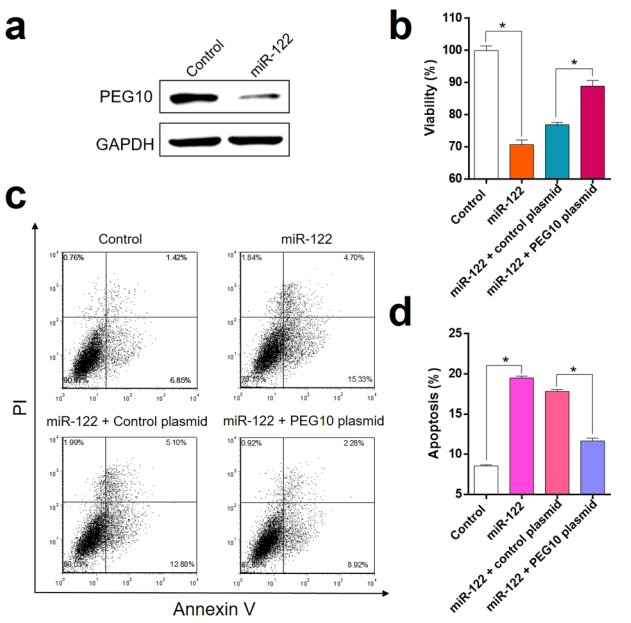


Figure 6. miR-122 promotes apoptosis of HepG2 cells through targeting PEG10. (a) Western blotting analysis of PEG10 in HepG2 cells after transfection with miR-122 (100 nM); (b) MTT assay of viability. (c and d) Flow cytometry analysis of apoptosis on HepG2 cells after transfection with miR-122 (100 nM), control plasmid (0.25 μg/mL), or PEG10 plasmid (0.25 μg/mL). (c) Representative images; (d) quantitative analysis. Lipofectamine 2000 was used as the control. Data are shown as mean ± SEM (n = 3–4). *, P < 0.05.

expression was significantly inhibited in the miR-122 overexpressed HepG2 cells. Meanwhile, MTT test revealed much lower cell viability (~70%, Figure 6b) of the miR-122 overexpressed HepG2 cells compared with normal HepG2 cells. Flow cytometry analysis also indicated that apoptosis of HepG2 cells was able to be promoted by the overexpression of miR-122 (~20%, Figure 6c,d). Moreover, simultaneous overexpression of miR-122 and PEG10 remarkably alleviated miR-122-induced inhibition on cell viability (~88%, Figure 6b) and its promotion of cell apoptosis (~12%, Figure 6c,d). These results and all the control experiments (Figure S10 and S11) collectively confirmed this new miR-122 involved regulatory pathway, that is, miR-122 promotes cell apoptosis through the direct repression on PEG10 expression. The application of the photoclickable miRNAs to identify new miRNA targets and reveal unknown intracellular regulatory pathway was thus successfully demonstrated. We expect reveal more miRNA-involved cellular regulation pathways by using different **TetI**-miRNAs to identify possible miRNA targets with pathological importance.

CONCLUSIONS

We demonstrated the first example of using the biocompatible photoclick reaction to develop photoclickable miRNAs for intracellular target identification. The 3′-modification of miRNAs with tetrazole as pretag did not affect the intracellular biological function of miRNAs to repress target gene expression, which is superior to the direct miRNA modification with biotin tag. The rapid intermolecular photoclick reaction between the tetrazole tag and MF-Biotin allowed postlabeling of the miRNAs-bound species in the extracted cell lysate with biotin for further enrichment, isolation, and identification. With photoclickable miRNAs as probes, we validated their ability to isolate confirmed target genes for miR-106a and miR-122. Then we identified several unknown target genes of miR-122 and revealed a new pathway in HepG2 cells by which miR-122 promotes cell apoptosis by targeting PEG10. The use of different photoclickable miRNA probes in combination with high-throughput screening techniques such as microarray or proteomics may reveal more unknown miRNA-involved cellular regulation pathways. Simultaneous use of miRNAs modified with different bio-orthogonal functional groups for sequential identification of target genes for different miRNAs is now underway in our group.

EXPERIMENTAL SECTION

Cell Culture. HepG2, HeLa, 293T, A549, and C2C12 cells were cultured in high-glucose DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin and maintained in 5% CO_2 at 37 °C.

Luciferase Assay. Cells were seeded in 24-well plates and transfected the following day with 0.5 μg of luciferase reporter plasmids and 0.3 μg of β-galactosidase expressing plasmids by using Lipofectamine 2000 according to manufacturer's protocol. For miR-122, HepG2 cells were used. For miR-21, miR-27, and miR-106a, HeLa cells were used. For miR-34a, A549 cells were used. For miR-133a, C2C12 cells were used. β-Galactosidase was used as the internal control. After 4 h, miRNA mimics (30 nM), **TetI**-miRNAs (30 nM), or **Biotin**-miRNAs (30 nM) were further transfected into corresponding cells by using Lipofectamine 2000 according to manufacturer's protocol. Luciferase signals were measured after 48 h by using luciferase assay kits according to manufacturer's instruction.

Pull-Down Assay. **TetI**-NC and **TetI**-miRNA were transfected into corresponding cells (~1.5×10^7 cells) with a concentration of 100 nM using Lipofectamine 2000 according to the manufacture's protocol. After 24 h, cells were pelleted, resuspended in 150 μL of PBS buffer (with 50 U/mL RNase inhibitor), and lysed by repeated freeze–thaw using liquid nitrogen. After centrifugation, the supernatant was collected. MF-Biotin was then added into **TetI**-NC and **TetI**-miRNA treated cell lysate with a final concentration of 1 mM. The reaction mixtures were then irradiated under a 302 nm UV light for 10 min, followed by centrifuging to remove free biotin using Amicon centrifugal filter (molecular weight cutoff 3000) for 5 times according to manufacturer's instructions. The treated cell lysate was then further incubated with streptavidin magnetic beads at 25 °C for 2 h. After washing with the wash/binding buffer (0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 U/mL RNase inhibitor) four times and cold low-salt buffer (0.15 M NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 U/mL RNase inhibitor) one time, biotinylated samples were eluted from the magnetic beads with elution buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 U/mL RNase inhibitor). The eluted samples were then subjected to reverse transcription and quantified by qRT-PCR on Applied Biosystem 7300 Real-time PCR system for analyzing the enrichment of miRNA or mRNAs. The primers for amplification of mRNAs were listed in Table S1. GAPDH was used as the internal control for quantification of miRNA targets. For control experiments, the same amounts of **TetI**-NC, miR-122, **TetI**-miR122, or MF-biotin was used.

Western Blotting Analysis. For purification of protein, 1× protease inhibitor cocktail was added to PBS buffer used in the pull-down assay to prevent protein degradation. Protein extract from pull-down samples diluted in 1× SDS loading buffer was predenatured and resolved in 10% SDS-PAGE, transferred to PVDF membrane, blocked in 5% nonfat milk in TBST, and blotted with primary antibodies for AGO2. A total of 30 μg of total protein was used for input control. After washing, the membrane was further incubated with the appropriate secondary antibody and finally visualized using ECL reagents.

DOI: 10.1021/jacs.6b08521
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To analyze PEG10 expression, HepG2 cells were transfected with 0.25 μg/mL control plasmid, 0.25 μg/mL PEG10 plasmid, or 100 nM miR-122 using Lipofectamine 2000 according to manufacturer's protocol. After 48 h, cell lysates were extracted using RIPA lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 1% NP40, 1% deoxycholate, 0.1% SDS, 1× protease inhibitor cocktail). Protein concentration was determined using BCA protein assay kit. A total of 30 μg of protein extract in 1× SDS loading buffer was predenatured and resolved by 10% SDS-PAGE, transferred to PVDF membrane, blocked in 5% nonfat milk in TBST, and blotted with primary antibodies for PEG10 and GAPDH. After washing, the membrane was further incubated with the appropriate secondary antibody and finally visualized using ECL reagents.

Northern Blotting Analysis. 293T cells were seeded in 6-well plates. Upon reaching 80% confluence, miR-122 and **TetI**-miR122 were transfected with a concentration of 30 nM using Lipofectamine 2000 according to manufacturer's protocol. Then, 24 h later, total RNA was isolated using TRIZOL reagent according to manufacturer's instruction. For each sample, 1 μg of RNA was used and separated on a 15% denatured polyacrylamide gel and electrophoretically transferred to Hybond-N^+ membrane. DIG-labeled DNA oligonucleotides complementary to miR-122 were used to hybridize overnight at 50 °C following the manufacturer's protocol. After washing, the membrane was further incubated with anti-DIG antibody and finally visualized using DIG luminescent detection kit for nucleic acids. U6 was used as the internal control.

MTT Assay. HepG2 cells were seeded in 96-well plates at a concentration of 5000 cells per well. Then, 24 h later, 100 nM miR-122 was transfected into the cells using Lipofectamine 2000 according to manufacturer's protocol. Control plasmid (0.25 μg/mL) or PEG10 plasmid (0.25 μg/mL) was transfected to overexpress PEG10 using Lipofectamine 2000 according to manufacturer's protocol. For the control, the same amount of Lipofectamine 2000 was used. MTT assay was carried out after 2 days of culture. For each well, 20 μL of MTT solution (final concentration: 1 mg/mL) was added. Then, 150 μL DMSO was added after 4 h of incubation, and absorbance at 490 nm was measured to indicate the cell viability.

Flow Cytometry Analysis. HepG2 cells were cultured in 6-well plates. When reaching ~80% confluence, HepG2 cells were transfected with 100 nM miR-122, 0.25 μg/mL control plasmid, or 0.25 μg/mL PEG10 plasmid by using Lipofectamine 2000 according to manufacturer's protocol. For the control, the same amount of Lipofectamine 2000 was used. The cells were cultured for 48 h and harvested for apoptosis analysis. The apoptotic cells were identified through flow cytometry by using an Annexin V-FITC/PI staining kit according to manufacturer's protocol.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b08521.

Experimental procedures for chemical synthesis, supporting figures, characterization data of tetrazole substrates and photoclickable miRNAs (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to acknowledge the financial support from National Natural Science Foundation of China (21572102, 21372115, 21672103, and 21302093) and the National Basic Research Program of China (2014CB542300).

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DOI: 10.1021/jacs.6b08521
J. Am. Chem. Soc. 2016, 138, 15943−15949