MicroRNA-181a modulates gene expression of zinc finger family members by directly targeting their coding regions

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ABSTRACT

MicroRNAs (miRNAs) are small endogenous, non-coding RNAs that specifically bind to the 3′ untranslated region (3′UTR) of target genes in animals. However, some recent studies have demonstrated that miRNAs also target the coding regions of mammalian genes. Here, we show that miRNA-181a downregulates the expression of a large number of zinc finger genes (ZNFs). Bioinformatics analysis revealed that these ZNFs contain many miR-181a seed-matched sites within their coding sequences (CDS). In particular, miR-181a 8-mer-matched sequences were mostly localized to the regions coding for the ZNF C2H2 domain. A series of reporter assays confirmed that miR-181a inhibits the expression of ZNFs by directly targeting their CDS. These inhibitory effects might be due to the multiple target sites located within the ZNF genes. In conclusion, our findings indicate that some miRNA species may regulate gene family by targeting their coding regions, thus providing an important and novel perspective for decoding the complex mechanism of miRNA/mRNA interplay.

INTRODUCTION

MicroRNAs (miRNAs) are small endogenous, non-coding, single-stranded RNAs that have been identified as post-transcriptional regulators of gene expression (1,2). Computational studies suggest that miRNAs regulate at least 30% of human genes, and miRNAs have been implicated in the regulation of a wide range of biological processes (3,4). In plants, most miRNAs hybridize to target mRNAs with near-perfect complementarity and mediate an endonucleolytic cleavage event by a mechanism that is similar to that used in the small interfering RNA (siRNA) pathway (5). In contrast, animal miRNAs have been shown to mediate either translational repression or degradation of target transcripts through imperfect base pairing with mRNA sequences. Extensive binding of nucleotides 2–7, the miRNA 'seed', to the target mRNA is considered to play a key role in target recognition (3,6). Almost all of the discovered miRNA-binding sites are located in the 3′ untranslated region (3′UTR) of target genes in animals. However, some recent reports have shown that miRNAs also target the coding sequences (CDS) of mammalian genes (7–9).

Zinc finger genes (ZNFs) are one of the largest gene families in mammals. ZNF proteins contain a number of zinc finger (ZNF) domains, ranging from 1 to 40, which are frequently arranged in groups or clusters of tandem repeats (10). ZNFs are known as the most abundant DNA-recognition domain and are stabilized by the coordinated binding of a zinc ion. Although there are ~20 different types of ZNF domains, the most common is the Cys2-His2 (C2H2) class (11,12). The subfamily of C2H2 ZNFs consists of a large number of ZNF proteins containing the consensus sequence $(F/Y)\text{-}X\text{-}C\text{-}X_{2\text{-}5}\text{-}C\text{-}X_3\text{-}(F/Y)\text{-}X_5\text{-}W\text{-}X_2\text{-}H\text{-}X_{3\text{-}4}\text{-}H$, where X is any amino acid and W is any hydrophobic residue (13). This motif, which self-folds to form a $\beta\beta\alpha$ structure, obtains its name from the coordinated binding of a zinc ion by the two conserved cysteine and histidine residues. The C2H2 ZNF proteins are thought to influence downstream gene expression by facilitating the interaction between DNA sequences and regulatory proteins. This class of ZNFs has been implicated in physiological processes and pathways, such

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as development and cell proliferation, and in complex pathological phenotypes (14–20). Yet, to the best of our knowledge, the regulation of ZNF expression is still largely unknown.

In this study, we found that the expression of a large number of ZNFs is regulated by miRNA-181a. Bioinformatics analysis further revealed that the protein coding regions of these ZNFs contain many miR-181a seed-matched sequences. In particular, miR-181a 8-mer-matched sites were mostly localised to the ZNF regions coding for the C2H2 domain. In addition, haemagglutinin (HA)-tagged construct and luciferase reporter assays demonstrated that miR-181a inhibits the expression of ZNFs by directly targeting their CDS.

EXPERIMENTAL PROCEDURES

Cell culture. HepG2, Huh-7 and HEK 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin.

Vector constructs. To generate a miR-181a expression vector, the pre-miR-181a sequence was amplified by polymerase chain reaction (PCR) from genomic DNA. The amplified fragment was cloned into the pWPT-GFP vector (a generous gift from Dr T. Didier, University of Geneva, Geneva, Switzerland) by replacing GFP to generate pWPT-miR-181a. The ZNF37A, ZNF83 and ZNF180 coding regions were amplified by PCR from plasmids (Proteintech Group, Wuhan, China) and then cloned into the pCDNA3.0 vector (Invitrogen, CA, USA) in frame with an HA tag. To create the luciferase reporters, the amplified fragments were cloned as a 3′UTR downstream of a cytomegalovirus (CMV) promoter-driven firefly luciferase cassette in the pCDNA3.0 vector. To introduce mutations into the three miR-181a 8-mer target sites in the ZNF37A coding region, primers were designed for site-directed mutagenesis that resulted in the destruction of the miR-181a target site without altering the amino acid sequence of ZNF37A. The sites were mutated as follows: before mutagenesis, TAt GAa TGt; after mutagenesis, TAc GAg TGc; translation, Y E C. Mutagenesis was performed with the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All primers used are shown in Supplementary Table S3.

Lentivirus production and transduction. Viruses were harvested 48 h after the transfection of HEK 293T cells with pWPT-miR-181a, the packaging plasmid, psPAX2 and the G-protein of vesicular stomatitis virus (VSV-G) envelope plasmid, pMD2.G (a generous gift from Dr D. Trono). Lipofectamine 2000 reagent was used for transfections (Invitrogen). HepG2 cells (1×10^5) were infected with 1×10^6 recombinant lentivirus-transducing units and exposed to 6 μg/ml of polybrene (Sigma, MA, USA).

mRNA array. HepG2 cells stably transduced with lentiviruses overexpressing mature miR-181a or with empty viruses were harvested and total RNA was extracted using Trizol reagent (Invitrogen). Hybridization with the Agilent Whole Human Genome Oligo Microarray was performed according to the manufacturer's protocol (Shanghai Biochip Co., Ltd, China). Microarray images were analysed using GenePix Pro 4.0, and data were normalized by the Lowess method. The genes that were decreased by at least 30% (Log2 ratio <-0.51) and $P<0.01$ were considered to be downregulated genes. Microarray analysis was performed using one biological replicate.

Bioinformatics analysis. The human full-length cDNAs of ZNFs were obtained from the NBCI Genbank database. MiR-181a seed-matched sites were classified as 8mer (TG AATGTA), 7-mer-m8 (TGAATGT, 7m8) and 7-mer-A1 (GAATGTA, 7A1). All ZNFs genes (Supplementary Data) were derived from the Agilent array and NBCI Genbank databases. A program was developed and performed to identify miR-181a seed-matched sites in the entire CDS/UTR of the ZNF transcripts.

Oligonucleotide transfection and quantitative real-time PCR. MiR-181a mimics and miR-181a inhibitors (2′-O-methyl modified) were synthesised by Ribobio (Guangzhou, China). The sequence of the mutant miR-181a mimic is as follows: aGcaCucGacgcugucggu gagu. Oligonucleotide transfection was performed with Lipofectamine 2000 reagent.

Total RNA was extracted using Trizol reagent. cDNA was synthesised using the PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan), and real-time PCR was performed using the SYBR Premix Ex Taq (TaKaRa). The β-actin was used as an internal control. The primers used are shown in Supplementary Table S4.

Western-blot analysis. HEK 293T cells were seeded into 6-well plates and co-transfected with 1.5 μg of either pWPT-GFP or pWPT-miR-181a and 0.5 μg of plasmids encoding HA-tagged ZNF proteins using Lipofectamine 2000 transfection reagent. Total protein was harvested after 48 h transfection. Proteins were separated by 12% SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, USA). The membrane was blocked with 5% non-fat milk and was incubated with mouse anti-HA mAb (Santa Cruz Biotechnology, CA, USA) or mouse anti-β-actin mAb (Sigma, St. Louis, USA). After extensive washing, goat anti-mouse secondary antibody (Pierce, IL, USA) was added, according to the manufacturer's instructions. The signal was detected using ECL (Pierce), and quantification was performed with Image-Pro Plus software (Olympus Corp., Tokyo, Japan).

Luciferase assay. HEK 293T cells were seeded into 96-well plates and co-transfected with 150 ng of either pWPT-GFP or pWPT-miR-181a, 50 ng of the luciferase reporter and 5 ng of the pRL-CMV internal control plasmid that encodes 'Renilla' luciferase using Lipofectamine 2000 transfection reagent. Cell lysates were harvested 48 h after transfection, and luciferase

activity was measured using the dual-luciferase reporter assay system (Promega, WI, USA).

RESULTS

MiR-181a has been identified as a key modulator of cellular differentiation (21–23). In our previous study, we showed that miR-181a is differentially expressed in hepatocellular carcinoma (HCC) (24). To find the potential targets of miR-181a, we infected cultured HepG2 cells with miRNA-containing or control lentiviruses and used microarrays to monitor miR-181a-induced alterations in gene expression. To our surprise, a large number of ZNF genes were downregulated in miR-181a-expressing cells compared with control cells (Figure 1, bottom and Supplementary Table S1). In fact, ZNF genes made up more than ten percent of the downregulated genes (50/451). This phenomenon was not caused by the expression of other miRNAs (miR-151, miR-125b or miR-30d) in these cells (data not shown). The results were further confirmed by quantitative real-time PCR (qRT-PCR) for these ZNFs. As shown in Supplementary Figure S1, most

of the ZNF genes are downregulated (45 out of the 50 ZNFs, Log2 ratio <−0.3) in HepG2 cells in the presence of miR-181a. In examining microarray data from a recent paper (25), similar results were also found in HeLa cells transfected with miR-181a mimics (Supplementary Table S1). Furthermore, we synthesized anti-sense oligonucleotides of miR-181a and transfected them into Huh-7 cells, which display relatively high expression of miR-181a (Supplementary Figure S2). The 31 ZNF genes that were downregulated by at least 30% (Log2 ratio <−0.5) in Supplementary Figure S1 were examined by qRT-PCR analysis. The results showed that miR-181a inhibition led to the increased expression of most of the examined ZNF genes (Figure 2). We also investigated the endogenous protein levels of some of these ZNFs by western blot but failed to get clear bands (data not shown). Taken together, our findings indicate that miR-181a indeed reduces the expression levels of ZNF genes.

To investigate whether these ZNFs were the targets of miR-181a, we searched the full length of these transcripts for miR-181a seed-matched sites, which can be classified as 8-mer (TGAATGTA), 7-mer-m8 (TGAATGT, 7m8)
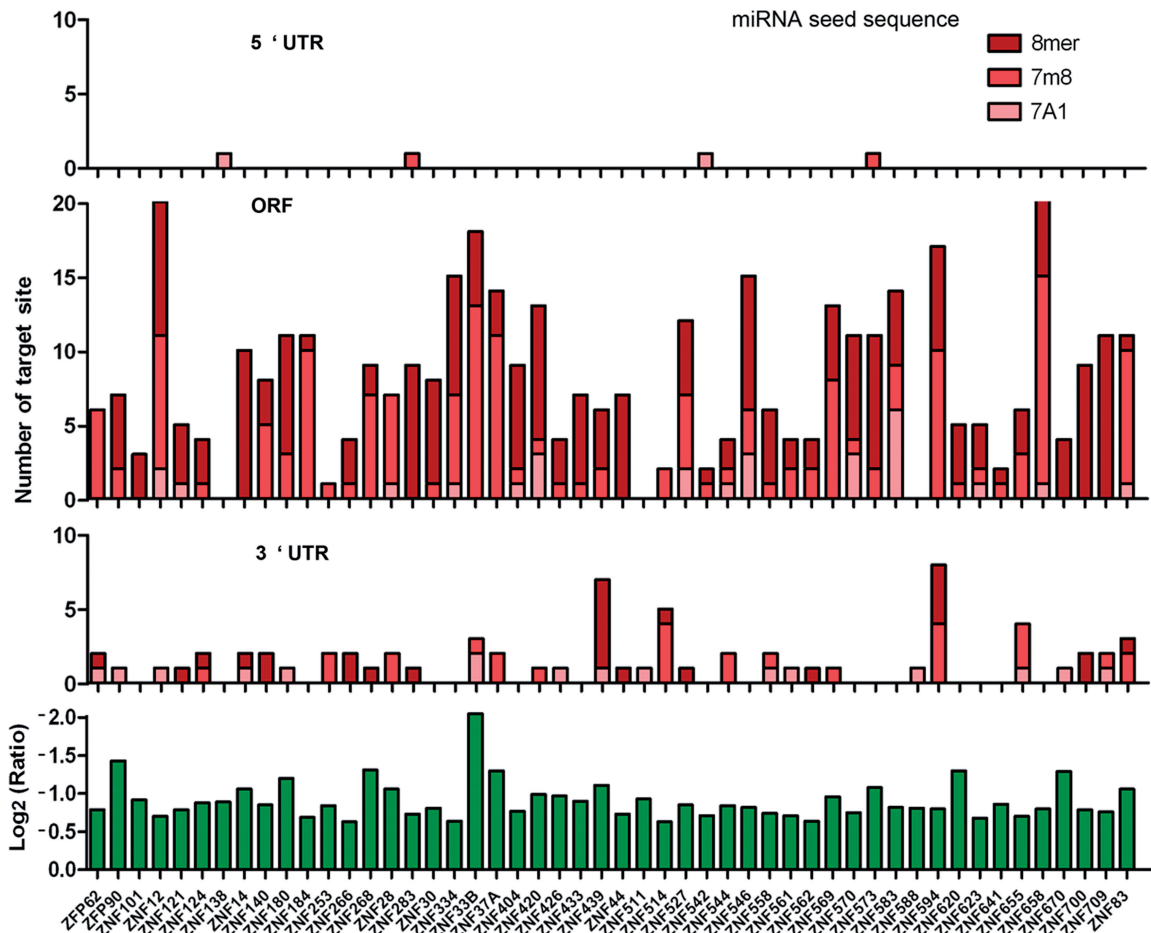

Figure 1. Distribution of miR-181a seed-matched sites in the ZNFs downregulated by miR-181a. Top: the number of miR-181a seed-matched sites within the transcripts of these ZNFs (including the 5′UTR, ORF and 3′UTR). MiR-181a seed-matched sequences are classified as 8-mer (TGAATGTA), 7-mer-m8 (TGAATGT, 7m8) and 7-mer-A1 (GAATGTA, 7A1). Note: ZNF658 has 14 8-mer-matched sites that are not fully shown in the figure. Bottom: quantitation of mRNA levels of ZNFs in miR-181a-transfused HepG2 cells relative to control cells according to the mRNA profile. The values were converted to the Log2 ratio of miR-181a versus control.
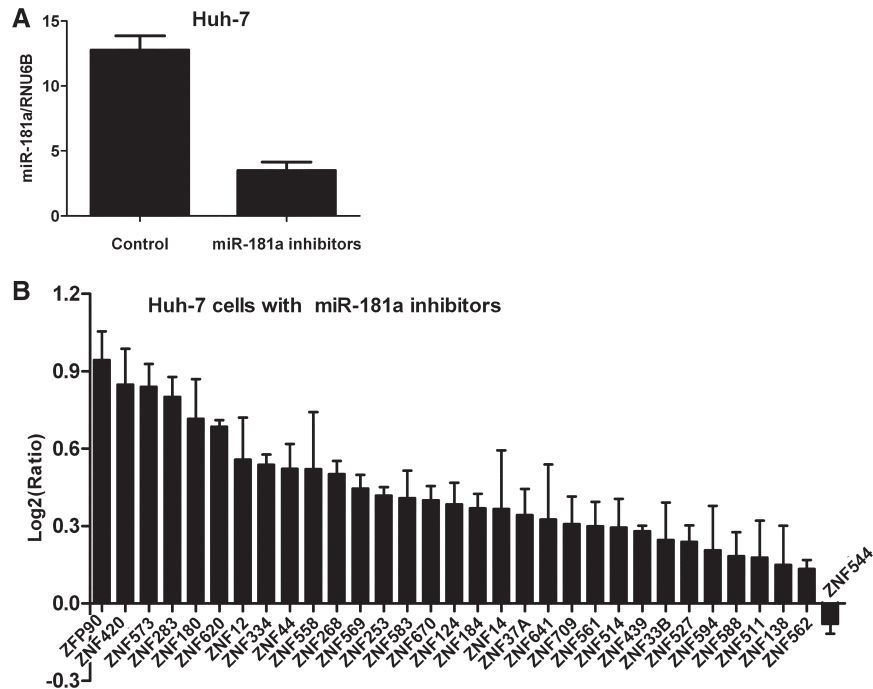
Figure 2. Inhibition of miR-181a increases the expression levels of the ZNF genes in Huh-7 cells. (**A**) The relative expression of mature miR-181a was determined by the ABI TaqMan miRNA assay in Huh-7 cells transfected with either miR-181a inhibitors or negative control. U6 snRNA served as an internal control. (**B**) The relative expression of the ZNF genes was determined by SYBR real-time PCR in Huh-7 cells transfected with either miR-181a inhibitors or negative control. The β-actin served as an internal control. The values were converted to the Log2 ratio of miR-181a inhibitors versus control. Results are representatives of three independent experiments. Error bars represent the SEM.

and 7-mer-A1 (GAATGTA, 7A1). Interestingly, there are many more miR-181a target sites in the open reading frame (ORF) of these genes than in the 5′ or 3′UTR ($P < 0.0001$, Mann–Whitney test) (Figure 1 and Supplementary Table S1). This finding suggested that miR-181a might target these ZNFs by directly recognising the seed-matched sequences within their coding regions. Furthermore, we examined the entire CDS/UTR matched to the miR-181a seed region within the ZNF genes and found that the coding regions of more than half of the ZNFs (378/707) contained at least one miR-181a seed-matched site, with 184 ZNFs containing miR-181a 8-mer-matched sequences, 303 ZNFs containing 7m8-matched sequences and 93 ZNFs containing 7A1-matched sequences (Figure 3A and Supplementary Table S2). The number of target sites was also significantly larger in the CDS than in the 5′ or 3′UTR ($P < 0.0001$). To rule out the possibility that differences in CDS or UTR length caused this discrepancy, the number of target sites was further normalised by the length. (The details of CDS and UTR lengths are provided in Supplementary Table 2.) This analysis revealed that the difference in localization was still statistically significant (number of normalized target sites in CDS > 5′ or 3′UTR, $P < 0.0001$). Notably, 8-mer-matched sequences were mostly located in the regions coding for C2H2 domains, particularly in the nucleotides encoding first cysteine residue (Figure 3B). The 8-mer-matched sequence encodes Glutamic acid (GAA) and Cysteine (TGT). In that case, such ZNF domain does not exist in all the members of ZNF family as the Glutamic acid (E) does not necessarily belong to be part of
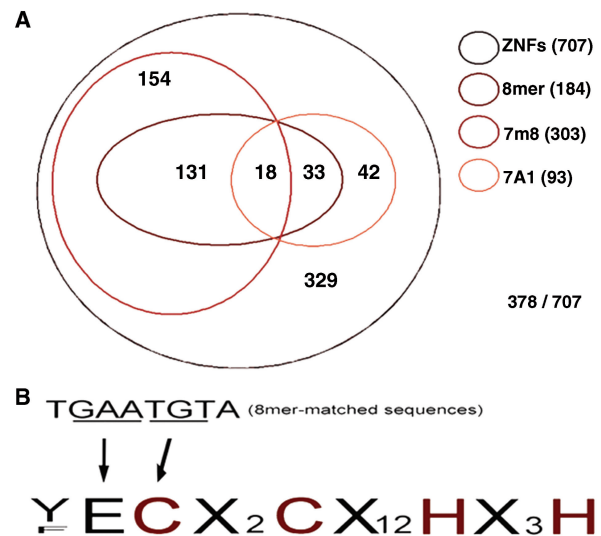

Figure 3. The miR-181a seed-matched sites in the coding regions of ZNFs. (**A**) The number of miR-181a seed-matched sites within the coding regions of 709 ZNFs. MiR-181a seed-matched sequences are classified as 8-mer (TGAATGTA), 7-mer-m8 (TGAATGT, 7m8) and 7-mer-A1 (GAATGTA, 7A1). (**B**) MiR-181a 8-mer-matched sites in ZNFs were mostly located in the regions coding for the C2H2 domain. The sequence GAA encodes glutamic acid (E), and TGT encodes cysteine (C). Y is tyrosine, F is phenylalanine, H is histidine and X is any amino acid.

the C2H2 domain. However, this domain is consistent in the ZNF family genes that potentially targeted by miR-181a.

To determine whether miR-181a could directly target ZNFs within their CDS, we first created several constructs

that express wild-type ZNFs (ZNF83, ZNF37A and ZNF180) tagged with HA, These constructs were co-transfected with a miR-181a expression vector or mature miR-181a mimics into HEK 293T cells. Immuno-blot analysis of HA showed a marked reduction in HA-ZNF expression in cells expressing miR-181a but not of co-transfected HA-RhoGDIA (no miR-181a seed-matched sites) (Figure 4A). No significant changes were found in cells transfected with the negative controls (empty vector, Ser control oligomer or miR-151 non-targeting miRNA) (Figure 4A). Because miR-181a did not significantly affect HEK 293T cell growth in these experiments (Supplementary Figure S3), we ruled out the possibility that the observed suppression was due to decreased cell proliferation.

In addition, we cloned full-length CDS fragments of ZNF genes (ZNF37A, ZNF83 and ZNF180) into an expression plasmid immediately downstream of sequences encoding a luciferase reporter gene. For luciferase activity assays, HEK 293T cells were co-transfected with miR-181a, miR-151 or Scr, with the luciferase reporter constructs above and with the 'Renilla' luciferase reporter plasmid (the internal control). In these experiments, miR-181a dramatically reduced the relative luciferase activities of the luciferase-ZNF reporters, whereas miR-151 and Scr did not have significant effects (Figure 4B). Taken together, these results indicate that miR-181a directly targets ZNFs within their CDS.

To better understand the potential role of miR-181a in the regulation of the ZNF CDS, we further created different length constructs of the ZNF37A CDS tagged with HA. The ZNF37A CDS contains three 8-mer-matched sites and eleven 7m8-matched sites with ten C2H2 domains. Our constructs contained varying numbers of miR-181a seed-matched sites and were denoted as p-HA-1686, p-HA-1500, p-HA-1350, p-HA-1260,

p-HA-1200, p-HA-900, p-HA-801 and p-HA-1201-1686 (Figure 5A). Translational fusion products of the appropriate size were detected by immuno-blot analysis (Figure 5B). Further, each construct was co-transfected with miR-181a expression vector or control vector into HEK 293T cells. As shown in Figure 5C, miR-181a significantly downregulated the expression of the p-HA-1686, p-HA-1500, p-HA-1350, p-HA-1260 and p-HA-1201-1686 constructs. This result suggests that 8-mer-matched sites have the major contributions to the effect of miR-181a on the expression of ZNF37A CDS, although the expression of the constructs containing 7m8-matched sites can also be downregulated by miR-181a despite of the mild inhibitory effect. We proposed that the effects of miR-181a on ZNF37A expression are largely mediated by the 8-mer-matched sites in the CDS of ZNF37A. Notably, the inhibitory effect became attenuated when the number of target sites decreased, indicating that suppression of ZNF expression by miR-181a might be due to the multiple target sites in the ZNF CDS. Furthermore, we used site-directed mutagenesis to create several variants of the ZNF37A construct in which the miR-181a target sites (three 8-mer sites) are mutated, while leaving the ZNF37A amino acid sequence intact ('Experimental Procedures' section). In experiments using these constructs, wild-type ZNF37A was strongly downregulated by miR-181a (Figure 5D, lane 2), whereas miR-181a had a minimal effect on the expression of ZNF37A when all three 8-mer sites were mutated (Figure 5D, lane 10). Notably, miR-181a still downregulated the expression of the variants with single 8-mer site mutations (Figure 5D, lanes 4, 6 and 8) but to a lesser extent than wild-type ZNF37A was silenced. We also synthesized a miRNA mimic containing a mutated seed region which matched the mutation in the ZNF37A CDS constructs ('Experimental Procedures' section).
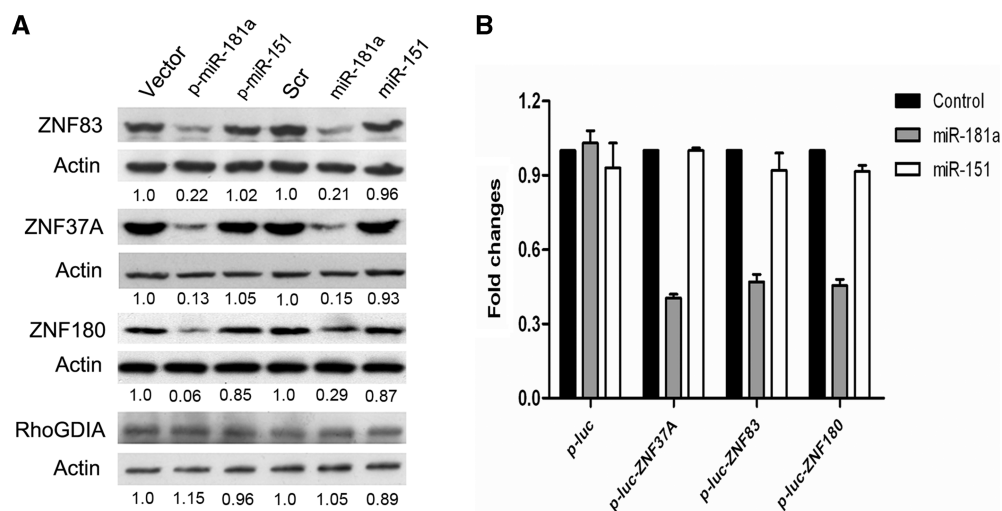

Figure 4. MiR-181a directly targets ZNFs within the CDS as determined by HA-tagged construct and luciferase reporter assays. (**A**) Vectors encoding the wild-type CDS of ZNF83, ZNF37A, ZNF180 and RhoGDI tagged with HA were constructed and co-transfected with pri-miR-181a/151 vectors or mature miR-181a/151 mimics into HEK 293T cells. After 48 h, protein was extracted and probed with HA antibody. (**B**) MiR-181a inhibits luciferase activity when the ZNF CDS region is located in the luciferase gene 3′UTR. HEK 293T cells were seeded in 96-well plates and co-transfected with pWPT-GFP or pWPT-miR-181a, luciferase reporter (p-luc, p-luc-ZNF37A, p-luc-ZNF83 or p-luc-ZNF180) and 'Renilla' pRL-CMV internal control plasmid using Lipofectamine 2000. Luciferase activity was measured by the dual-luciferase reporter assay system after 48 h transfection. Results are representatives of three independent experiments. Error bars represent the SEM.
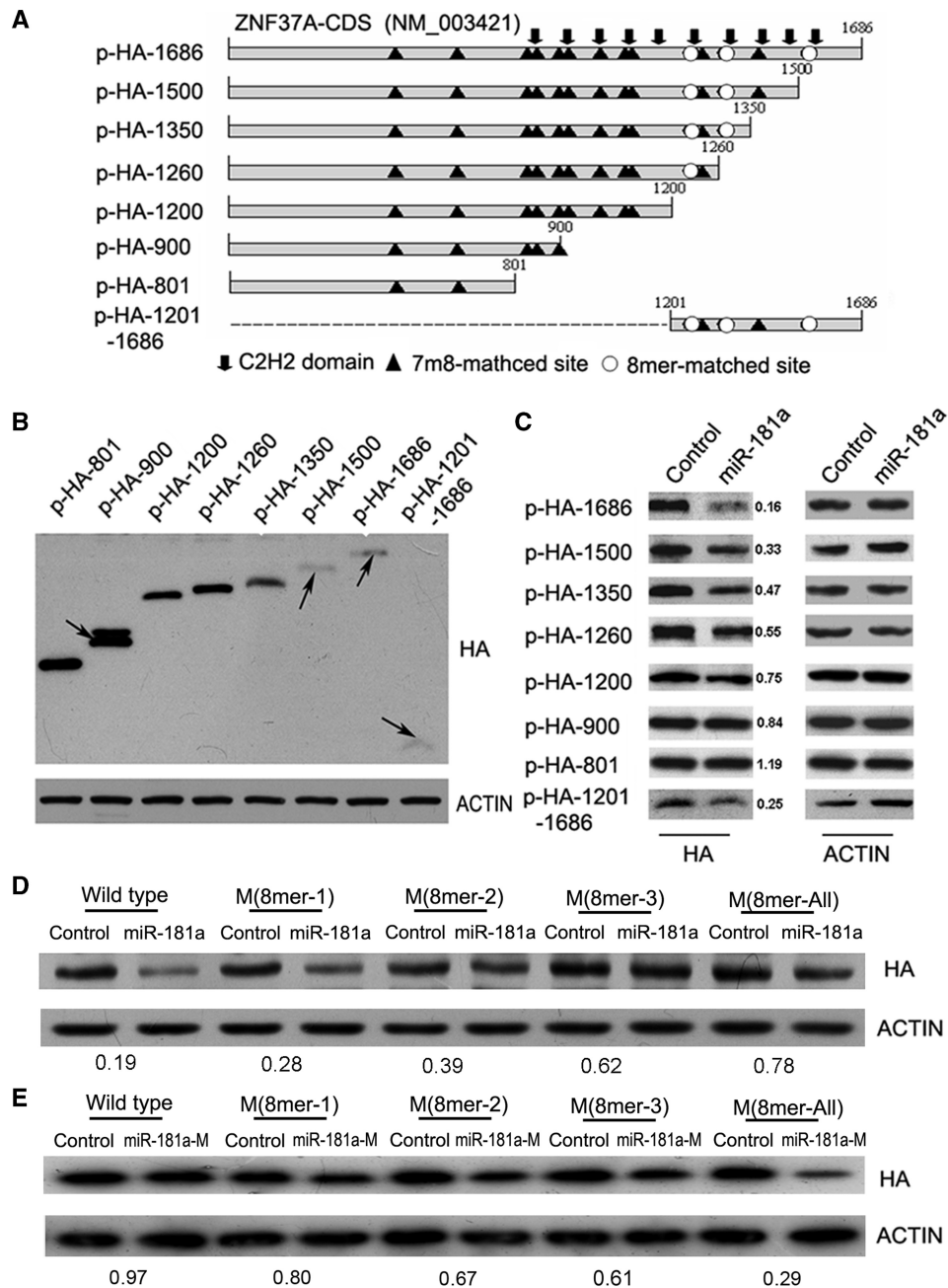
Figure 5. The effects of miR-181a on the expression of ZNF37A CDS variants. (**A**) Schematic diagram of various ZNF37A CDS constructs tagged with HA. (**B–E**) Western-blot analysis of HA-tagged protein expression in HEK 293T cells transfected with (B) various ZNF37A CDS fragments; (C) either pWPT-GFP or pWPT-miR-181a and various fragments of the ZNF37A CDS; (D) either control or miR-181a mimics and mutated variants of the ZNF37A CDS; and (E) either control or miR-181a mutated mimics and mutated variants of the ZNF37A CDS. β-actin was used as an internal control. Quantification was performed with Image-Pro Plus software.

As shown in Figure 5E, co-transfecting the mutated ZNF constructs with the mutated miRNA mimic restored repression. These findings further confirm that miR-181a directly targets ZNF genes within their CDS.

DISCUSSION

In the present study, we have demonstrated for the first time that a single miRNA can target a large number of genes within a specific family by targeting their coding regions. Most ZNFs include multiple miR-181a target sites within their CDS, especially in sequences coding for the C2H2 domain. Our findings show that miR-181a downregulates the expression of many ZNFs by directly targeting these coding regions.

The localization of most miRNA target sites was originally found to be in the non-coding regions (3'UTR) of mRNAs in animals. However, some recent reports have shown that miRNAs can also target the coding regions of mammalian genes. Duursma *et al.* (7) reported that

miR-148 targets the human DNMT3b protein-coding region, which has the potential to interact with all 22 nt of miR-148 (leaving only one gap), therefore resembling the miRNA-target interactions observed in plants. In a computational screen for highly conserved motifs within coding regions, Forman *et al.* (8) experimentally demonstrated that let-7 miRNA directly targets the miRNA-processing enzyme, Dicer, within its CD. By using rna22 miRNA prediction programs, Tay *et al.* (9) demonstrated the existence of many naturally occurring miRNA target sites within the CDS regions of the mouse Nanog, Oct4 and Sox2 genes. In this study, we found that miR-181a targets the CDS of a number of ZNF genes, which provides new evidence that miRNAs might target specific gene families within their coding regions. These findings also support the notion that the CDS are nearly optimal for providing additional information in parallel to the genetic code itself (26). Analysing ZNF mRNA sequences, we found that there are many miR-181a seed-matched sites within their coding regions. This result might indicate a characteristic of ZNF sequences, which contain many ZNF domains composed of multiple cysteines. TGT, the codon for cysteine, is complementary to part of the miR-181a seed sequence. The multiple target sites could have synergistic effects on the regulation of ZNF expression by miR-181a.

MiR-181a has been described as a key modulator of cellular differentiation, including during hematopoietic lineage differentiation (21), myoblast differentiation (22) and T-cell sensitivity and selection (23). More recently, miR-181a was reported to have a potentially important role in maintaining the stem cell phenotype of liver cancer cells (27). In these previous reports, some functional targets of miR-181a had been elucidated, but the mechanism of silencing remained unclear. Meanwhile, accumulating evidence has proven that ZNFs play crucial roles in multiple physiological and pathological processes (14–20), although the exact function and molecular mechanism of most ZNFs have not been well established. Thus, we postulate that these ZNFs that are directly downregulated by miR-181a might have roles in mediating the function of miR-181a in development and disease. This idea remains to be further explored.

In conclusion, our study presents the first evidence that ZNFs are regulated at the post-transcriptional level by a non-coding miRNA, miR-181a, which directly targets ZNFs within their coding regions. This finding provides an important and novel perspective for decoding the complex mechanism of miRNA/mRNA interplay.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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