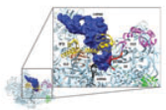
RNA Biology



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TECHNICAL PAPER

Comparing two approaches of miR-34a target identification, biotinylated-miRNA pulldown vs miRNA overexpression

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ABSTRACT

microRNAs (miRNAs) are critical regulators of gene expression. For elucidating functional roles of miRNAs, it is critical to identify their direct targets. There are debates about whether pulldown of biotinylated miRNA mimics can be used to identify miRNA targets or not. Here we show that biotin-labelled miR-34a can be loaded to AGO2, and AGO2 immunoprecipitation can pulldown biotinylated miR-34a (Bio-miR pulldown). RNA-sequencing (RNA-seq) of the Bio-miR pulldown RNAs efficiently identified miR-34a mRNA targets, which could be verified with luciferase assays. In contrast to the approach of Bio-miR pulldown, RNA-seq of miR-34a overexpression samples had limited value in identifying direct targets of miR-34a. It seems that pulldown of 3′-Biotin-tagged miRNA can identify *bona fide* microRNA targets at least for miR-34a.

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Biotinylated miRNA; Argonaut 2; microRNA targets; RNA pull down; RNA-seq

Introduction

microRNAs (miRNAs) are class of small noncoding RNAs (ncRNAs) of ~22 nt length and by far most well studied class of ncRNAs.[1,2] It is estimated that miRNAs comprise up to 0.02% of total cellular RNA, and regulate more than 60% of coding genes post transcriptionally.[3-5] Each miRNA generally regulates the expression of target genes by binding to their 3′ untranslated region (UTR), thereby repressing gene expression by mRNA degradation and/or translation inhibition.[1,6,7] Target recognition takes place when miRNA associates with Argonaute2 (AGO2), which is the core component of RNA induced silencing complex (RISC).[1] AGO2 is highly conserved and exhibits slicing activity on mRNAs or noncoding RNAs targeted by miRNAs or siRNAs.[8,9] Formation of AGO2-miRNA complex depends upon the recognition of 5′ and 3′ ends of miRNA by MID and PAZ domains of AGO2 respectively.[10]

miRNA target identification is important in understanding its biological function. *In silico* tools are helpful in predicting miRNA targets. These tools rely on exact base pairing of seed region (nucleotides 2-9) of miRNA and binding site in 3′ UTR of mRNA. Many experimentally verified miRNA-mRNA interactions, however, do not follow *in silico* rules, since imperfect seed match between miRNA and target mRNA, and targeting to sites outside of the 3′ UTR can also exist.[11-15] As a result, computational tools predict hundreds of miRNA targets that are often biologically irrelevant since false positives of computational prediction tools are estimated to be up to 70%.[6,16-18] Therefore, in addition to *in silico* methods, high throughput experimental methods have to be developed.

Multiple experimental approaches are now available to determine genuine miRNA targets, each having its own merits and demerits.[18] Overexpression of miRNA by means of expression vectors or use of synthetic miRNA mimics followed by high-throughput analysis of change in gene expression either by microarray of mRNAs or RNA-Sequencing (RNA-seq) can give a direct assessment of target genes.[19] However, these overexpression approaches have two limitations. The first is the inability to distinguish between direct and indirect targets.[20] Secondly, gene regulation at the level of translation occurring without substantial change in the mRNA levels will not be identified.[18] Affinity purification, another method to identify miRNA targets, has been used in many studies, where synthetic miRNA duplexes typically biotinylated at the 3′ end of the guide strand are used.[21-27] The duplexes are transfected into appropriate cell type, where biotinylated miRNA (Bio-miRNA) presumably gets incorporated into RISC to form miRNA-mRNA complex. Following cell lysis, tagged miRNAs are captured on streptavidin beads followed by mRNA isolation and identification. There are debates about whether pulldown of biotinylated miRNA mimics can be used to identify miRNA targets, and the argument is the presence of biotin moiety at the 3′ terminus of miRNA may hamper its ability to interact with AGO2.[28]

In this study, we showed that biotin-labelled miR-34a could be loaded to AGO2, and conversely immunoprecipitation of AGO2 could pulldown biotinylated miR-34a. By combining the transcriptome profiling after miR-34a overexpression and affinity pulldown of Bio-miR approaches together, we identified direct miR-34a targets with high-confidence.

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Results

Biotinylated miRNA interacted with AGO2

We started with transfection of 3′-biotinlyated-miRNA-34a into cells, and examined whether it could be loaded into RISC or not. Co-immunoprecipitation (Co-IP) of miRNA from cell lysate using anti-AGO2 antibody provides good assessment of miRNA-AGO2 interaction.[28] HEK293T cells were transfected with Bio-miR-34a duplex or a control with scrambled sequences (Bio-miR-Scr) (Fig. 1A). 24 h post transfection, AGO2 complexes were IPed (Fig. 1B). Bio-miR-Scr and Bio-miR-34a were both successfully co-IPed with AGO2 (Fig. 1C), indicating that biotinylated miRNAs were loaded into RISC and interacted with the core protein.

In a reciprocal experiment, cell lysates were subjected to pulldown of biotinylated-RNA with streptavidin beads (Bio-miR pulldown) (Fig. 2A). Two bands could be detected when bio-miR-34a was transfected. One possibility could be that one nucleotide at the 3′ end maybe, since biotin was labelled at the 5′ end, of a small portion of the transfected bio-miR-34a was somehow removed inside cells. AGO2 protein was co-pulled down with either Bio-miR-34a or Bio-miR-Scr (Fig. 2B). These results together demonstrated that biotinylated miRNA could interact with AGO2, and highly possible got loaded in the RISC.

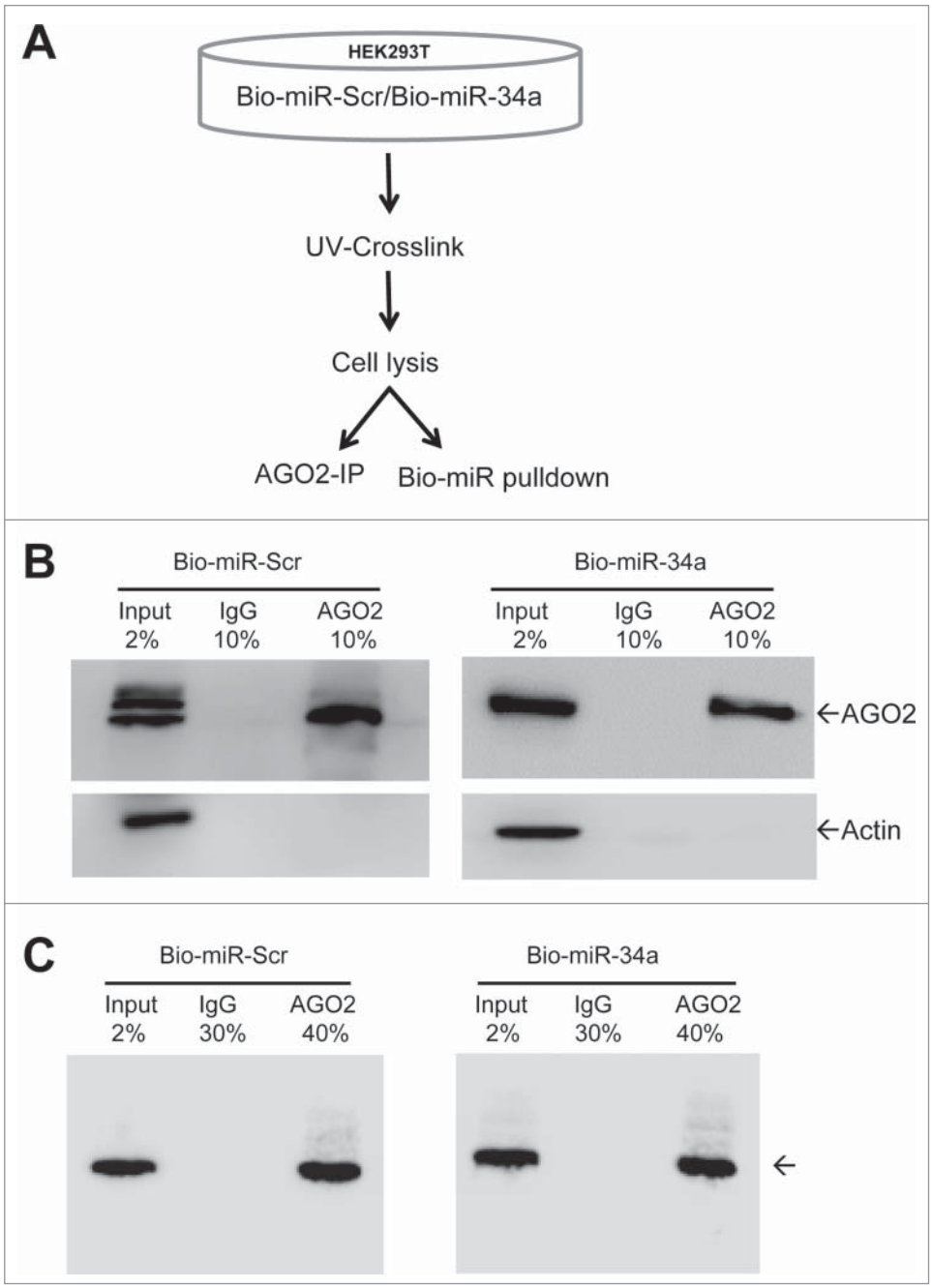

Figure 1. Biotin-labelled miRNA co-immunoprecipitates with AGO2: (A) Schematic of AGO2 immunoprecipitation (IP) and biotin-labelled miRNA detection. (B) AGO2 IP of Bio-miR-Scr and Bio-miR-34a transfected HEK293T cells. Western blots of AGO2 showing successful IP, and Actin was used as a negative control. (C) Bio-miR-Scr and Bio-miR-34a both co-IPed with AGO2 but not with IgG. Arrow points towards biotinylated miRNA (bands were probed with streptavidin-HRP).

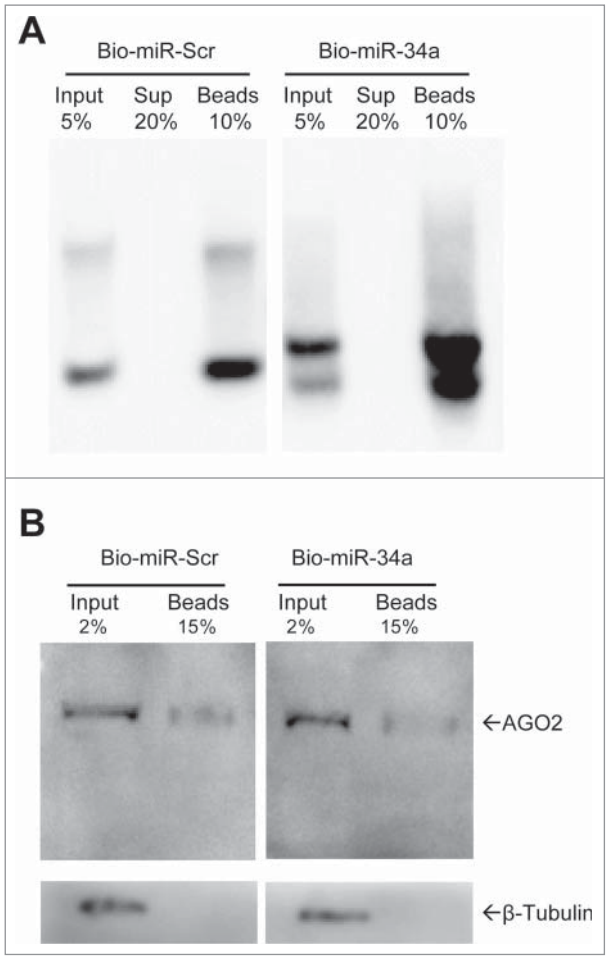

Figure 2. AGO2 co-immunoprecipitates with biotin-labelled miRNA: (A) RNA pulldown of Bio-miR-Scr and Bio-miR-34a in HEK293T cells. Bands were probed with streptavidin-HRP. (B) AGO2 co-pulled down with both Bio-miR-Scr and Bio-miR-34a. Western blots of AGO2 were performed, and β-tubulin was used as a negative control.

RNA sequencing of Bio-miR pulldown and miR-34a overexpression samples

Next, Bio-miR-Scr and Bio-miR-34a duplexes were transfected in HEK293T cells (Fig. 3A). Total RNAs were extracted and prepared for RNA sequencing (RNA-seq). At the same time, cell lysates were prepared and subjected to Bio-miR pulldown. RNAs pulled down were also sequenced. Analyses of Bio-miR pulldown reads identified 9290 mRNAs, 473 lncRNAs and 419 circRNAs (count backsplicing junctions,[29,30]) (Fig. 3B). There were 633 mRNAs and 29 lncRNAs enriched in Bio-miR-34a pulldown compared to RNAs pulldown with biotinylated control of scrambled sequences (Bio-miR-Scr) (cutoff of two-fold enrichment and p value less than 0.05) (Fig. 3C). It seems that the pulldown & RNA-seq method can get substantial number of nonspecific targets, and really requires normalization with scramble control. 110 mRNAs enriched in the Bio-miR-34a pulldown harbored 3′ UTR match with miR-34a seed region (Fig. 3C, Table S1). On the other hand, RNA-seq data from mir-34a overexpression revealed that 853 mRNAs and 103 lncRNAs were significantly downregulated (cutoff of two-fold decrease and p value less than 0.05) (Fig. 3D). Not much overlap was present between RNAs from Bio-miR pulldown and miR-34a overexpression (Fig. 3E). All the overlapped 22 mRNAs have miR-34a seed sequence in the 3′ UTR. In the 853 mRNAs significantly suppressed by miR-34a overexpression, 139 had miR-34a guide strand seed match in their 3′ UTR (Table S2); 64 of them had match in their 3′ UTR to the seed region of miR-34a passenger strand (Fig. 3F). These 185 mRNAs (some with 3′ UTR seed matches to both guide and passenger strand) were potentially direct mRNA targets identified by miR-34a overexpression. Very small number of overlaps were identified between Bio-miR-34a pulldown targets and miR-34a overexpression targets (Fig. 3F, G). Interestingly, Bio-miR-34a pulldown targets still showed significantly decreased levels in miR-34a overexpression (Fig. 3H).

Validation of potential miR-34a targets

To verify if the pulled down candidates were true miR-34a targets, fourteen targets were randomly selected for luciferase reporter assay, from the list of pulled down targets with cutoff of two-fold enrichment and p value less than 0.05 (Table S1). Compared to control group, reduction in the luciferase activity was observed in 11 targets (Fig. 4A). Next, randomly chosen nine targets from miR-34a overexpression data (cutoff of two-fold decrease and p value less than 0.05) were subjected to luciferase assay (Table S2). Eight of them showed significant reduction in the luciferase activity as compared to control group (Fig. 4B). Relative luciferase activity of 3 out of randomly selected 4 targets, which were common in both pulldown and overexpression data, showed reduction in luciferase activity (Fig. 4C). This data suggested that most miR-34a 3′ UTR seed bearing targets identified by either Bio-miR-34a pulldown or miR-34a overexpression were genuine miR-34a targets.

Discussion

miRNAs are known to play pivotal roles in myriad of cellular functions ranging from development to disease and response to endogenous or exogenous stimuli.[31-35] To assess the involvement of a miRNA in a biological process, computational methods and molecular biology approaches are routinely used. Computational methods generally rely on phylogenetically conserved miRNA binding sites in the target gene and free energy of binding.[36] A limitation to this method is high false positive rates, making such predictions biologically extraneous. As an example, p21Cip1/Waf1, whose function is to regulate cell cycle progression at G1 phase, was predicted to be targeted by at least 266 miRNAs. Experimentally, however, only 28 miRNAs significantly reduced the luciferase activity in cells stably expressing luciferase-3′-UTR reporter gene.[37] Similarly, miR-3648 targeted only one gene in contrast to 13 predicted targets.[34] Therefore, experimental approach to identify true miRNA targets is indispensable. Biochemical approaches such as AGO2-IP, high-throughput sequencing coupled with UV cross-linking and immunoprecipitation (HITS-CLIP), photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP), 3′ end biotin-tagged miRNA, miRNP immunopurification, or a recently developed screening system to identify miRNA targets are available.[18,38,39] Among these methods, 3′ end biotin-tagged miRNA based approach is being used in

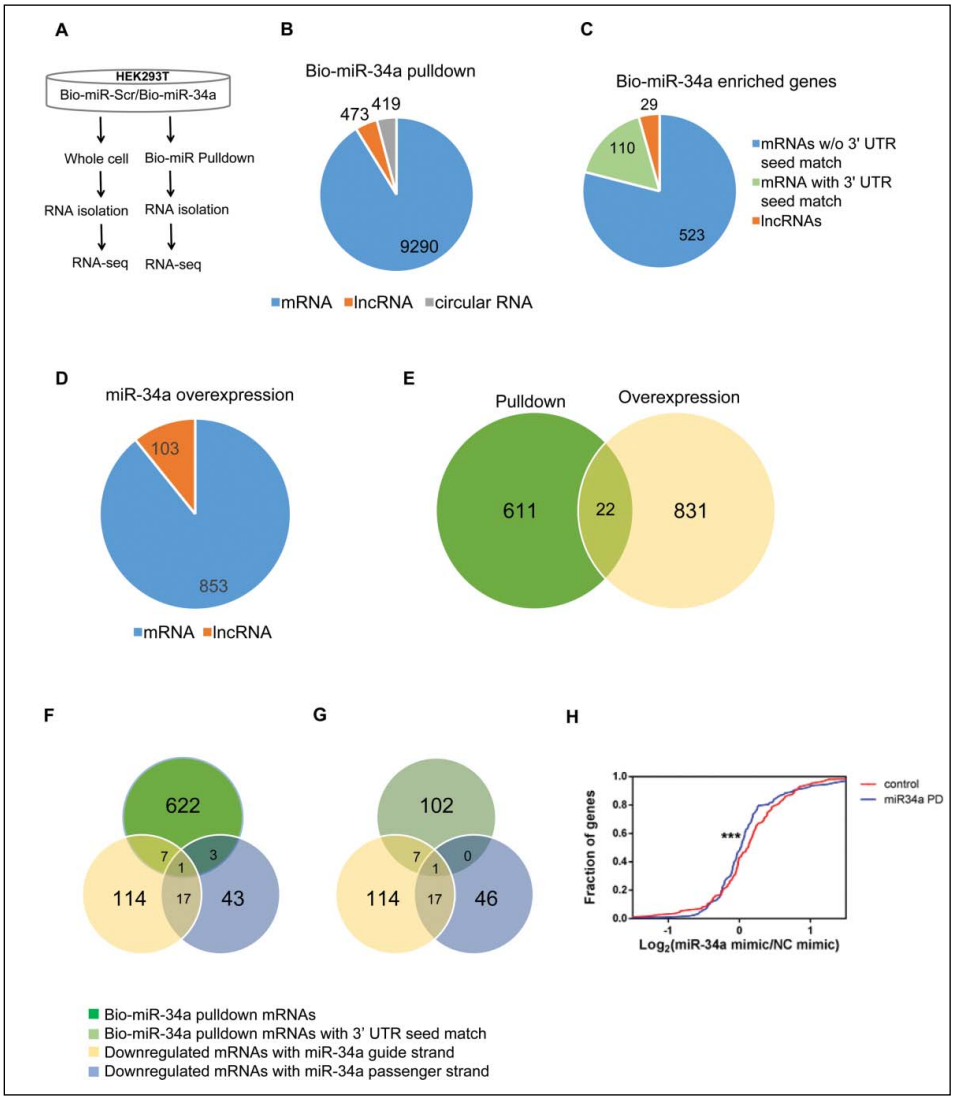

Figure 3. RNA-Sequencing data analyses: (A) Schematic of sample preparation for RNA-seq. (B) Total number of pulled down mRNAs and lncRNAs from Bio-miR-34a pull down and RNA-seq. (C) Number of pulled down mRNAs and lncRNAs after normalizing with Bio-miR-scramble pulldown. (D) Number of mRNAs and lncRNAs downregulated after miR-34a overexpression. (E) Comparison of pulled down and downregulated mRNAs in search of common targets. (F) Comparison of pulled down targets with downregulated mRNAs due to guide strand (miR-34a-5p) and passenger strand (miR-34a-3p). (G) Pulled down targets with 3′ UTR seed match compared with downregulated mRNAs due to guide strand (miR-34a-5p) and passenger strand (miR-34a-3p). (H) Cumulative distribution plots for Bio- miR-34a pull down targets ($n = 139$, $p < 0.05$) and scramble control pull down targets (randomly chosen 103 genes, $p > 0.05$) showing percentage of genes with average $\log_2$ (miR-34a mimic/scramble control) indicated on the x-axis.

many studies since target affinity between two miRNAs, with even a single nucleotide difference, can be measured.[40]

In this study we have shown biotin-tagged miRNA duplex pull down method worked well, at least for miR-34a, since both Bio-miR-34a and Bio-miR-Scr was co-IPed with AGO2 (Fig. 1). Similarly, pulldown of biotin labelled miR-34a and scramble control also pulled down AGO2 (Fig. 2). To rule out the possibility of detecting endogenous miRNA, biotin detection kit was used to specifically visualize miRNAs labelled with biotin. On the contrary, some studies argued that biotin moiety when attached to the 3′ end of the miRNA impede its association with AGO2.[41] Hence, biotin-labelled miRNA was not co-IPed with AGO2.[28] The negative result could be due to low transfection efficiency or post-translational modification of AGO2 that can hamper miRNA-AGO2 interaction.[42] Another possible justification could be sequence- or context-specific reasons since pulldown did not worked well for miR-21 in some cell lines.[43]

Furthermore, in this study, we tried to combine two approaches together; biotin-labelled miR-34a pulldown and overexpression followed by transcriptome profiling. RNA-seq data followed by luciferase reporter assay showed most of the randomly selected pulldown enriched and downregulated genes when miR-34a was overexpressed were true targets (Fig. 4). However, the number of common targets between pulldown enriched targets and downregulated genes after miR-34a overexpression were very small (Fig. 3), likely due to different ranking of target genes after computational analysis and/or effect of miRNA on protein synthesis rather than affecting mRNA levels. Since microRNAs may directly or indirectly affect the activity of multiple transcription factors, which in turn can have profound effects on transcription that are not the direct result of microRNA interaction with mRNA targets, such indirect influences by miR-34a overexpression should also be considered. All 22 mRNAs overlapped targets by the two methods have miR-34a 3′

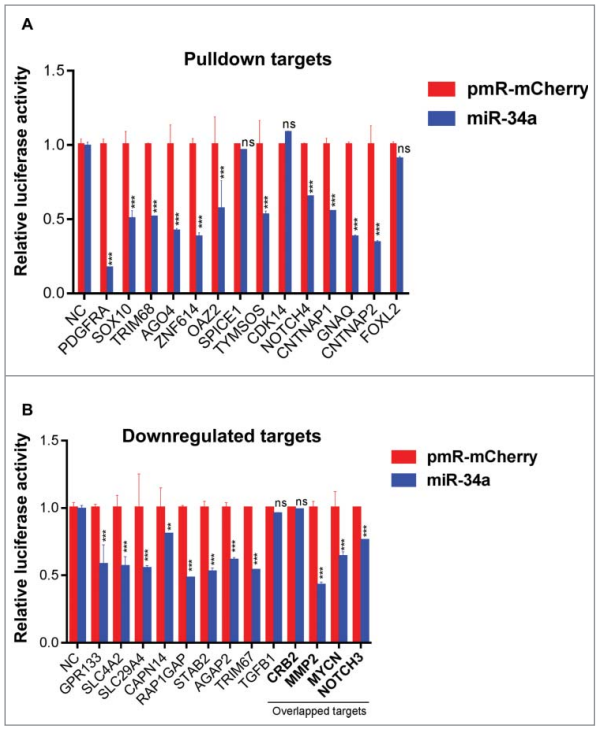

Figure 4. Validation of potential miR-34a targets: Firefly luciferase activity of; (A) randomly chosen pulldown targets, (B) randomly chosen downregulated mRNAs in RNA-seq data after miR-34a overexpression and, (C) overlapped targets, in HEK293T cells relative to Renilla luciferase. Data represents values from triplicates. P values were generated using two-tailed Student's t-test. ns, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars represents S.D.

UTR seed sequence (Fig. 3E), and the combination of the two methods might help to narrow down target candidates.

Collectively, based on our data and a recent study,[26] we can conclude that biotinylated miRNAs can be used to identify miRNA targets. The sensitivity of this method can be increased with the addition of a scramble control mimic. Both methods, however, are not perfect and experimental validation of miRNA targets is indispensable.

Materials and methods

miRNA mimic synthesis

Chemically modified miR-34a mimic and scramble control duplexes were chemically synthesized from Bioneer. Sequence given in Table S3.

Cell culture and transfection

HEK293T were cultured in DMEM (Hyclone) with 10% FBS. Cells were cultured at 37 °C in a humidified 5% (v/v) CO_2 incubator. Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Cell lysis for IP and bio-miR pulldown

Cells were washed with 1X ice-cold PBS and cross-linked in a UV cross-linker at 1200 mJ/cm2 for 2 min. Cells were lysed in the lysis buffer [0.02 M Tris-HCl (pH 7.5), 0.1 M KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5% NP-40, 60 U/ml RNase inhibitor (Promega), 1X protease inhibitor EDTA-free (Sigma, 05892791001)] for 20 min after which lysates were sonicated for 2 min at 20% amplitude. Lysates were cleared off cell debris by centrifugation at 13000 rpm for 10 min. 10% lysate was saved as input (5% for RNA and 5% for protein detection) while remaining lysate was incubated with respective magnetic beads for IP or bio-miR pulldown.

Immunoprecipitation

60 μl of Dynabeads Protein G beads (Life Technologies, 10004D) per 10 cm plate were washed three times with lysis buffer and incubated with 6 μg of rat-anti-AGO2 monoclonal antibody (Sigma-Aldrich, SAB4200085) or rat-normal-IgG antibody (Santa Cruz, sc-2026) in 10 μg/ml RNase-free BSA for 30 min at room temperature. Blocked beads were washed three times with lysis buffer and cell lysate was incubated for 2 h at room temperature. Also beads-only pulldown experiments were performed, and no AGO2 protein could be detected in the pulldown materials.

Western blot

Proteins either from cell lysate or isolated from IP beads, were separated on SDS–PAGE gels and then transferred to PVDF membranes (Millipore). Membranes were processed according to the ECL western blotting protocol (GE Healthcare). The following antibodies were used in western blots: rat-antiAGO2 primary antibody (Sigma-Aldrich, SAB4200085), anti-actin (Signalway antibody, 21338) and anti-tubulin (Transgen, HC101).

Biotinylated miRNA pulldown

Final concentration of Bio-miR-34a or Bio-miR-Scr was 20 μM with standard protocol of RNA oligos (Lipofectamine 2000, Invitrogen). Transfection was done for 24 hours. For a 10 cm plate, 20 μl Dynabeads M-280 Streptavidin beads (Life Technologies, 11206D) were activated according to manufacturer's protocol. The beads were blocked with 10 μg/ml RNase-free BSA and yeast tRNA (Sigma-Aldrich, R8508) for 30 min at 4 °C. After washing the beads with lysis buffer, lysates as prepared in "Cell lysis for IP and bio-miR pulldown" were incubated with blocked beads and incubated at room temperature for 2 h followed by RNA extraction.

RNA isolation

RNAs from either whole cells or pulldown materials were isolated with Trizol reagent (Invitrogen, 15596026) according to manufacturer's instructions, then treated with RNase-free DNase I (Promega, M6101) and extracted with ethanol precipitation.

Detection of biotinylated miRNA on membrane

RNA samples were separated on a 10% Urea-PAGE gel and electrophoretically transferred onto Hybond^{TM}-N+ membrane (GE Healthcare). Biotinylated miRNA was detected on membrane using Chemiluminescent Biotin-labeled Nucleic Acid

Detection Kit (Beyotime, D3308) following the manufacturer's protocol. Membrane was visualized using ImageQuant LAS4000 Biomolecular Imager (GE Healthcare).

RNA-sequencing

For high-throughput sequencing, the RNA enriched from pulldown assay or poly (A)-tailed mRNA was iron-fragmented at 95 °C and then subjected to end repair and 5′-adaptor ligation. The cDNAs, reverse-transcribed with random primers containing 3′ adaptor sequences and randomized hexamers were purified and amplified, and PCR products of 200–500 bp were purified, quantified and stored at −80 °C until sequencing. The libraries were prepared according to the manufacturer's instructions and subjected to 151 nt paired-end sequencing with an Illumina HiSeq 2500 system.

Bioinformatics analyses of RNA-seq data

In pulldown deep sequencing data, 27,380,065 and 39,445,653 reads from the control (Bio-miR-Scr) library and the pulldown (Bio-miR-34a) library were obtained, where 87.3% and 88.6% of the reads were aligned, respectively. In overexpression deep sequencing data, 28,909,157 and 26,871,903 reads from the NC mimic and miR-34a mimic samples were obtained, where 79.05% and 79.77% of the reads were aligned respectively. All the reads were aligned to the reference transcriptome with bowtie2, no mismatch allowed. Differential analysis was performed with edgeR package. Differentially expressed genes were selected by the logFC > 1 and p value <0.05. The statistical significance and p values in Fig. 3H were evaluated by Wilcoxon signed rank test. For circular RNA analysis, only best matched junction reads were considered.[30,44]

Plasmid construction

Plasmids were constructed by restriction digestion (Thermo Scientific) and recombination method (Vazyme C112-01). For the functional analysis of miRNA, partial segments of the mRNA 3′ UTR containing the miRNA binding sequences for respective candidate targets were PCR amplified from cDNA prepared from RNA of HEK293T cells. The PCR product was then sub-cloned into the *XbaI* site downstream of the stop codon in the pGL3-control firefly luciferase reporter vector. miRNA expression plasmids were constructed by inserting DNA fragment containing pre-miR-34a coding sequence between the *HindIII* and *BamHI* sites of pmR-mCherry (Clontech, Mountain View, USA). The correct orientation of 3′ UTR fragments and pre-miRNA coding sequences in plasmid DNA constructs were confirmed by sequencing. Primers used for plasmid construction are given in Table S3.

Plasmid transfection and luciferase activity assay

Plasmid transfection was performed with Lipofectamine 2000 (Invitrogen) according to the supplier's instructions. For luciferase activity assay, cells were transfected in 6-well plates with miRNA expression plasmids (1 μg/well) or pmR-mCherry control plasmids (1 μg/well), pRL-null (Renilla plasmid) (10 ng/well), and the firefly luciferase constructs carrying the corresponding 3′ UTR (1 μg/well). The luciferase activity was measured 42 h post transfection using the Dual Luciferase Reporter assay system (Promega) based on the manufacturer's protocol. Briefly, cells were lysed with passive lysis buffer at room temperature for 15 min. The luciferase assay buffer II was then added, and firefly luciferase (F-luc) activity was immediately read using a Fluoroskan Ascent FL microplate reader (Thermo Scientific, Waltham, MA, USA). Next, Stop & Glo Buffers with Stop & Glo substrates were added and mixed briefly. Renilla luciferase (R-luc) activity was immediately read. F-luc activity was normalized to R-luc activity to account for variation in transfection efficiency.

Statistical analysis

The values reported in this study represents averages of three independent experiments, with error bars showing standard deviation. After analysis of variance by F test, the statistical significance and P values were evaluated by Student's t-test.

Data availability

GEO accession number of RNA-seq data will be available upon the acceptance of the paper.

Disclosure of potential conflicts of interest

The authors declare that there is no conflict of interest.

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References

1. Bartel DP. MicroRNA Target Recognition and Regulatory Functions. Cell. 2009;136(2):215-33. doi:10.1016/j.cell.2009.01.002.
2. Esteller M. Non-coding RNAs in human disease. Nat Rev Genet. 2011;12(12):861-74. doi:10.1038/nrg3074.
3. Bissels U, Wild S, Tomiuk S, Holste A, Hafner M, Tuschl T, Bosio A. Absolute quantification of microRNAs by using a universal reference. RNA. 2009;15(12):2375-84. doi:10.1261/rna.1754109.
4. Palazzo AF, Lee ES. Non-coding RNA: What is functional and what is junk? Front Genet. 2015;6:235. doi:10.3389/fgene.2015.00002.
5. Tang Z, Yang Y, Wang Z, Zhao S, Mu Y, Li K. Integrated analysis of miRNA and mRNA paired expression profiling of prenatal skeletal muscle development in three genotype pigs. Sci Rep. 2015;5:15544. doi:10.1038/srep15544.

6. Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature. 2008;455(7209):64-71. doi:10.1038/nature07242.
7. Djuranovic S, Nahvi A, Green R. miRNA-Mediated Gene Silencing by Translational Repression Followed by mRNA Deadenylation and Decay. Science. 2012;336(6078):237-40. doi:10.1126/science.1215691.
8. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ. Argonaute2 is the catalytic engine of mammalian RNAi. Science. 2004;305(5689):1437-41. doi:10.1126/science.1102513.
9. Huang C, Wang X, Liu X, Cao S, Shan G. RNAi pathway participates into chromosome segregation in mammalian cells. Cell Discov. 2015;1:15029. doi:10.1038/celldisc.2015.29.
10. Kuhn CD, Joshua-Tor L. Eukaryotic Argonautes come into focus. Trends Biochem Sci. 2013;38(5):263-71. doi:10.1016/j.tibs.2013.02.008.
11. Thomas M, Lieberman J, Lal A. Desperately seeking microRNA targets. Nat Struct Mol Biol. 2010;17(10):1169-74. doi:10.1038/nsmb.1921.
12. Pasquinelli AE. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet. 2012;13(4):271-82.
13. Shin C, Nam JW, Farh KKH, Chiang HR, Shkumatava A, Bartel DP. Expanding the microRNA targeting code: functional sites with centered pairing. Mol Cell. 2010;38(6):789-802. doi:10.1016/j.molcel.2010.06.005.
14. Loeb GB, Khan AA, Canner D, Hiatt JB, Shendure J, Darnell RB, Leslie CS, Rudensky AY. Transcriptome-wide miR-155 Binding Map Reveals Widespread Noncanonical MicroRNA Targeting. Mol Cell. 2012;48(5):760-70. doi:10.1016/j.molcel.2012.10.002.
15. Chi SW, Hannon G J, Darnell R B. An alternative mode of microRNA target recognition. Nat Struct Mol Biol. 2012;19(3):321-7. doi:10.1038/nsmb.2230.
16. Pinzón N, Li B, Martinez L, Sergeeva A, Presumey J, Apparailly F, Seitz H. microRNA target prediction programs predict many false positives. Genome Res. 2017;27(2):234-45. doi:10.1101/gr.205146.116.
17. Bentwich I. Prediction and validation of microRNAs and their targets. FEBS Lett. 2005;579(26):5904-10. doi:10.1016/j.febslet.2005.09.040.
18. Thomson DW, Bracken CP, Goodall GJ. Experimental strategies for microRNA target identification. Nucleic Acids Res. 2011;39(16):6845-53. doi:10.1093/nar/gkr330.
19. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castie J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature. 2005;433(7027):769-773. doi:10.1038/nature03315.
20. Thomas M, Liberman J, Lal A. Desperately seeking microRNA targets. Nat Struct Mol Biol. 2010;17(10):1169-1174. doi:10.1038/nsmb.1921.
21. Ørom UA, Lund AH. Isolation of microRNA targets using biotinylated synthetic microRNAs. Methods. 2007;43(2):162-65. doi:10.1016/j.ymeth.2007.04.007.
22. Suh MR, Lee Y, Kim JY, Kim SK, Moon SH, Lee JY, Cha KY, Chung HM, Yoon HS, Moon SY, et al. Human embryonic stem cells express a unique set of microRNAs. Dev Biol. 2004;270(2):488-98. doi:10.1016/j.ydbio.2004.02.019.
23. Lal A, Thomas MP, Altschuler G, Navarro F, O'Day E, Li XL, Concepcion C, Han YC, Thiery J, Rajani DK, et al. Capture of microRNA-bound mRNAs identifies the tumor suppressor miR-34a as a regulator of growth factor signaling. PLoS Genet. 2011;7(11):e1002363. doi:10.1371/journal.pgen.1002363.
24. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. Nature. 2013;495(7441):384-88. doi:10.1038/nature11993.
25. Kim HS, Lee KS, Bae HJ, Eun JW, Shen Q, Park SJ, Shin WC, Yang HD, Park M, Park WS, et al. MicroRNA-31 functions as a tumor suppressor by regulating cell cycle and epithelial-mesenchymal transition regulatory proteins in liver cancer. Oncotarget. 2015;6(10):8089-102. doi:10.18632/oncotarget.3512.
26. Stark MS, Tom LN, Boyle GM, Bonazzi VF, Soyer HP, Herington AC, Pollock PM, Hayward NK. The "Melanoma-enriched" microRNA miR-4731-5p acts as a tumour suppressor. Oncotarget. 2016;7(31):49677-87. doi:10.18632/oncotarget.10109.
27. Li G, Wu X, Qian W, Cai H, Sun X, Zhang W, Tan S, Wu Z, Qian P, Ding K, et al. CCAR1 5′ UTR as a natural miRancer of miR-1254 overrides tamoxifen resistance. Cell Res. 2016;26(6):655-73. doi:10.1038/cr.2016.32.
28. Guo YE, Steitz JA. 3′-Biotin-tagged microRNA-27 does not associate with Argonaute proteins in cells. RNA. 2014;20(7):985-8. doi:10.1261/rna.045054.114.
29. Chen L, Huang C, Wang X, Shan G. Circular RNAs in eukaryotic cells. Curr Genomics. 2015;16(5):312-8. doi:10.2174/1389202916666150707161554.
30. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, Mackowiak SD, Gregersen LH, Munschauer M, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. Nature. 2013;495(7441):333-8. doi:10.1038/nature11928.
31. Meng L, Chen L, Li Z, Wu ZX, Shan G. Roles of MicroRNAs in the Caenorhabditis elegans nervous system. J. Genet Genomics. 2013;40(9):445-52. doi:10.1016/j.jgg.2013.07.002.
32. Liu H, Wang X, Wang HD, Wu J, Ren J, Meng L, Wu Q, Dong H, Wu J, Kao TY, et al. Escherichia coli noncoding RNAs can affect gene expression and physiology of Caenorhabditis elegans. Nat Commun. 2012;3:2071. doi:10.1038/ncomms2071.
33. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: Biomarkers, functions and therapy. Trends Mol Med. 2014;20(8):460-69. doi:10.1016/j.molmed.2014.06.005.
34. Rashid F, Awan HM, Shan A, Chen L, Shan G. Induction of miR-3648 upon ER stress and its regulatory role in cell proliferation. Int J Mol Sci. 2017;18(7):doi:10.3390/ijms18071375. doi:10.3390/ijms18071375.
35. Dai L, Huang C, Chen L, Shan G, Li Z. Altered expression of microRNAs in the response to ER stress. Sci Bull. 2015;60(2):202-9. doi:10.1007/s11434-014-0657-z.
36. Martin HC, Wani S, Steptoe AL, Krishnan K, Nones K, Nourbakhsh E, Vlassov A, Grimmond SM, Cloonan N. Imperfect centered miRNA binding sites are common and can mediate repression of target mRNAs. Genome Biol. 2014;15(3):R51. doi:10.1186/gb-2014-15-3-r51.
37. Wu S, Huang S, Ding J, Zhao Y, Liang L, Liu T, Zhan R, He X. Multiple microRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3′ untranslated region. Oncogene. 2010;29(15):2302-08. doi:10.1038/onc.2010.34.
38. Easow G, Teleman AA, Cohen SM. Isolation of microRNA targets by miRNP immunopurification. RNA. 2007;13(8):1198-204. doi:10.1261/rna.563707.
39. Ito Y, Inoue A, Seers T, Hato Y, Igarashi A, Toyama T, Taganov KD, Boldin MP, Asahara H. Identification of targets of tumor suppressor microRNA-34a using a reporter library system. Proc Natl Acad Sci U S A. 2017;114(15):3927-32. doi:10.1073/pnas.1620019114.
40. Cloonan N, Wani S, Xu Q, Gu J, Lea K, Heater S, Barbacioru C, Steptoe AL, Martin HC, Nourbakhsh E, et al. MicroRNAs and their isomiRs function cooperatively to target common biological pathways. Genome Biol. 2011;12(12):R126. doi:10.1186/gb-2011-12-12-r126.
41. Ma JB, Ye K, Patel DJ. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Nature. 2004;429(6989):318-22. doi:10.1038/nature02519.
42. Meister G. Argonaute proteins: functional insights and emerging roles. Nat Rev Genet. 2013;14(7):447-59. doi:10.1038/nrg3462.
43. Tan SM, Liberman J. Capture and identification of miRNA targets by biotin pulldown and RNA-seq. In: Dassi E, editor. Post-transcriptional gene regulation, 2nd ed. New York: Humana Press; 2016. p. 211-28.
44. Li Z, Huang C, Bao C, Chen L, Lin M, Wang X, Zhong G, Yu B, Hu W, Dail L, et al. Exon-intron circular RNAs regulate transcription in the nucleus. Nat Struct Mol Biol. 2015;22(3):256-64. doi:10.1038/nsmb.2959.