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Research Paper

Identification of Human microRNA Targets From Isolated Argonaute Protein Complexes

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KEY WORDS

RNAi, RNA interference, microRNAs, Argonaute, gene silencing, non-coding RNAs, translation, gene regulation

ABBREVIATIONS

ds	double stranded
nt	nucleotide
Ago	Argonaute proteins
miRNAs	microRNAs
UTR	untranslated region
IRES	internal ribosome entry site

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ABSTRACT

MicroRNAs (miRNAs) constitute a class of small non-coding RNAs that regulate gene expression on the level of translation and/or mRNA stability. Mammalian miRNAs associate with members of the Argonaute (Ago) protein family and bind to partially complementary sequences in the 3' untranslated region (UTR) of specific target mRNAs. Computer algorithms based on factors such as free binding energy or sequence conservation have been used to predict miRNA target mRNAs. Based on such predictions, up to one third of all mammalian mRNAs seem to be under miRNA regulation. However, due to the low degree of complementarity between the miRNA and its target, such computer programs are often imprecise and therefore not very reliable. Here we report the first biochemical identification approach of miRNA targets from human cells. Using highly specific monoclonal antibodies against members of the Ago protein family, we co-immunoprecipitate Ago-bound mRNAs and identify them by cloning. Interestingly, most of the identified targets are also predicted by different computer programs. Moreover, we randomly analyzed six different target candidates and were able to experimentally validate five as miRNA targets. Our data clearly indicate that miRNA targets can be experimentally identified from Ago complexes and therefore provide a new tool to directly analyze miRNA function.

INTRODUCTION

MiRNAs form a highly conserved class of small non-coding RNAs with regulatory functions in processes as diverse as development, cell differentiation or apoptosis.[1-3] MiRNA genes are often organized in genomic clusters, which give rise to one primary transcript containing multiple miRNAs. MiRNAs, however, also derive from one individual transcript or frequently originate from intronic sequences.[1-3] MiRNA genes are transcribed by RNA polymerases II or III to generate primary miRNA transcripts that are poly-adenylated and capped.[4,5] Primary miRNA transcripts are processed by the nuclear microprocessor complex, which contains the RNase III enzyme Drosha and its co-factor DGCR8.[6-8] Drosha produces stem-loop structured miRNA precursors (pre-miRNAs) with characteristic two nucleotide (nt) 3' overhangs.[9] The pre-miRNA hairpin is further transported to the cytoplasm by the export receptor Exportin 5 where it is further processed by the RNase III enzyme Dicer.[3,10,11] Dicer most likely recognizes the two nt overhangs and cleaves 21 nt from the ends to produce a short-lived double stranded (ds) miRNA/miRNA* RNA which is subsequently unwound and only one strand is incorporated into miRNA-protein complexes (miRNPs) and gives rise to the mature miRNA. [3,10,11]

MiRNAs guide members of the Argonaute protein family to partially complementary sequences within the 3' UTR of target mRNAs to either regulate their translation into protein or their stability.[12,13] Argonaute proteins are highly conserved and contain PAZ (PIWI-Argonaute-zwille) and PIWI domains. Structural studies have demonstrated that the PAZ domain forms a highly specific binding module for 2 nt 3' overhangs generated by RNase III enzymes. In contrast, PIWI domains fold similar to RNase H and may therefore function as endonucleases.[14-16] Indeed, it has been demonstrated for some Ago proteins that they are endonucleolytically active and such proteins have therefore been termed "Slicer". Interestingly, in mammalian cells Ago2 is the only member of the Ago protein family, which has endonuclease activity, although the critical amino acids are conserved in some other Ago proteins as well.[17,18]

MiRNAs recognize partially complementary binding sites located in the 3' UTR of target mRNAs and function as guides for protein effectors such as Ago proteins.[13] It has been shown that miRNP-binding to specific target mRNAs can regulate gene expression in different ways. On target sites with a high degree of complementarity, miRNAs function alike siRNAs and guide sequence-specific cleavage of the target mRNA.[19] Targets with low degree of complementarity can either be destabilized by recruiting de-capping and de-polymerization enzymes or their translation is repressed without altering mRNA levels.[12] While sequence-specific cleavage of target RNAs is well understood, the mechanism of how miRNAs guide translational repression or destabilization is only poorly understood.

The mode of miRNA-guided translational repression is still a matter of debate. Initial studies in *Caenorhabditis elegans* (*C.elegans*) have shown that miRNAs as well as target mRNAs associate with polysomes in sucrose gradients and it has been suggested that miRNAs function after initiation and presumably at the elongation steps of translation.[20,21] Later on, it has been reported that also mammalian miRNAs can be found on polysomes and a ribosome drop-off model has been suggested, which describes the rapid ribosome drop-off from mRNAs that are targeted by miRNAs.[22-24] Controversially, it has been demonstrated that miRNAs interfere with translation initiation steps. Moreover, cap-independent translation mediated by internal ribosome entry sites (IRES) is not sensitive to miRNA-guided regulation, indicating that miRNAs inhibit translation initiation.[25,26] Very recently, two reports supported this model. First, miRNPs associate with the 60S ribosomal subunit and eIF6, a protein known to prevent assembly of 80S ribosomes.[27] Second, in a *Drosophila* in vitro translation system, miRNAs induce the formation of dense miRNPs that co-migrate with polysomes.[28] These structures have been termed pseudo-polysomes and may reflect the structures that have been interpreted as polysomes in other studies.

Novel mechanistic insights into miRNA function came from recent studies on Ago proteins. Mourelatos and co-workers identified a motif within Ago proteins, which specifically recognizes and binds the m7G cap of mRNAs, thus preventing binding of eIF4E binding. This Ago-m7G cap interaction therefore leads to the inhibition of translational initiation.[29]

Due to the low degree of complementarity between miRNAs and target mRNAs, only a few mammalian target mRNAs have been discovered and validated thus far. Computer algorithms have been developed to predict putative miRNA targets and it has been found that seven to eight nt at the 5' end of the miRNA is the most important determinant for target specificity. Such sequences are also called "seed sequence".[30-33] Based on seed sequence conservation and free binding energy, miRNA targets have been predicted in a variety of different organisms. The different prediction programs find about 100 to 150 different targets for one miRNA. Some algorithms even predict one third of all human mRNAs as miRNA targets. Computer programs usually predict targets on a genome wide scale irrespective of tissue-specific mRNA and miRNA expression patterns. Therefore, it is very difficult to find miRNA targets that are specific to tissues or cell lines. Interestingly, it has been proposed earlier, that miRNA-mRNA interactions are restricted to specific tissues in *C. elegans*.[34] It is becoming more and more apparent that it is very important to develop biochemical tools to identify only subsets of miRNA targets from specific tissue material.

Here we report the first biochemical miRNA target identification approach. Using specific monoclonal antibodies against human Ago1 and Ago2, we succeeded to co-immunoprecipitate Ago-bound mRNAs. We generated a cDNA library from the Ago-associated mRNAs and identified a significant number by sequencing. We further validated six Ago-bound mRNAs either by inhibiting or over-expressing miRNAs that have been predicted to regulate the respective mRNAs.

MATERIALS AND METHODS

Reporter constructs. To generate miRNA reporter plasmids which express the firefly luciferase mRNA with a 3'UTR sequence of interest and renilla luciferase as a transfection control, pMIR-REPORT (Ambion) was modified as follows: The renilla luciferase gene with a SV40 promoter and a poly(A) site was PCR-amplified from the pRL-SV40 plasmid (Promega) and inserted into the SspI site of pMIR-REPORT. Additionally, an HSV-TK promoter was PCR-cloned from pRL-TK (Promega) and inserted into pMIR-REPORT to replace the CMV promoter of the firefly luciferase gene.

Luciferase expression constructs were generated by cloning of the 3'UTRs of STMN1, HMGB1, Raver2, SERBP1, DNAJB11 and SFPQ into the modified pMIR vector (pMIR-RL) described above. The 3'UTRs were cloned via PCR amplification from cDNA libraries and ligated into the corresponding SacI and NaeI sites of pMIR-RL. The primer sequences are:
SERBP1, 5'-CGCTGAGCTCCTGGATGCCATAAGACAACCCT, 5'-CGCTGCCGGCTTAACTGGTACACACTGTTCAC;
Raver2, 5'-CGCTGAGCTCGTTAAGCTCTCTCCTAATAACC, 5'-CGCTGCCGGCTCCCAATAAAAGTTTATTTATG;
SFPQ, 5'-CGCTGAGCTCATGTGATATTTAGGCTTTCATT, 5'-CGCTGCCGGCTCATGTTTAACATCTTTAATTC;
DNAJB11,5'-CGCTGAGCTCGAGTGAATAAAATTGGACTTTG, 5'-CGCTGCCGGCTTCATGAAAAAAATAACAACAA;
HMGB1, 5'-CGCTGAGCTCGTTGGTTCTAGCGCAGTTTTTT, 5'-CGCTGCCGGCTTACTGCAATTATTAGTTTATT;
STMN1, 5'-CGCTGAGCTCTTTGTTCTGAGAACTGACTTTC, 5'-CGCTGCCGGCCTACAGCAGTACATAAAGTTTT.
The sequences of miR-let7a, miR-26a, miR-26b, miR-29b, miR-99a, miR-100 and miR-141 were cloned as oligonucleotides into pSuper (oligoengiene) according to the manufacturer's protocol.
miR-let7a, 5'-GATCCCTGGGATGAGGTAGTAGGTTGTATAG TTTTAGGGTCACACCCACCACTGGGAGATAACTATACAAT CTACTGTCTTTCCTATTTTTA;
miR-26a, 5'-GATCCCGTGGCCTCGTTCAAGTAATCCAGGAT AGGCTGTGCAGGTCCCAATGGGCCTATTCTTGGTTACTT GCACGGGGACGCTTTTTA;
miR-26b; 5'-GATCCCCCGGGACCCAGTTCAAGTAATTCAG GATAGGTTGTGTGCTGTCCAGCCTGTTCTCCATTACTTG GCTCGGGGACCGGTTTTTA;
miR-29b, 5'-GATCCCCTTCAGGAAGCTGGTTTCATATGGT GGTTTAGATTTAAATAGTGATTGTCTAGCACCATTTGAAA TCAGTGTTCTTGGGGGTTTTTA;
miR-99a, 5'-GATCCCCCCATTGGCATAAACCCGTAGATCCG ATCTTGTGGTGAAGTGGACCGCACAAGCTCGCTTCTATG GGTCTGTGTCAGTGTGTTTTTA;
miR-100, 5'-GATCCCCCTGTTGCCACAAACCCGTAGATCC GAACTTGTGGTATTAGTCCGCACAAGCTTGTATCTATAGG TATGTGTCTGTTAGGTTTTTA;
miR-141, 5'-GATCCCCGGCCGGCCCTGGGTCCATCTTCC AGTACAGTGTTGGATGGTCTAATTGTGAAGCTCCTAACA CTGTCTGGTAAAGATGGCTCCCGGGTGGGTTCTTTTTA.
2'O-methylated miRNA inhibitors were designed as antisense oligos

to the mature miRNAs according to the miRNA registry (microrna.sanger.ac.uk/sequences/index.shtml).

Transfections and luciferase assays. Plasmid transfections for luciferase assays were performed with 50 ng pMIR-RL 3'UTR and 200 ng pSuper-miRNA vector or 40 pmol 2'OMe-miRNA-inhibitor per 2x104 cells in a 48-well plate using EscortV transfection reagent (Sigma) as described by the manufacturer. For Co-transfection of DNA and 2'OMe-miRNA-inhibitors, pMIR-RL 3'UTR were transfected 6 h after transfection with the 2'OMe-miRNA-inhibitors. Luciferase activity was measured 48 h after transfection using a dual luciferase reporter system as described by the manufacturer (Promega).

Cell lysates and immunoprecipitations. HEK 293 cells were lysed in buffer containing 25 mM Tris HCl pH 7.4, 150 mM KCl, 0.5% NP-40, 2 mM EDTA, 1 mM NaF, 0.5 mM DTT and protease inhibitors (Roche) and centrifuged at 10000 g for 10 min at 4°C. For immunoprecipitations 5 ml of hybridoma supernatant from monoclonal anti-Ago1-4B8 and anti-Ago2-5D4 antibodies were coupled to approx. 80 µl Protein-G-Sepharose (GE Healthcare). Beads were subsequently incubated with 10 ml of HEK 293 lysate (approx. 10 µg/µl) for 5 h under constant rotation at 4°C. After incubation, the beads were washed three times with washing buffer (300 mM KCl, 50 mM Tris-HCl pH 7.4, 1 mM MgCl2, 0.1 % NP-40). Finally, the beads were washed once with PBS. Co-precipitated RNA was extracted using one volume of phenol and subsequently precipitated from the aqueous phase using three volumes of Ethanol. The RNA pellet was used for oligo-dT purification and library generation.

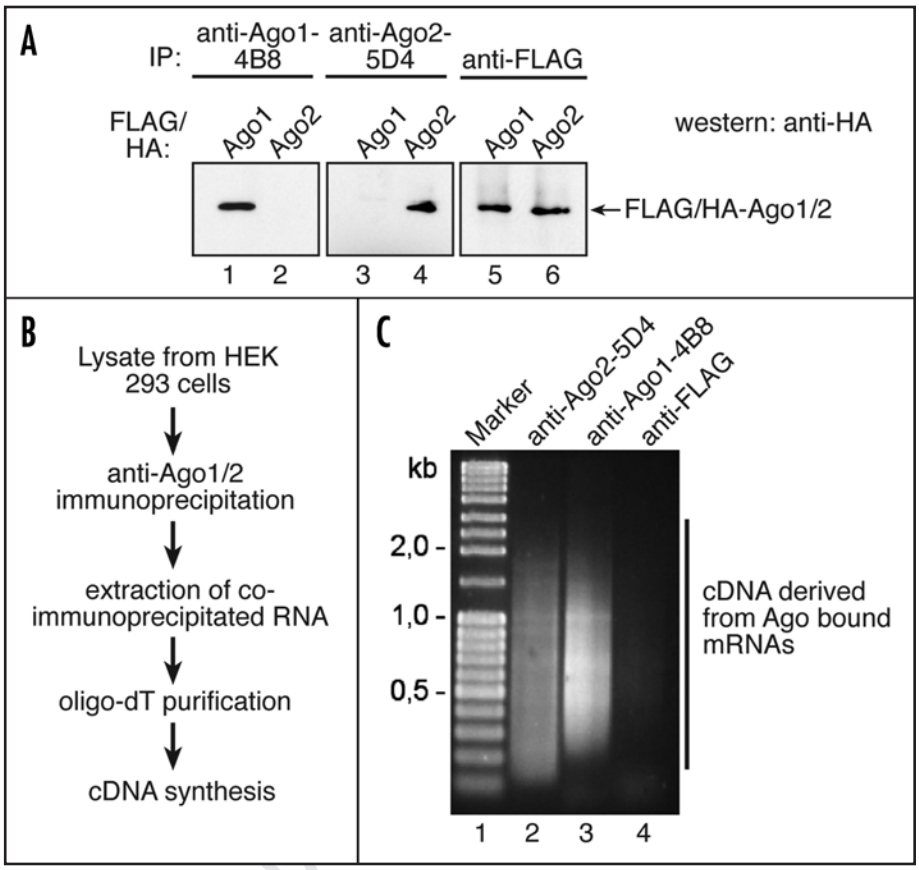

Figure 1: Biochemical isolation of mRNAs that are associated with human Ago1 or Ago2. (A) Monoclonal antibodies against Ago1 (4B8, lanes 1 and 2), against Ago2 (5D4, lanes 3 and 4) and the FLAG tag (lanes 5 and 6) were bound to Protein-G Sepharose beads and used for immunoprecipitations of FLAG/HA-tagged Ago1 (lanes 1, 3 and 5) and FLAG/HA-tagged Ago2 (lanes 2, 4 and 6). Immunoprecipitated proteins were analyzed by western blotting using anti-HA antibodies. (B) Schematic presentation of the Ago1- and Ago2-associated mRNP purification protocol. (C) Monoclonal antibodies Ago1-4B8 (lane 3), Ago2-5D4 (lane 2) or a monoclonal antibody against the FLAG-tag (lane 4) were coupled to Protein-Sepharose beads and incubated with HEK 293 lysates. Co-immunoprecipitated mRNAs were reverse transcribed and second-strand cDNA synthesis was performed using random hexamers. Lane 1 shows a DNA size marker.

cDNA synthesis and library generation. The cDNA library was generated by Vertis Biotechnology (Weihenstephan, Germany). Briefly, immunoprecipitated mRNAs were subjected to oligo-dT purification. The purified mRNAs were reverse transcribed using reverse transcriptase and oligo-dT as primer. The single stranded cDNA was used for second-strand synthesis using random hexamers as primers. The resulting cDNA was cloned into a TOPO vector (Invitrogen) according to the manufacturer's instructions. The plasmids were subsequently transformed into E.coli to generate cDNA libraries. In order to identify individual cDNAs from the libraries, bacteria were separated on agar plates and individual colonies were picked for plasmid DNA preparation. Plasmids were sequenced in a 96 well format by LARK (UK).

Monoclonal antibodies. For monoclonal antibody production Lou/C rats were immunized subcutaneously and intraperitoneally with a mixture of Ago1-GST or Ago2-GST fusion protein (50 µg), 5 nmol CPG oligonucleotide (ODN 2006, TIB Molbiol, Berlin, Germany), 500 µl PBS and 500 µl IFA. After a six-week interval a final boost without adjuvant was given three days before fusion of the rat spleen cells with the murine myeloma cell line P3X63-Ag8.653 (ZIT). Hybridoma supernatants were tested in an ELISA using bacterially expressed Ago1 or Ago2 fusion protein or an irrelevant GST fusion protein. mAbs reacting only with Ago1-GST or Ago2-GST fusion proteins were analyzed in western blotting and immunoprecipitation. Only Ago1-4B8 (rat IgG2a) recognized the protein specifically in western blotting. However, both Ago1-4B8 and Ago2-5D4 recognized only Ago1 or Ago2 specifically and were used in this study.

RESULTS

Isolation of Ago-bound mRNAs. Based on structural studies on archaeal Ago proteins, it is very likely that Ago proteins do not only bind to small RNAs but also directly contact the regulated target RNAs.[35,36] We therefore hypothesized that Ago proteins may form stable Ago mRNPs that can be biochemically analyzed. We generated highly specific monoclonal antibodies against human Ago1 and Ago2. Clone Ago1-4B8 recognized specifically tagged Ago1 and a 100 kDa band in human cell extracts (data not shown). Moreover, Ago1-4B8 immunoprecipitated only tagged Ago1 but not Ago2 (Fig. 1A). Clone Ago2-5D4 did not show any signal in western blots but specifically immunoprecipitated tagged Ago2 but not Ago1

Table 1 **Ago1-associated mRNAs**

mRNA			# of clones	miRanda	TargetScan	picTAR
Nuclear-encoded						
ANKRD47	ankyrin repeat domain 47	NM_198471.1	2	-	-	-
APPBP1	amyloid beta precursor protein binding protein 1	NM_003905.3	2	-	+	-
ATF4	activating transcription factor 4	NM_001675.2	3	-	-	-
ATIC	5-aminoimidazole-4-carboxamide ribonucleotide formy transferase/ IMP cyclohydrolase	NM_004044.4	2	-	-	-
ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1, nuclear gene, mitochondrial protein	NM_001688.4	3	-	-	-
BTF3L4	basic transcription factor 3-like 4	NM_152265.1	2	-	+	-
C10orf84	Chromosome 10 open reading frame 84	NM_022063.1	2	-	-	-
C19orf2	Chromosome 19 open reading frame 2	NM_003796.2	2	+	+	+
C21orf51	Chromosome 21 open reading frame 51	NM_058182.2	2	-	-	-
C2orf25	Chromosome 2 open reading frame 25	NM_015702.1	3	-	+	-
C6orf48	Chromosome 6 open reading frame 48	NM_001040438.1	2	-	-	-
CALM2	calmodulin 2	NM_001743.3	2	-	+	+
CCDC28A	Coiled-coil domain cont. 28a	NM_015439.2	3	+	-	+
CCNB1IP1	Cyclin B1 interac. Protein	NM_021178.3	2	-	-	-
CDC26	cell division cycle 26 homolog	NM_139286.3	3	-	-	-
CPVL	carboxypeptidase, vitellogenic-like	NM_019029.2	2	-	-	-
CTNNAL1	catenin (cadherin-associated protein)	NM_003798.1	2	-	+	+
CUL1	Cullin 1	NM_003592.2	2	+	+	+
DEXI	dexamethasone-induced transcript	NM_014015.3	2	-	+	+
DHFR	Dihydrofolate reductase	NM_000791.3	2	+	-	-
DNAJB11	Hsp40/ DNAJ	NM_016306.4	3	+	+	+
EEF1A1	eucaryotic translation elongation factor 1 alpha	NM_001402.5	4	+	+	+
EEF1G	eukaryotic translation elongation factor 1 gamma	NM_001404.4	2	+	-	-
EIF4A2	eukaryotic translation initiation factor 4A, isoform 2	NM_001967.3	2	+	+	+
Fam18B	family with sequence similarity 18, member B	NM_016078.3	2	+	-	-
FBLN1	fibulin 1 variant c	NM_001996.2	2	+	-	-
FHL1	four and a half LIM domains 1	NM_001449.3	2	+	+	-
GNAS	GNAS complex locus variant 1	NM_000516.3	2	+	+	+
GNL2	Guanine nucleotide binding protein like 2	NM_013285.1	2	-	-	-
HEBP2	heme binding protein 2	NM_014320.2		-	-	-
HMGB1	High mobility group box 1	NM_002128.3	6	+	+	+
HNRPA2B1	heterogenous nuclear ribonucleoprotein A2/ B1	NM_002137.2	4	+	+	+
HNRPD	heterogenous nuclear ribonucleoprotein D AU rich element RNA-binding protein 1	NM_031370.2	2	+	+	-
HNRPH1	heterogenous nuclear ribonucleoprotein H1	NM_005520.1	2	+	+	+
IARS	isoleucin-tRNA synthetase	NM_002161.3	2	-	-	-
IMPDH2	IMP (inosine monophosphate) dehydrogenase 2	NM_000884.2	2	-	-	-
KIF22	kinesin family member 22	NM_007317.1	2	-	-	-
LDLR	low density lipoprotein receptor (familial hypercholesterolemia)	NM_000527.2	2	+	+	-
LOC388181	hypothetical protein LOC388181	XR_019273.1	2	-	-	-
LTK	leukocyte tyrosine kinase	NM_002344.3	2	+	-	-
Myc	v myc myelocytomatosis viral oncogene homolog	NM_002467.3	2	+	+	+
NDUFV3	NADH dehydrogenase (ubiquinone) flaovoprotein 3, nuclear protein encoding mitochondrial protein	NM_021075.3	2	-	-	-
NM23B	non metastatic cells 2 protein	NM_001018139.1	2	-	-	-
NOLA2	Nucleolar protein	NM_001034833.1	3	+	-	-
NOP5/ NOP8	nuclear protein NOP5/ NOP8	NM_015934.3	3	-	-	-
NPM1	Nucleophosmin	NM_002520.5	6	+	+	+
NRD1	nardilysin	NM_002525.1	2	-	-	-
NT5C3L	5'-nucleotidase, cytosolic III-like	NM_052935.2	2	-	-	
PABPC1	poly A binding protein, cytoplasm	NM_002568.3	4	+	+	+
PAIBP1 SERBP1	Pai mRNA binding protein	NM_015640.3	2	+	-	+
POMP	proteasome maturation protein	NM_015932.2	2	+	-	-
POUF2F	POU domain TF	NM_002698.1	6	+	+	+
PSAT1	phosphoserine aminotransferase 1	NM_021154.3	3	-	-	-
PSMA4	proteasome (prosome, macropain) subunit, alpha type, 4	NM_002789.3	2	-	-	-
PTMA	prothymosin alpha(gene sequence 28)	NM_002823.2	2	-	+	-
PTP4A2	predicted protein tyrosine phosphatase type iVA	XM_001132357.1	2	+	+	+
RAB13	Ras oncogene family	NM_002870.2	2	-	-	-
RasL10B	ras like family 10 member B	NM_033315.2	2	+	+	+
RAVER2	PTB binding 2	NM_018211.2	2	+	+	-
RPL11	ribosomal protein L11	NM_000975.2	2	-	-	-
RPL15	ribosomal protein L15	NM_002948.2	3	+	+	-
RPL24	ribosomal protein L24	NM_000986.3	2	-	-	-

Table 1 continued

RPL29	ribosomal protein L29	NM_000992.2	3	-	-	-
RPL3	ribosomal protein L3	NM_00967.3	3	-	-	-
RPL41	ribosomal protein L41	NM_021104.1	4	-	-	+
RPL6	ribosomal protein L6	NM_000970.3	5	+	-	-
RPL7	ribosomal protein L7	NM_000971.3	3	+	-	-
RPL9	ribosomal protein L9	NM_000661.4	2	+	-	-
RPS11	ribosomal protein S11	NM_001015.3	2	+	-	-
RPS14	ribosomal protein S14	NM_005617.3	2	+	-	-
RPS16	ribosomal protein S16	NM_001020.4	2	+	-	-
RPS24	ribosomal protein S24	NM_033022.2	3	-	-	-
RPS27A	ribosomal protein S27A	NM_002954.3	2	-	-	-
RPS27L	ribosomal protein S27L	NM_015920.3	2	-	-	-
RPS3A	Ribosomal protein S3A	NM_001006.3	2	-	-	-
RPS6	ribosomal protein S6	NM_001010.2	6	-	-	-
RUVBL1	RuvB-like1	NM_003707.1	2	-	-	-
SCD	stearoyl-CoA desaturase	NM_005063.4	2	+	+	+
SFPQ	PTB associated	NM_005066.1	2	+	+	+
SNAPC5	Small nuclear RNA activating complex	NM_006049.2	2	+	-	-
SSB	Sjorgen syndrome antigen B	NM_003142.2	2	-	-	-
STMN1	Stathmin1/ oncoprotein 18	NM_005563.3	4	+	+	+
TCEAL4	transcription elongation factor A (SII) like 4	NM_024863.4	2	-	-	-
TCEB1	transcription elongation factor B SIII	NM_005648.2	2	-	+	-
TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	NM_016639.1	2	+	-	-
TPT1	tumor protein translationally-controlled 1	NM_003295.1	2	+	+	-
VDAC2	Voltage-dependent anion channel	NM_003375.2	3	+	+	+
WDSOF1	WD repeats and SOF1 domain containing	NM_015420.4	2	-	-	-
WIBG	within bgcn homolog	NM_032345.1	2	-	+	-
YBX-1	YB	NM_004559.3	5	+	+	+
YIPF5	YIP1 domain family	NM_030799.6	2	-	+	-
Mitochondrion-encoded						
ATP6	ATP-synthase F0 subunit 2	NC_001807.4	3	-	-	-
Cox1	cytochrome c oxidase subunit I	NC_001807.4	2	-	-	-
Cox3	cytochrome c oxidase subunit III	NC_001807.4	5	-	-	-
ND1	NADH dehydrogenase subunit2	NC_001807.4	3	-	-	-

(Fig. 1A). Therefore, we used the antibodies for affinity purification of Ago complexes (Fig. 1). Monoclonal antibodies Ago1-4B8 or Ago2-5D4 were immobilized on Protein-G-Sepharose beads and incubated with HEK 293 cell lysates. After stringent washing, the co-immunoprecipitated Ago-bound RNAs were extracted and subjected to oligo-dT purification to purify mRNAs from other Ago-associated RNA species (Fig. 1B). The isolated mRNAs were reverse transcribed, cloned and subjected to second-strand synthesis using random hexamer oligos (Fig. 1C). Interestingly, both the Ago1 and the Ago2-specific monoclonal antibodies pull-down mRNAs indicated by a RNA smear over a broad range of the agarose gel (lanes 2 and 3). A monoclonal antibody directed against the FLAG-tag was used as a control and did not immunoprecipitate visible amounts of mRNAs (lane 4), indicating that the Ago1 and Ago2 mRNA signal is specific. Taken together, we have demonstrated that Ago1 and Ago2 form stable mRNPs in human cells that can be biochemically isolated.

Cloning and identification of Ago bound mRNAs. In order to identify and further analyze the mRNA pools that were isolated from Ago1 and Ago2 mRNPs, the cDNAs described above were cloned and transformed into *E. coli* to produce Ago-associated mRNA libraries. About 600 colonies from each of the libraries were picked and the corresponding plasmids were sequenced (Table 1 and Table 2). Since it cannot be excluded that the libraries contain a limited number of mRNAs that are unspecifically bound to the beads, we removed all the single hits from the list and considered only enriched mRNAs as miRNA target candidates (all cloned mRNAs are shown in supplementary tables 1 and 2). To further analyze the list of Ago-bound mRNAs that we have obtained, we employed three target prediction algorithms that are widely used (Miranda, targetScan, pictar). Surprisingly, only about 60% of the Ago1-bound mRNAs and about 50% of the Ago2–bound mRNAs are predicted as miRNA targets. Moreover, we also find high numbers of messages that are encoded by the mitochondrion indicating that pools of cellular mRNAs that have not been analyzed by target prediction programs so far are subject to miRNA regulation as well. In order to gain insight into Ago1 and Ago2 function, we compared the Ago1-bound mRNAs with the Ago2-bound mRNAs (supplementary table 3). We find that only a limited number of mRNA overlap between Ago1 and Ago2 suggesting that many miRNA targets might be specific to one Ago protein.

Taken together, we have demonstrated that mRNAs specifically co-purify with human Ago1 and Ago2 complexes. Interestingly, prediction algorithms have not identified many of the identified Ago-bound mRNAs. Therefore, our data suggest that larger numbers of mRNAs as previously anticipated might be under the control of the miRNA pathway.

Validation of identified mRNAs. For detailed analysis of the specificity of the Ago-bound mRNAs, we validated a number of mRNAs by different experimental approaches. We chose six mRNAs from the Ago1-bound cDNA library that are also predicted as miRNA targets by different algorithms and cloned the 3' UTRs behind a firefly luciferase reporter gene (for predictions see supplementary figure 1). Notably, all miRNAs that have been tested are also associated with Ago1 in HEK 293 cells (data not shown, submitted elsewhere). The reporter plasmids were co-transfected with 2'O-methylated antisense inhibitors against endogenous miRNAs that are predicted to bind to the respective 3'UTRs (Figure 2, left

Table 2 **Ago2-associated mRNAs**

mRNA			# of clones	miRanda	Target scan	pictar
Nuclear-encoded						
ASNS	asparagine synthetase (ASNS), transcript variant 3	NM_183356.1	4	-	-	-
CCDC72	coiled-coil domain containing 72	NM_015933.2	2	-	-	-
CNBP	CCHC-type zinc finger, nucleic acid binding protein (CNBP)	NM_003418.1	2	+	-	-
CTPS	CTP synthase	NM_001905.1	2	-	-	-
DDX48	DEAD (Asp-Glu-Ala-Asp) box polypeptide 48 (DDX48)	NM_014740.2	3	-	-	-
DPM1	dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit (DPM1)	NM_003859.1	2	-	-	-
EBNA1BP2	EBNA1 binding protein 2 (EBNA1BP2)	NM_006824.1	2	-	-	-
EEF1A1	eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)	NM_001402.5	2	+	+	+
EEF1G	eukaryotic translation elongation factor 1 gamma	NM_001404.4	2	+	-	-
EIF1	eukaryotic translation initiation factor 1	NM_005801.3	2	+	-	-
EIF3S3	eukaryotic translation initiation factor 3, subunit 3 gamma, 40kDa	NM_003756.2	2	-	-	-
ENY2	enhancer of yellow 2 homolog (Drosophila) (ENY2)	NM_020189.4	2	-	+	+
FABP5	fatty acid binding protein 5 (psoriasis-associated) (FABP5)	NM_001444.1	2	-	-	-
GJA1	gap junction protein, alpha 1, 43kDa (connexin 43) (GJA1)	NM_000165.2	2	+	+	+
GLUL	glutamate-ammonia ligase (glutamine synthetase), transcript variant 1	NM_002065.4	2	+	-	-
GMNN	geminin, DNA replication inhibitor	BC005185.1	2	-	-	-
HADH2	hydroxyacyl-Coenzyme A dehydrogenase, type II (HADH2), nuclear gene encoding mitochondrial protein, transcript variant 1	NM_004493.2	2	-	-	-
HAX1	HCLS1 associated protein X-1 (HAX1), transcript variant 1	NM_006118.3	2	-	-	-
HMGB1	high-mobility group box 1 (HMGB1)	NM_002128.3	2	+	+	+
HNRPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1 (HNRPA2B1), transcript variant B1	NM_031243.1	2	+	+	+
IARS	isoleucine-tRNA synthetase, transcript variant l	NM_013417.2	5	-	-	-
NAP1L1	nucleosome assembly protein 1-like 1, transcript variant 1	NM_139207.1	4	+	+	-
NP	nucleoside phosphorylase (NP)	NM_000270.1	2	-	-	-
NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin) (NPM1), transcript variant 1	NM_002520.5	2	+	+	+
OAZ1	ornithine decarboxylase antizyme 1 (OAZ1)	NM_004152.2	2	-	-	-
PABPC1	poly(A) binding protein, cytoplasmic 1	NM_002568.3	4	+	+	+
PABPN1	poly(A) binding protein, nuclear 1 (PABPN1)	NM_004643.1	3	+	+	+
PDCD10	programmed cell death 10 (PDCD10), transcript variant 2	NM_145859.1	2	+	+	+
PRDX3	peroxiredoxin 3 (PRDX3), nuclear gene encoding mitochondrial protein, transcript variant 1	NM_006793.2	2	+	-	-
PSMB6	proteasome (prosome, macropain) subunit, beta type, 6	NM_002798.1	2	-	-	-
RNF166	ring finger protein 166 (RNF166)	NM_178841.2	2	-	-	-
RPL3	ribosomal protein L3 (RPL3), transcript variant 1	NM_000967.3	2	-	-	+
RPL36A	ribosomal protein L36a (RPL36A)	NM_021029.4	2	-	-	-
RPL39	ribosomal protein L39 (RPL39)	NM_001000.2	2	-	-	-
RPL4	ribosomal protein L4	NM_000968.2	3	-	-	-
RPL41	ribosomal protein L41 (RPL41), transcript variant 1	NM_021104.1	4	-	-	+
RPL7	ribosomal protein L7 (RPL7)	NM_000971.3	3	+	-	-
RPS27A	ribosomal protein S27a (RPS27A)	NM_002954.3	3	-	-	-
RPS3A	ribosomal protein S3A (RPS3A)	NM_001006.3	5	-	-	-
RPS4x	ribosomal protein S4, X-linked (RPS4X)	NM_001007.3	2	-	-	-
SET	SET translocation (myeloid leukemia-associated) (SET)	NM_003011.1	4	+	+	-
STMN1	stathmin 1/oncoprotein 18 (STMN1), transcript variant 3	NM_005563.3	2	+	+	+
TOMM20	translocase of outer mitochondrial membrane 20 homolog	NM_014765.1	3	+	+	+
TPI1	triosephosphate isomerase 1	NM_000365.4	2	+	+	+
TPT1	tumor protein, translationally-controlled 1 (TPT1)	NM_003295.1	2	+	-	-
TUBB	tubulin, beta (TUBB)	NM_178014.2	2	+	+	+
VDAC2	voltage-dependent anion channel 2 (VDAC2)	NM_003375.2	2	+	+	+
Mitochondrion-encoded						
COX3	cytochrome c oxidase subunit III	NC_001807.4	3	-	-	-
TNRK	mitochondrially encoded tRNA lysine	NC_001807.4	2	-	-	-

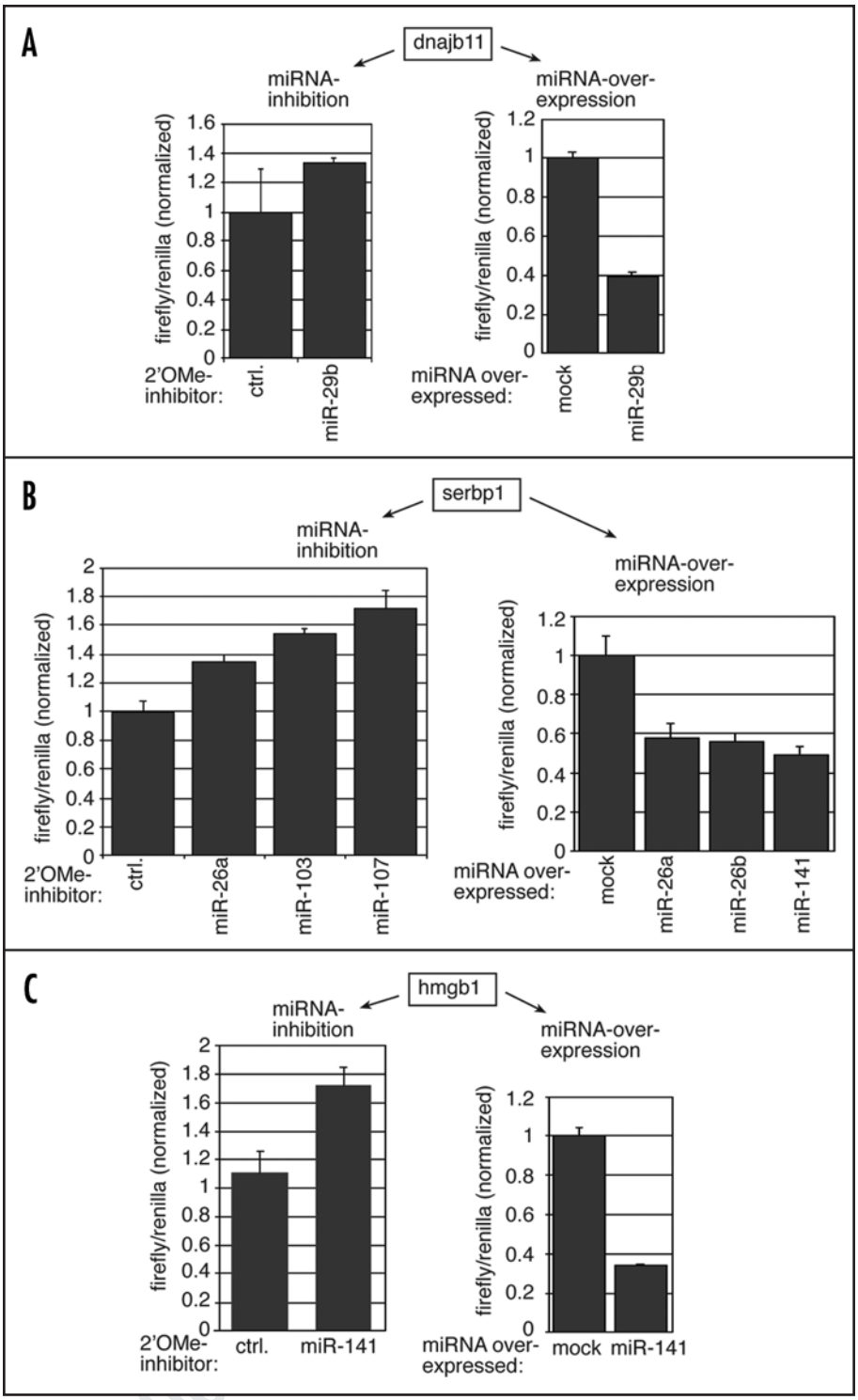

Figure 2. For legend, see page e8.

panels). Inhibitors against miRNAs that are not predicted to regulate the analyzed mRNAs served as controls. Strikingly, inhibition of miR-29b led to an enhanced luciferase activity compared to the control transfection when fused to the full length 3'UTR of dnajb11, an mRNA that encodes for a hsp40 variant indicating the dnajb11 is indeed a miRNA target (Fig. 2A). Similar results were obtained when inhibitors against miRNAs that have been predicted to regulate serbp1 (Fig. 3B), hmgb1 (Fig. 3C), raver2 (Fig. 3D) and sfpq (Fig. 3E) were co-transfected with reporter plasmids containing the full length 3'UTRs of these mRNAs. Notably, we were not able to validate stmn1 as miRNA target (data not shown). Therefore, our data suggest that the majority of the Ago1-bound mRNAs that have been validated are indeed under the control of endogenous miRNAs.

Inhibition of specific endogenous miRNAs resulted in enhanced expression of the above-mentioned Ago1-bound mRNAs. If these mRNAs are indeed under the control of the miRNA pathway, we hypothesized that forced over-expression of those miRNAs may lead to a decreased expression of Ago1-bound mRNAs. We cloned different miRNA hairpins into the pSUPER vector to allow for over-expression. Strikingly, when we express miR-29b the expression of a luciferase reporter containing the full-length dnajb11 3'UTR was reduced to about 40% indicating that dnajb11 is indeed regulated by miR-29b (Fig. 2A, right panel). Similarly, when over-expressing miR-26a, miR-26b and miR-141 the expression of a luciferase reporter containing the full-length 3'UTR of serbp1 was strongly reduced indicating that serbp1 is under the control of the miRNA pathway (Fig. 2B). Similar results were obtained with hmgb1 (Fig. 2C), raver2 (Fig. 2D) and sfpq (Fig. 2E).

In summary, two independent validation approaches clearly demonstrate that all Ago1-bound mRNAs that have been tested are indeed miRNA targets suggesting a high number of miRNA targets in our libraries. We also provide a novel tool to specifically identify miRNA targets from individual human cell lines or tissues.

DISCUSSION

Ago proteins specifically recognize and bind both ends of small non-coding RNAs and are therefore considered as key factors in RNA silencing 14-16. Moreover, structural studies demonstrated that Ago proteins might not only interact with the small RNA but also with target RNAs that are regulated. Using a biochemical approach, we purified Ago-mRNPs from human cells and identified about 600 Ago1-bound and about 600 Ago2-bound mRNAs. Since it cannot be excluded that our libraries also contain mRNAs that un-specifically co-purify, we eliminated the single hits form the list to obtain all mRNAs that are enriched in the libraries. Using a luciferase-based reporter system, we have analyzed six Ago1-bound mRNAs and have validated five of them as miRNA targets. It is therefore tempting to speculate that a high percentage of our purified mRNAs are indeed miRNA targets. Notably, we have not validated Ago2-bound mRNAs yet. However, due to the specificity of the antibodies (Fig. 1A), it is likely that miRNA targets are enriched in the Ago2-associated mRNA fraction as well. Large-scale validation

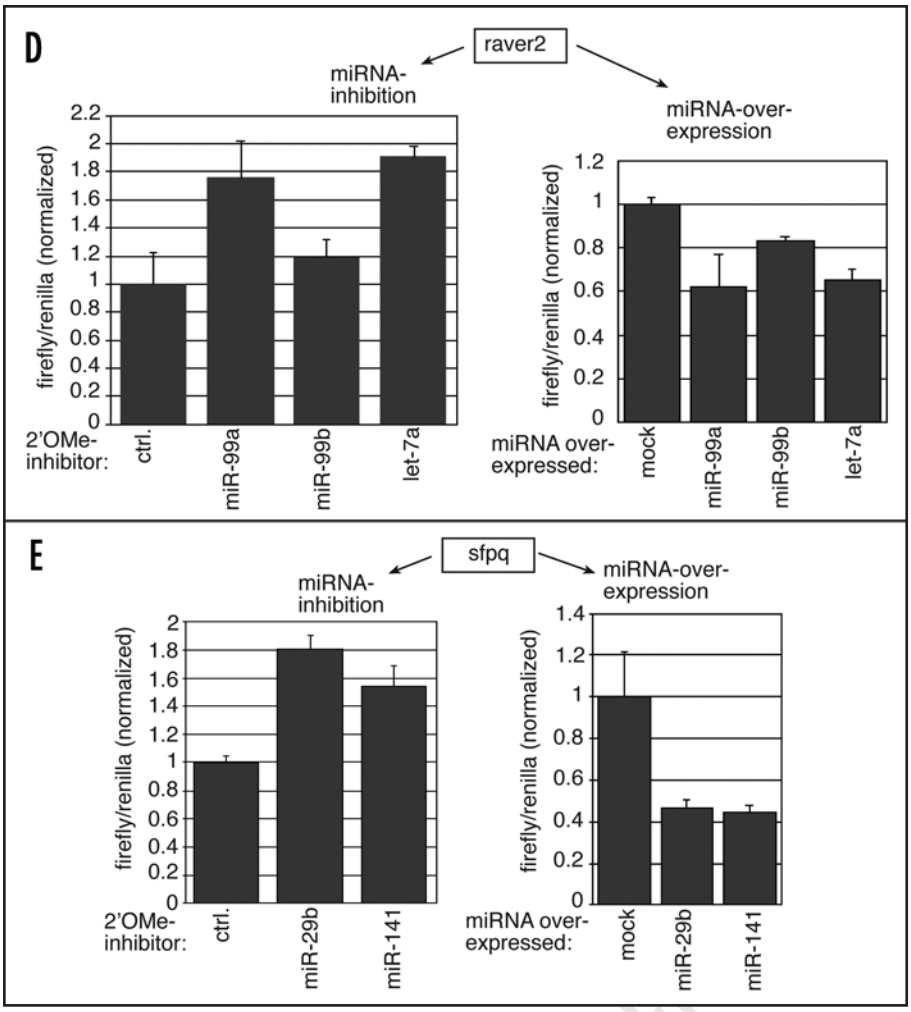

Figure 2. Validation of miRNA targets that are associated with human Ago1 mRNPs. (A) A luciferase reporter construct containing the full length 3'UTR of dnajb11 was co-transfected either with a 2'O-methylated inhibitor antisense to miR-29b (left panel) or a plasmid over-expressing miR-29b (right panel). The empty vector served as control. Firefly luciferase expression was normalized to Renilla luciferase expression. Error bars are derived from three individual experiments. (B) Experiments were performed as in (A). The full-length 3'UTR of serbp1 was fused to firefly luciferase. Inhibitors against miR-26a, miR-103 and miR-107 were co-transfected (left panel). MiR-26a, miR-26b and miR-141 were over-expressed (right panel). (C) Experiments were performed as in (A). The full length 3'UTR of hmgb1 was fused to firefly luciferase. An inhibitor against miR-141 was co-transfected (left panel). MiR-141 was over-expressed (right panel). (D) Experiments were performed as in (A). The full-length 3'UTR of raver2 was fused to firefly luciferase. Inhibitors against miR-99a, miR-99b and let-7a were co-transfected (left panel). MiR-99a, miR-99b and let-7a were over-expressed (right panel). (E) Experiments were performed as in (A). The full-length 3'UTR of sfpq was fused to firefly luciferase. Inhibitors against miR-29b and miR-141 were co-transfected (left panel). MiR-29b and miR-141 were overexpressed (right panel).

approaches will be needed to clearly show that the identified Ago-bound mRNAs are regulated by the miRNA pathway.

Among our purifications we find very abundant mRNA including many that encode for ribosomal proteins. Such highly abundant mRNAs very often co-precipitate un-specifically. However, several lines of evidence argue against high background in our purifications. First, we do not find visible amounts of mRNAs that co-purify with the monoclonal anti-FLAG antibody (Fig. 1B) indicating that most of the Ago1 and Ago2-associated mRNAs are specific and not just bound to the beads. Second, among the Ago1 and Ago2 mRNP purifications we find low abundant mRNAs with higher frequency than for example messages for ribosomal proteins. Third, beside a few overlapping mRNAs in the Ago1- and Ago2-bound mRNA fractions, we find many mRNAs that are specific to one or the other Ago protein again arguing against general mRNA background in our purifications.

The limited number of overlapping mRNAs could be considered as general background. However, it is equally reasonable that those mRNAs are targeted both by Ago1 and Ago2 since it has been suggested by computer predictions that individual mRNAs might be targeted by multiple miRNAs. An exhaustive sequencing approach of Ago-bound cDNA libraries in conjunction with large-scale validation approaches will be a powerful tool to identify all mRNAs that are targeted by the miRNA pathway. Analysis of target mRNAs that are not predicted as miRNA targets yet will also help to understand how the miRNA pathway in general and individual Ago proteins in particular function in gene silencing.

Most interestingly, only about 60% of the Ago-bound mRNAs, which have been identified in this study, are predicted by the three most widely used prediction algorithms Miranda, targetScan and pictar (tables 1 and 2).[30-32] Assuming that the mRNA background in our purifications is low, our data suggest that the currently used miRNA target prediction algorithms may miss a significant number of miRNA targets. Moreover, many prediction programs do not cover all cellular RNAs including mitochondrial RNAs due to incomplete databases. It is therefore reasonable that we find mRNAs in our purifications that are not predicted as miRNA targets. However, only a comprehensive validation of the isolated mRNAs will allow for a conclusive comparison of bioinformatics and biochemical miRNA target identification approaches.

Detailed bioinformatic as well as biochemical analysis of biochemically-identified mRNAs will lead to a better understanding of how miRNPs function and how such particles are embedded into larger regulatory networks. It is reasonable that other protein factors bind to miRNA targets and either inhibit miRNA association or enhance miRNA and therefore Ago binding. Such factors might be specific to individual mRNAs that are targeted by miRNAs. The limited number of putative miRNA targets that we provide in this study is a perfect starting point to analyze consensus sequences for RNA binding proteins in the vicinity of predicted miRNA binding site.

Note

Supplemental materials can be found at:
www.landesbioscience.com/supplement/beitzingerRNA4-2-sup.pdf

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