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A Novel Method to Detect Functional MicroRNA Targets

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MicroRNA (miRNA) molecules are non-coding RNAs, 19 to 24 nt in length that have been identified recently as important regulators of gene expression. Several computational methods have been developed to describe the target recognition mechanism by miRNA. We propose here a novel method to detect miRNA–mRNA complexes in eukaryotic cells. As a first step, we synthesize cDNA on an mRNA template using miRNAs as the endogenous cytoplasmic primer. This step extends miRNA and overcomes the problem of low complementary binding of miRNAs to their targets. Purified hybrid 3′-cDNA–miRNA-5′ molecules are used in a second round of reverse transcription to anneal to target mRNA in a highly gene-specific manner. The 5′-end analysis of these cDNA molecules demonstrated that primers for cDNAs were "signatures" of miRNA molecules, and over-expression of their full-length mature miRNAs resulted in functional inhibition of target protein expression.

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Introduction

Over the past two decades, it has become clear that a new layer of gene regulation, under the control of small RNAs, exerts numerous cellular functions. In 1993, two groups of investigators suggested that, in the worm *Caenorhabditis elegans*, a small RNA, *lin-4*, regulated the expression of another gene, *lin-14*, through direct interactions with its mRNA.[1,2] Since then, short RNAs have been shown to serve as powerful regulatory agents in many eukaryotes, with a versatile repertoire of transcriptional and post-transcriptional effects. Animal microRNAs (miRNAs) appear to play a regulatory role in a diverse array of biological processes, including apoptosis, development, metabolism, gene imprinting, hematopoietic development, and may play a role in some cancers.[3,3–18] Nonetheless, the regulatory role of most miRNAs remains elusive.

The miRNA genes are believed to constitute ~1% of the predicted genes in humans, flies and worms.[19] A "mature" miRNA is derived from a larger precursor primary miRNA (pri-miRNA) that folds into an imperfect stem–loop structure. In animals, including humans, these precursors appear to be derived from cleavage of the pri-miRNA transcript by a nuclear multiprotein complex, the "microprocessor", of which two examples currently identified include Drosha RNase III and Pasha (partner of Drosha).[20–22] After cleavage, miRNA precursors, pre-miRNAs, are exported into the cytoplasm, where they are cleaved into double-stranded RNA duplexes (composed of the mature miRNA and its complement, designated miRNA*) by an endonuclease, Dicer. The mature miRNA strand is incorporated into an effector complex, the RNA-induced silencing complex. Within the RNA-induced silencing complex, miRNAs appear to regulate gene expression by either inducing mRNA cleavage or causing translational repression, the former mechanism being predominant in plants and the latter in animal cells. A majority of the known mammalian miRNA genes overlap with transcription units in the introns of protein-coding genes as well as the exons and introns of mRNA-like coding genes.[23–26]

miRNA molecules bind to complementary strands of target mRNA, with subsequent endonucleolytic digestion or translational repression.[27,28] The fate of the targeted mRNA message is determined by the extent of complementary pairing between targeted mRNA and the miRNA.[29] Weak complementarities usually stand a better chance of repressing translation from mRNA, while higher degrees of complementary binding increase the chances for cleavage and digestion significantly.

Abbreviations used: miRNA, microRNA; pri-miRNA, primary miRNA; pre-miRNA, miRNA precursor; GSP, gene-specific primer.

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The results of several studies that seek to identify possible miRNA targets have been published. Using modern bioinformatics approaches, thousands of mRNAs were tested for interaction with known sets of miRNAs. The method introduced by Stark *et al.* led to validation of six targets for two *Drosophila melanogaster* miRNAs.[30] The strategy presented by Lewis *et al.* predicted numerous mammalian targets; experimentally, 11 out of 15 tested were validated.[31] Many predicted targets identified by this technique contained multiple miRNA-binding sites for the same miRNA molecule or were found to associate with more than one miRNA. More recently, Kiriakidou *et al*. developed a powerful computational algorithm to predict a larger set of miRNA targets.[32] Other methods introduced recently have improved the prediction efficiency to some degree.[19,33–39] However, there is no simple method to detect miRNA targets or to validate them reproducibly, efficiently, and inexpensively.

We propose here a novel and potentially near-universal method to detect miRNA–mRNA complexes *in vivo*. First, we use reverse transcription of a cytoplasmic extract to increase the length of an miRNA by extending it with cDNA on the template of a target mRNA. This step minimizes non-specific annealing in a second round of reverse transcription, which in turn creates cDNA molecules long enough for sequencing and analysis. The miRNA molecules we detected were confirmed in miRNA database searches and were shown to be functional by repressing target protein synthesis *in vivo* and in a heterologous reporter system. This method appears broadly applicable for the detection of miRNA molecules on their target messenger RNAs.

Results

miRNA as a primer for cDNA synthesis

The question that we wanted to answer was how to detect miRNA–mRNA complexes or their derivatives and to define an inexpensive, tractable, reproducible system to achieve this goal. It has been suggested that in many cases miRNAs are fully or almost nearly complementary to their target mRNA 3′-end (for example, see Figure 2 of Yeckta *et al*., Figure 4 of Johns *et al.* and Figures 4 and 5 of Kiriakidou *et al.*).[19,32,40] On the basis of these data, we hypothesized that miRNA bound to its target can be used as a primer to drive synthesis of cDNA on an mRNA template. We assumed that the 3′-end may form a temporarily stable substrate that facilitates initiation of cDNA synthesis in cases of weak complementarity between the 3′-end of miRNA and the target mRNA. The resultant cDNA, with miRNA on its 5′-end, can be cloned and sequenced easily. However, the challenge remains of how to accomplish this while minimizing background amplification of unwanted or non-existent miRNA species.

Purified RNA generally cannot be used to detect miRNA targets using the proposed model, because techniques to isolate RNA employ mixtures with increased pH as well as highly denaturing agents, which disrupt most secondary structures in intact RNA. Short strands of miRNAs will anneal to any possible sites in most mRNAs, even to sites that were protected from miRNA binding *in vivo* by preformed secondary structures or protein complexes. Second, many miRNAs form functional complexes in the 3′-untranslated regions of mRNA,[1,40–43] which may contain some ubiquitous translational controlling elements. In purified mixtures of miRNAs and mRNAs, miRNAs will tend to anneal (at proper temperatures) to even slightly acceptable RNA species; this feature renders them useless, due to a high background of non-functional complexes.

Therefore, we took advantage of the fact that cytoplasmic molecular complexes that already exist *in vivo* can be used to achieve our goal. We surmised that reverse transcriptase can initiate cDNA synthesis only from naturally preformed, double-stranded complexes between miRNA and mRNA (plus supportive protein machinery) using miRNA as a primer. Non-specific annealing is controlled by preserving the secondary structures of mRNAs and mRNA–protein complexes using RNase and protease inhibitors, thereby avoiding cDNA synthesis from altered double-stranded molecules.

Reverse transcriptase, like other DNA polymerases, does not initiate DNA synthesis without a primer strand to extend and a template strand to copy. Three decades ago, Harada and colleagues demonstrated that reverse transcriptase using an RNA molecule would successfully initiate Rous sarcoma virus DNA synthesis *in vitro*.[44] Thus, it seemed reasonable to assume that commercially available reverse transcriptases would initiate synthesis of cDNA molecules on a template of target mRNA, using miRNA(s) as a primer strand.

The major purpose of a first round of reverse transcription (RT#1) is to increase the homology of miRNA molecules to the targets by simply increasing their length, thereby creating 3′-cDNA–miRNA-5′ hybrid molecules. To avoid RNA degradation (Figure 1(a)), we divided the cytoplasmic extract into two equal parts, using the first part for routine RNA isolation and the second to create target specific 3′-cDNA–miRNA-5′ molecules, followed by the same RNA isolation procedure (Figure 1(a)). The RNA isolated from this second part was called primeRNA to reflect its possibly increased gene-specific priming activity.

In a second round of reverse transcription (RT#2), we used the 3′-cDNA–miRNA-5′ hybrid molecule to drive synthesis of cDNA from target mRNAs, therefore increasing the length of cDNA and making it suitable for detection (Figure 1(b)). Intact RNA and primeRNA are mixed and incubated at 96 °C for 5–10 min to denature any non-specific

duplexes between mRNA and other available polynucleotides. Next, the RNA mix is cooled carefully to 58 °C, allowing the molecules to re-anneal and to form double-stranded molecules between mRNA and long cDNA–miRNA hybrids. All other, non-specific short polynucleotides cannot form stable double-stranded complexes with target RNA, because they need lower temperatures to do so. According to published data, the target mRNA can carry multiple sites for single or even different miRNAs.[1,2,45,46] In this case, to detect all possible sites of miRNA interaction with its target, the ratio in the mRNA–primeRNA mix must be in favor of mRNA molecules, since competition for the target between hybrid molecules will complicate detection or even eliminate some existing miRNA–mRNA complexes. To maximize the interaction of primeRNAs, we used 10–20 parts of intact RNA mixed with one part of primeRNA.

After the denaturing/re-annealing step, the doubly concentrated enzyme mix containing reverse transcriptase, pre-warmed to 58 °C, was added to warm (58 °C) RNA mix, mixed quickly, and incubated for 30 min at 58 °C to extend the 3′-cDNA–miRNA-5′ hybrid molecule to the length suitable for detection by gene-specific PCR reaction (Figure 1(b)). The resulting mix of cDNA molecules was analyzed by several standard molecular and genetic techniques.

These results indicate that a 3′-cDNA–primeRNA-5′ hybrid molecule can be used as a primer to drive cDNA synthesis in a gene-specific manner (Figure 1(c)). Some genes, like *K-Ras* and *N-Ras*, appear to require a more prolonged RT#1 step (30 min). This may reflect the formation of complex secondary structures between mRNA, miRNA, and protein complexes. These hypothetical structures gradually open due to a high level of unblocked nuclease and proteinase activities. Other genes, like β-*actin*, are accessed more easily by the reverse transcriptase and PCR products can be obtained after 5 min of RT#1 (Figure 1(c)). As a negative control, we used cytoplasmic extract from cells treated with actinomycin D, which binds to DNA duplexes and interferes with the action of enzymes engaged in replication and transcription. We expected that actinomycin D-treated cells would have fewer mature miRNA molecules and, as a consequence, the priming activity of RNA in second round of RT would be decreased. Furthermore, lack of *de novo* RNA synthesis will lead to degradation of the available pool of mRNAs. This experiment may serve as a control to estimate self-priming resulting from RNA degradation. We used real-time PCR to estimate the priming activity on the ubiquitously expressed β-actin mRNAs. The primeRNA was prepared in an actinomycin D-treated cytoplasmic extract. Untreated RNA was used in RT#2 in a mixture with treated primeRNA. We found significantly reduced priming activity in RT#2 of primeRNA obtained from actinomycin D-treated cytoplasmic extract (Figure 1(c)). These results demonstrate that degraded RNAs do not contribute significantly to the priming activity observed in cytoplasmic extract prepared from cells *in vitro* and allowed optimization of the number of cycles in PCR to minimize the impact of background self and random priming.

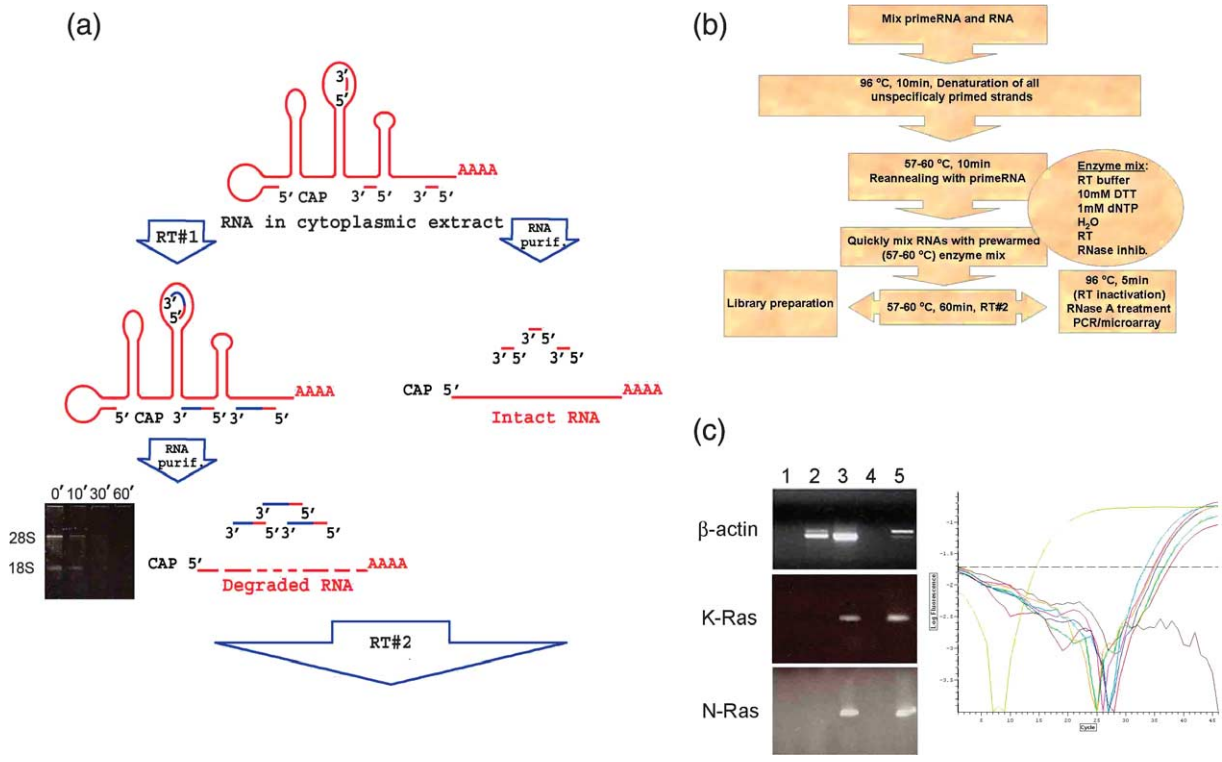

Figure 1 (*legend next page*)

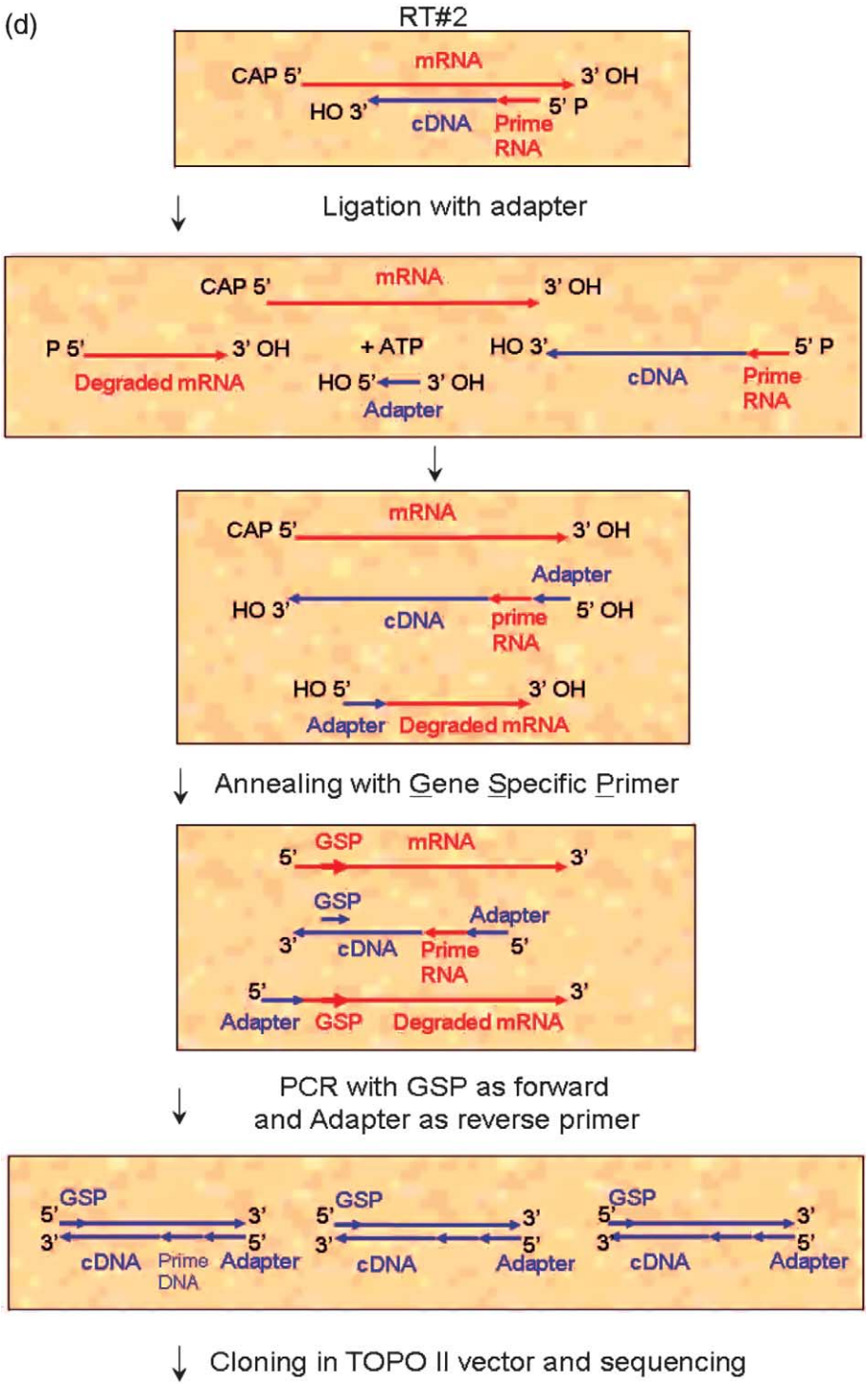

Figure 1. Schematic of the first and second reverse transcriptase reaction (RT#1 and RT#2). (a) RT#1 of fresh, cytoplasmic extracts creates 3′cDNA–miRNA-5′ hybids (left), while the other portion of the cytoplasmic extract is subjected to RNA purification for later use (right). (b) Second round of reverse transcription (RT#2) used to create long, gene-specific 3′-cDNA–miRNA-5′ hybrid molecules. (c) Left panel: detection of gene-specific 3′-cDNA–miRNA-5′ hybrid molecules after RT#2 by PCR for β-actin (top), K-Ras (middle), and N-Ras (bottom). In each gel, the following was done. Lane 1, control RT#2 was run with intact RNA combined with untranscribed sample (RT#1 without addition of reverse transcriptase in reaction mixture). Lane 2, experimental RT#2, 5 min. Lane 3, experimental RT#2 30 min. Lane 4, water. Lane 5, RT#2 with random primers, 30 min. (d) Strategy for 5′-end cloning of 3′-cDNA–miRNA-5′ hybrid molecules. (c) Right panel: real-time PCR detection of β-actin cDNA after standard protocol (RT#1/RT#2, yellow line) and using prime RNA from actinomycin D-treated cytoplasmic extract. The results of six different experiments are shown. The dark brown line is an empty (water alone) real-time PCR sample.

Strategy for 5′-end cloning of cDNA molecule

To demonstrate that cDNA synthesis was initiated from known, mature miRNAs and not from other polynucleotides unrelated to miRNAs, we used the following strategy to demonstrate that RT#1 was initiated from the miRNA primer on the template of a known, target mRNA molecule (Figure 1(d)). The total purified product of the RT#2 reaction was ligated to a known primer/oligonucleotide designated the Adapter. Intact mRNA molecules possess a CAP (m_7-Gppp) at the 5′-end and cannot ligate with the Adapter oligonucleotide. Mature miRNA molecules have 5′-phosphorylated ends without modification,[47,48] and can be ligated with Adapter. The resulting products start from the Adapter oligonucleotide on their 5′-end followed by miRNA, and then the gene-specific cDNA, as follows: 3′-cDNA–miRNA–Adapter-5′.

We used gene-specific primer (GSP) as the forward primer and primer sequence from the Adapter molecule to amplify the products of ligation in a gene-specific manner. Only cDNA molecules containing sequence complementary to GSP and ligated with Adapter become amplified (Figure 1(d)). It is important to recognize that Taq polymerase has a low, but sufficient reverse transcriptase activity to push synthesis of cDNA through a short stretch of a complementary RNA primer.[49] Products were cloned into the TOPO II vector and analyzed by restriction nuclease digestion, followed by direct sequencing. Using this approach, we determined the location of miRNA molecules, because cloned molecules became "polarized", beginning with Adapter, followed by the miRNA sequence, then the gene-specific cDNA, and terminating with the GSP. Furthermore, because miRNAs form partially complementary adducts with the target mRNA, one would expect to find single mismatches in the 5′-end of resulting cDNA molecules, which we did (Figure 2).

Signatures of miRNA molecules are detected on their targets

To validate this method of identifying miRNA molecules, we applied this technique to detect miRNAs that specifically bind to β-actin, K-Ras and N-Ras mRNAs. Figure 2 shows the informative sequences of cDNA molecules that originated after amplification of ligated products. These cDNAs were found to have 5′-ends in the region adjacent to the stop codon of tested mRNAs. To evaluate potential homologies of cDNA 5′-ends to known, mature miRNAs, we searched the Sanger miRNA website†.

As expected, homology between resultant cDNAs 5′-end and β-actin, K-Ras and N-Ras mRNAs was not perfect. We found a group of cDNAs with sequences almost identical with β-actin mRNA (ten nucleotides out of 12), except at nucleotides at positions 9 and 12, starting from the first non-Adapter nucleotide. A search of the miRNA database revealed that this 12 nt miRNA has homology to hsa-mir-129, with one mismatch at position 6 (hsa-mir-129 has guanine while the RNA deduced from cDNA has cytosine at this position (Figure 2)). The G at position 12 can form a hydrogen bond with U in β-actin mRNA. Analysis of another group of cDNAs, demonstrated that several cloned cDNAs have a 12 nucleotides stretch similar to the 3′ end of hsa-mir-32, with two mismatches (Figure 2).

Analysis of cDNAs for K-Ras mRNA revealed several clones highly similar to K-Ras cDNA. A search of the miRNA database revealed a 12 nt sequence with homology to hsa-mir-33, with two mismatches, at positions 1 and 8. This possible miRNA-binding site covers the stop codon of K-Ras mRNA, similar to hsa-mir-32 in β-actin mRNA. In addition, another cDNA set contained a 13 nt stretch similar to hsa-mir-137, with one mismatch at the 5′end (Figure 2). In accord with published data, we detected the signature of let-7a miRNA, represented by the first 11 nt of let-7a, starting from the 5′ end of the miRNA, as predicted.[50] Next, we checked whether there is a binding site for let-7a at the predicted position, distant from the stop codon; we detected the signature of let-7a miRNA at position 2708–2724, with 17 nt homology to let-7a.

Analysis of cDNAs after using gene-specific primers for N-Ras mRNA revealed several target cDNAs. Search of the miRNA database demonstrated one 13 nt miRNA conforming to hsa-mir-375. A second group of cDNAs revealed a 13 nt stretch with homology to the 5′-end of hsa-mir-452 (Figure 2). Finally, we detected the signature of let-7a miRNA, represented by the first 13 nt from the 5′ end of the miRNA, at the position predicted.[50] We did not detect let-7 miRNA or other miRNA members of the let-7 family at other, bioinformatically predicted positions. The detected sites contain let-7 miRNA in less strained conformations compared to other predicted complexes, which may be more difficult to detect by our proposed technique.

The divergence between the cloned cDNA and the miRNAs could be explained by either the presence of a functional miRNA polymorphism or by the existence of an as yet unidentified miRNA species with partial similarity to the detected miRNA signatures.[51,52] The percentage of cDNA sets with strong (let-7a like) or partial homology to species in the Sanger miRNA database was approximately 20%. Other groups of cloned cDNA molecules did not fit any published miRNA sequences and may represent either cDNA molecules that originate from self-priming (cDNA synthesis from broken mRNA itself) or miRNA molecules that have not been described.[51] Thus, our technique detected small but unique 10–14 nt portions of several known miRNAs, which we refer to as miRNA signatures.

† microrna.sanger.ac.uk

Figure 2. Deduced and sequence-verified miRNAs. The black font corresponds to β-actin cDNA, RefSeq# NM_001101, K-Ras, NM_004985 and N-Ras NM_002524. The blue font corresponds to cloned and analyzed cDNAs, the green font corresponds to deduced RNA sequences and corresponding miRNAs. Upper underlined nucleotides have no homology between target and experimental cDNAs. The lower underlined green sequences are RNAs complementary to cDNAs and show no homology between it and known miRNAs. Numbers in parentheses correspond to the number of cDNA molecules analyzed. Sequences highlighted with yellow are let-7 predicted sites.[50] The red font indicates a stop codon in mRNA.

Functional analysis

According to current concepts, interaction of miRNA molecules with their targets causes post-transcriptional silencing *via* transcript degradation or harnessing the translational machinery.[3] One would expect that imposed expression of miRNAs may diminish protein synthesis from target mRNA. To evaluate whether the miRNAs we identified were functional, we performed several validation tests. While the detected miRNA signatures are not fully identical with known miRNAs, to facilitate the functional analysis and to avoid possible complications with proper processing of artificial pre-miRNA, we decided to clone into an expression vector previously identified pre-miRNAs under control of the CMV promoter.

First, we sought to validate that transfection of our miRNAs produced detectable amounts of mature miRNA species. Plasmids were transfected in hTERT-RPE1 cells; four days later, RNA and protein fractions were isolated and analyzed.

Mature miRNAs were detected in all cases (Figure 3(a)). Next, we checked whether expression of target miRNAs altered mRNA levels of β-actin, K-Ras, and N-Ras (Figure 3(b) and (c)). Negative control samples (cells transfected with a neutral, miRNA-like molecule) displayed similar amounts of mRNA, while transfection with miRNAs decreased the levels of mRNA only minimally (Figure 3(b) and (c)). As a control, the level of GAPDH mRNA was checked; all control and

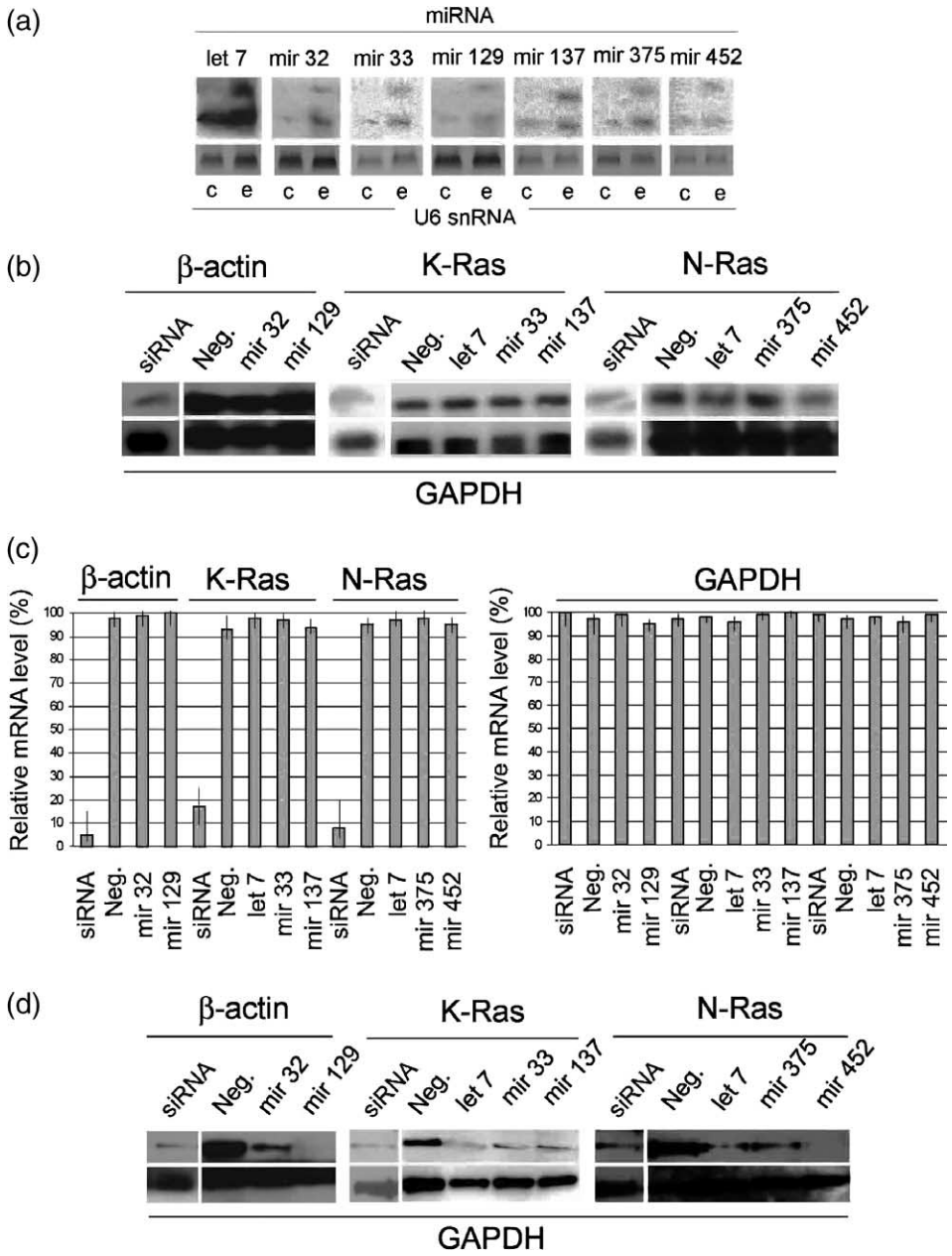

Figure 3. Regulation of β-actin, K-Ras and N-Ras expression by miRNA molecules caught on their mRNAs. (a) Detection by Northern blotting of mature miRNA and pre-miRNA molecules expressed from the indicated plasmid. Upper panels: detection of miRNA expression in control,c, untreated cells and in cells transfected with plasmid expressing corresponding to pre-miRNA, e. Lower panel: Detection of U6 snRNA in the same samples used as a loading control. (b) Upper panel: levels of β-actin, K-Ras and N-Ras mRNAs after transfection with siRNA targeted against the corresponding mRNA or miRNA expressing plasmids detected by Northern blotting. (b) Lower panel: RNA loading control, re-probing the same membrane with probe recognizing GAPDH mRNA revealed a similar amount of this transcript in all samples. (c) Levels of β-actin, K-Ras and N-Ras mRNAs detected by real-time PCR, data are normalized using 18 S rRNA levels. Left panel: detection of β-actin, K-Ras and N-Ras cDNA after transfection with siRNA targeting the corresponding mRNA or with miRNA-expressing plasmids. Right panel: the same samples with primers for amplification of GAPDH cDNA confirms equal amounts of transcript in all samples. (d) Upper panel: protein levels of β-actin, K-Ras and N-Ras after transfection with siRNA targeted against the corresponding mRNA or miRNA-expressing plasmids detected by Western blotting. Lower panel: control of protein loading, re-probing the same membranes with monoclonal antibodies against GAPDH.

experimental samples had similar amounts of GAPDH mRNA (Figure 3(b) and (c)).

We confirmed the functional activity of our target miRNAs at the protein level. β-Actin was readily down-regulated by over-expression of hsa-mir-32 or hsa-mir-129 (Figure 3(d)). The higher level of complementarity between hsa-mir-129 and its target mRNA appears to effect a higher degree of protein suppression than does hsa-mir-32. GAPDH protein levels remain stable in all conditions, confirming the specificity of the miRNAs detected (Figure 3(d)). Nonetheless, off-target effects of miRNAs have been described.[53,54] This prompted us to evaluate whether miRNA over-expression had a more global influence on the pattern of protein expression. To obtain a rough estimation of this phenomenon, we performed 2-D gel protein electrophoresis on control cells hTERT-RPE1 and cells transfected with hsa-mir-32 and hsa-mir-129 (Supplementary Data, Figure 1). We found only minor differences that are likely a consequence of β-actin protein down-regulation itself.

Transfection of cells with miRNAs targeted specifically to K-Ras and N-Ras mRNAs resulted in similar outcomes. Northern blot analysis did not display any change in mRNA levels of K-Ras or N-Ras. Western blot analysis showed that over-expression of all miRNAs detected by our method, including the previously characterized, functional let-7a miRNA, decreased the expression level of K-Ras and N-Ras proteins significantly (Figure 3(d)).

We then performed immuno-fluorescence staining of the cells transfected with these miRNA. Immuno-fluorescence staining of cells transfected with has-mir-32 and 129 revealed a significant decrease in β-actin levels as well as a profound spatial re-distribution of the sparse, remaining β-actin filaments when compared with cells transfected with negative control plasmid (Figure 4). Control cells possess well-organized β-actin filaments, which fill the cytoplasm. Transfection with plasmid expressing let-7a miRNA did not change the levels of β-actin protein or its intracellular distribution. A similar effect was observed after over-expression of miRNAs targeting K-Ras and N-Ras mRNAs. The antibodies against K-Ras or N-Ras could not detect any recognizable amount of K-Ras or N-Ras proteins, while control cells maintained normal levels of these proteins.

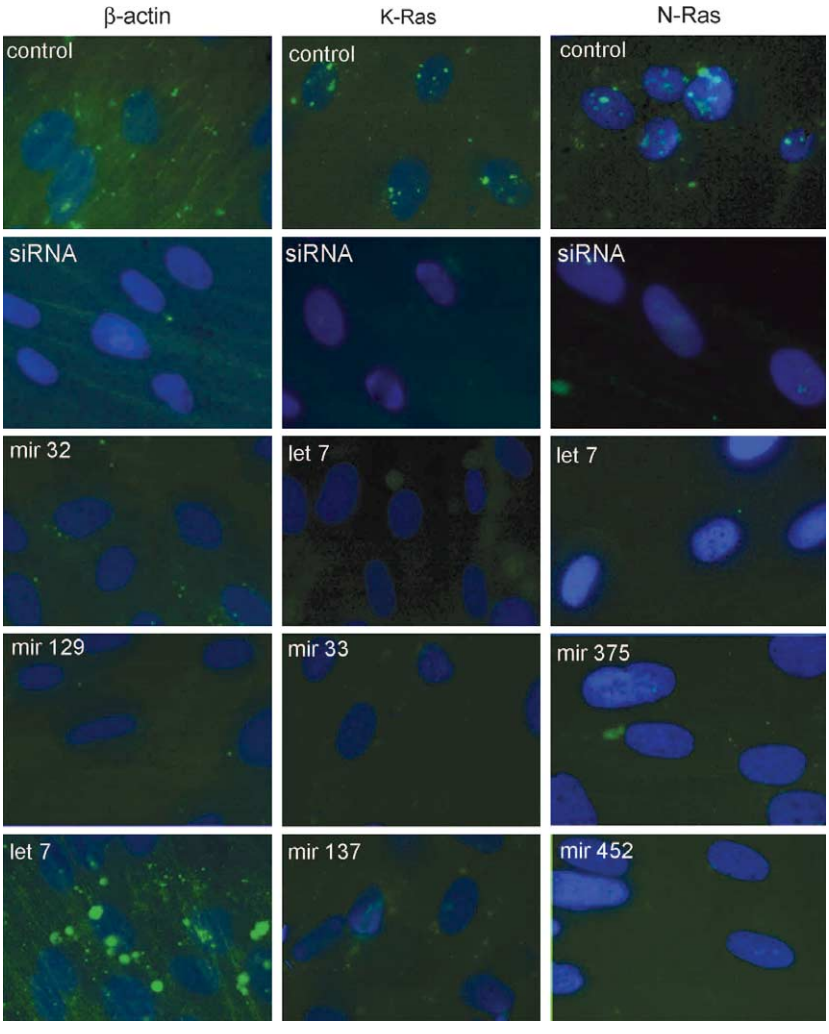

Figure 4. Immuno-fluorescence analysis of β-actin, K-Ras and N-Ras proteins expression after transfection with miRNA expressing plasmids. Left panel: staining with mouse-anti-β-actin monoclonal antibodies. Middle panel: staining with mouse-anti-K-Ras monoclonal antibodies. Right panel: staining with mouse-anti-N-Ras monoclonal antibodies. Secondary antibody in all cases was FITC-labeled goat-anti-mouse antibody. DAPI staining to display nuclei was overlaid on the immunofluorescence images. Top panels: negative control vector transfection.

As a final, important step to confirm the specificity of miRNA:mRNA target recognition, we performed a heterologous reporter assay (Figure 5). The identified miRNA target sites were cloned into a luciferase reporter gene construct, which was transfected into hTERT-RPE1 cells. First, we transfected luciferase constructs to evaluate whether miRNA can regulate the expression of luciferase from unmodified construct, without the addition of regulatory modules. We found that all miRNAs tested had no or minimal influence on the expression of luciferase protein expressed from unmodified mRNA (Figure 5). Next, we transfected constructs with luciferase and the corresponding miRNA regulatory site into cells along with co-transfection of plasmid expressing the corresponding miRNA molecule. In all cases, the level of luciferase activity was decreased, from 2.5-fold to sevenfold. These results indicate that miRNA target sites cloned behind a reporter are sufficient for miRNA-dependent regulation and demonstrate a direct effect of a particular miRNA on a target. In addition, we checked whether expression of endogenous miRNA could control the expression of the artificial construct. To our surprise, the luciferase activity was decreased, which indicates that the detected regulatory motifs are able to control the expression of luciferase on a background of normally expressed miRNA molecules. The ability of endogenous miRNA to regulate the expression of transfected, heterologous luciferase construct containing miRNA-binding sites has been demonstrated recently, which supports the results reported here.[55]

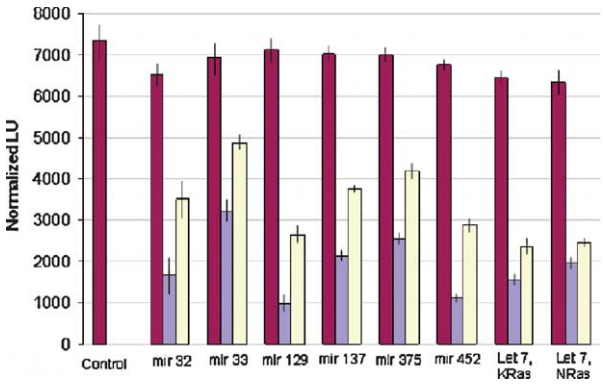

Figure 5. Effect of miRNA binding sites on expression of luciferase protein. Purple bars correspond to control luciferase construct without addition of miRNA binding sites: control, cells transfected with unmodified luciferase construct alone; mir 32–mir 452, let 7-co-transfection of unmodified luciferase construct with pre-miRNA-expressing vectors. Blue bars: co-transfection of luciferase construct with addition of miRNA-binding sites with corresponding pre-miRNA expressing plasmids. mir 32-hsa-mir-32 site and over-expressed hsa-mir-32, mir 33-hsa-mir-33 site and over-expressed hsa-mir-33, mir 129-hsa-mir-129 site and over-expressed hsa-mir-129, mir 137-hsa-mir-137 site and over-expressed hsa-mir-137, mir 375-hsa-mir-375 site and over-expressed hsa-mir-375, mir 452-hsa-mir-452 site and over-expressed hsa-mir-452, Let 7 K-Ras-let-7 site from K-Ras mRNA and over-expressed let-7a, Let 7, N-Ras–let 7 site from N-Ras mRNA and over-expressed let 7a. Yellow bars, transfection of luciferase construct with addition of miRNA-binding sites, evaluation of miRNA endogenous activity. The results are presented as the mean of assays performed in triplicate; error bars represent the standard error.

Discussion

We have described a novel method to detect functional miRNAs on their target mRNAs. The method is based on synthesis of cDNA on a template of target mRNA in freshly prepared cytoplasmic extract, using miRNA as a primer. The resultant 3′-cDNA–miRNA-5′ hybrid molecules are then used as a highly target-specific primer to initiate synthesis of detectable cDNA in a second reverse transcription reaction. This strategy bypasses the low-complementary interaction of mature miRNA with its target. The major purpose of the second round of reverse transcription (RT#2) is to increase the length of cDNA molecules created in RT#1, since conformational hindrances found in the preserved miRNA–mRNA–protein complex after the first round of reverse transcription generally precluded detection or cloning of the 3′-cDNA–miRNA-5′ constructs.

Pilot experiments demonstrated that this method generated cDNA molecules that were detected easily by PCR (Figures 1(c)). cDNA was never detected in intact RNA or RNA mixed with sample without addition of reverse transcriptase. We used this technique to detect the cDNAs of β-actin, K-Ras and N-Ras, which suggests that the expression of these genes might be controlled by miRNAs.

Our second goal was to confirm that cDNA synthesis was initiated from miRNAs (Figure 1(d)). Using the modified 5′-rapid amplification of cDNA ends (RACE) technique (Invitrogen), we developed a method to analyze the sequences of cDNA molecules originated after RT#2. We chose β-actin as a model mRNA to analyze first, for several reasons: its RNA and protein are abundant in nearly all cell types, its steady-state level of expression is consistent, and its mRNA sequence is relatively conserved during evolution, which makes the use of this method in other model systems relatively easy. On the other hand, we examined K-Ras and N-Ras, since expression of both has been shown recently to be functionally regulated by let-7 miRNA.[50] In agreement with previously published data showing that miRNAs bind their targets mostly in the region of the 3′-untranslated region,[1,2,56,57] we found that most of the cDNAs we identified were localized to the 3′-end of the mRNA studied, a region adjacent to the stop codon. While some experimental and bioinformatics evidence suggests miRNA 3′ends may not bind well to target mRNA, thereby limiting the efficiency of endogenous miRNA to serve as efficient primers for our RT reaction, we nonetheless found in a variety

of known, functional miRNAs that this was not the case, as suggested also in the recent report by Bagga *et al*.[58] We first searched the Sanger miRNA database and identified homology between several of the cDNAs we sequenced and known, mature miRNAs, including hsa-mir129 and hsa-mir-32, which appeared to target β-actin mRNA (Figure 2). While hsa-mir-32 binds β-actin mRNA exactly at the stop codon, the hsa-mir-129 binding site is found within the coding region, more than 100 nt upstream of the stop codon, a feature that may influence protein expression, as we found (Figures 3(c) and 4). We extended our method to analysis of K-Ras and N-Ras mRNAs. We detected cDNAs located close to the stop codons of both mRNAs and detected miRNAs of a let-7 family member, as others have suggested,[50] as well as several additional miRNAs, including hsa-mir-33 and 137, which interact with K-Ras mRNA near the stop codon at the 3′end, and hsa-mir-375 and 452 on N-Ras. All of these miRNAs identified with our method appear to have a functional effect on K-Ras and N-Ras.

Interestingly, our method detected miRNA signatures, 10–14 nt long that belonged to functional miRNA molecules. The miRNA signatures we observed may be the result of continued processing of miRNAs during interaction with their target. The 10–14 nt domains responsible for effectual interaction in the miRNA–mRNA complex that we identified can be localized to the middle, at the 3′ end and, possibly, at the 5′ end of the miRNA molecule. This signature may represent the functional seed of the miRNA molecule.[40] Alternatively, this 10–14 nt signature could be a result of interactions between messenger RNA and miRNA machinery presented by the RNA-induced silencing complex protein complex. In cases of partial homology of endogenous miRNA to target mRNA, smaller stretches, perhaps as small as the 10–14 nt signature we observed, may be responsible for post-transcriptional silencing mediated by miRNAs. These nucleotides likely represent the functional center responsible for target recognition, and miRNA–mRNA intermolecular interaction. Investigation of the potential correlation between signature content and miRNA functionality is in progress.

It must be mentioned that some of the miRNA signatures identified have minor mismatches with their possible mature, source miRNA molecules. We believe this discrepancy may arise from potential polymorphisms within a miRNA gene family or from possible underestimation of the number of such genes; our results with let-7 miRNA are consistent with these potential situations. According to recent work, the total number of miRNA molecules could be much greater than previously believed.[51] Several of the sets of cDNA sequences we obtained in our experiments were excluded from analysis due to lack of homology to known miRNAs. However, these may represent novel cDNAs derived from unidentified miRNA, although random self-priming is also a possibility. Due to difficulties in expressing some artificial mi/siRNA molecules, we plan to explore this question in future work.

Finally, functional analysis confirmed that our method detects the footprints of functional miRNA molecules (Figures 3–5). Western blotting, immunofluorescence staining, and luciferase reporter assays revealed significant protein down-regulation after over-expression of full-length, mature miRNAs. Over-expression of hsa-mir-129, located about 100 nucleotides 5′ of the stop codon, resulted in dramatic β-actin protein down-regulation. Over-expression of hsa-mir-32, which overlaps the stop codon, had a similar, but less-pronounced repressive effect on β-actin protein expression. Expression of hsa-mir-33, 137 and let-7a, whose target sites were detected on K-Ras, and hsa-mir-375, 452 and let-7a, which were found to interact with N-Ras, correspondingly repressed the translation from target mRNAs (Figure 3). These results were confirmed in a heterologous reporter assay, where 2.5-fold to sevenfold repression was seen. Furthermore, immuno-fluorescence staining showed an alteration in both the amount and distribution of protein following transfection of miRNAs. This appears to work at the level of translational repression, since Northern blotting revealed that β-actin mRNA levels are not affected by over-expression of hsa-mir-32 or hsa-mir-129 (Figure 3(b) and (c)). Meanwhile, similar decreases in protein expression were seen for both K-Ras and N-Ras after miRNA transfection (Figures 3(d) and 4). GAPDH mRNA and protein levels were unaffected by transfection of either miRNA or control plasmid.

In summary, this method identifies specific interactions of miRNA with its target accurately and reproducibly, and can identify functional 10–14 nt miRNA signatures, which are able to down-regulate target protein expression. This technique is a simple, efficient, and tractable method to identify miRNA targets in different cellular systems.

Materials and Methods

Reverse transcription reactions #1 and 2

hTERT-RPE1 cells (Clontech, Mountain View, CA) were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin/streptomycin/neomycin mix, Fungizone (Invitrogen, Carlsbad, CA). Before cytoplasmic extract isolation, 1×10^7 cells were washed twice with cold (4 °C) PBS; scraped from the tissue flask in 10 ml of 4 °C PBS; centrifuged for 10 min at 200*g* at 4 °C; re-suspended in 1 ml of cold PBS; centrifuged again at 200*g* at 4 °C. The supernatant was carefully removed. In some cases, for generation of negative control samples, cells were pretreated overnight with 5 μg ml^{-1} actinomycin D (Sigma-Aldrich, St. Louis, MO).

Cytoplasmic extract was isolated using the NE-PER kit (Nuclear and Cytoplasmic) (Pierce, Rockford, IL) according to the manufacturer's protocol. The final volume

was 210 μl. The extract was divided into three equal parts: one was used for RT#1, the second part was used as a control and the third was saved for isolation of intact template RNA. RT#1 was performed as follows: 70 μl of cytoplasmic extract were transferred into a chilled tube and successive reagents were added at the indicated final concentrations (or units of activities): RNaseOUT 2units, 5×RT buffer, 5 mM DTT, 0.5 mM dNTP, Superscript Reverse Transcriptase III 200units; all from Invitrogen. The reaction was run for 20 min at 37 °C. The control sample contained the same set of reagents except reverse transcriptase. At completion, 1 ml of TRIzol reagent (Invitrogen) was added. RNA was extracted according to the manufacturer's protocol simultaneously from all samples. The RNA pellets were re-suspended in 50 μl of diethyl pyrocarbonate-treated water.

RT#2 was performed next: 10 μg of intact RNA was mixed with 1 μg of either primeRNA or RNA isolated from the control sample. As an additional control of that RT reaction, intact RNA was mixed with 1 ng of random primers. RNA mixtures were heated to 96 °C for 5 min, and then chilled to 58 °C. Positive control samples containing RNAs with random primers were chilled to room temperature and keep for at least 10 min to allow annealing of random primers to RNA. While RNA mixtures were being denatured/annealed, enzyme mix was prepared and warmed at 58 °C for at least 5 min: 2units of RNase OUT in 2×RT buffer, 5 mM DTT, 0.5 mM dNTP, SuperScript Reverse Transcriptase III (all Invitrogen) and water. After that, RNA and pre-warmed enzyme cocktail was mixed quickly and thoroughly. The reaction was run for 30 min at 58 °C and terminated by heating (85 °C, 5 min). A sample (2 μl) of the reaction mix used for control PCR amplification was treated with 0.1 mg ml^{-1} RNase A (Roche, Indianapolis, IN) at room temperature for 10 min. The PCR mix was prepared as following: 2×B PCR buffer (Epicentre, Madison, WI), water, 2units of Tag-polymerase (Invitrogen), cDNA (0.05 of RT reaction volume). The procedure was: 94 °C for 5 min→(94 °C for 30 s, 65 °C for 30 s, 72 °C for 45 s)×40→72 °C for 5 min→storage at 4 °C. Primer sequences are available as Supplementary Data.

To clone cDNA–primeRNA hybrid molecules, polynucleotides from samples after RT#2 were purified using the QIAGEN PCR purification kit (QIAGEN, Valencia, CA). The ligation reaction was comprised of the following: primeRNA, 10 μM Adapter, 10× ligation buffer, 1 μM ATP, 10units of bacteriophage T4 RNA ligase (New England Biolab, Ipswich, MA), 10% (v/v) DMSO, water to a final volume of 20 μl, 37 °C for 2 h, and purified with the QIAGEN PCR purification kit. Pre-amplification was performed with forward GSP and primer from Adapter oligonucleotide. After 10–15 cycles, samples were treated with 0.1 mg ml^{-1} RNase A at room temperature for 10 min. The PCR reaction was performed with forward nested GSPs and nested primer from Adapter oligonucleotide as reverse primer: 94 °C for 5 min→(94 °C for 30 s, 62 °C for 45 s, 72 °C for 15 min)×40→72 °C for 5 min→storage at 4 °C. Primer sequences are available as Supplementary Data.

Plasmids for expression of mature miRNAs and transfection

The sequences of pre-miRNA molecules hsa-mir-32, 33, 129, 137, 375, 452 and let-7a were taken from the Sanger web site and cloned in pSilencer 4.1 plasmid (Ambion, Austin, TX) according to the manufacturer's protocol. The final products were sequenced before any further applications. Transfection of plasmids into the cells was performed using Fugene 6 reagent (Roche) according to the manufacturer's protocol. In brief, cells were placed in six-well tissue-culture plate (500,000 cells per well) and the next day 1 μg of negative control plasmid (Ambion) or plasmid, which contains pre-miRNA, was used for transfection. RNA and protein fractions were isolated simultaneously at 96 h after transfection using standard techniques.

Northern analysis

For Northern analysis of miRNA expression from the plasmids, 20 μg of RNA was separated on a 10% TBE-urea gel (Invitrogen). RNA was transferred to nitrocellulose membrane (Invitrogen) by semi-dry transfer technique in 0.5× TBE at 200 mA. The membrane was air-dried and hybridized with ^{32}P-labeled probe generated by 5′-end phosphorylation of oligonucleotides complementary to mature miRNA molecules or U6 snRNA in ULTRAHyb-oligo buffer (Ambion) overnight at 35 °C. After that, the membrane was washed in 2×SSC, 0.1% (w/v) SDS and the signal was detected on BioMax MS film (Kodak, Rochester, NY). To analyze the β-actin, K-Ras, N-Ras and GAPDH mRNAs expression, 2 μg of RNA was separated on 1% (w/v) agarose gel using a glyoxal-based system (Ambion). RNA was transferred to the nitrocellulose membrane in 2×SSC, cross-linked with UV and hybridized with isotope-labeled probes in ULTRHyb buffer (Ambion) generated using the random primer kit Prime-It II (Stratagene, Cedar Creek, TX) at 45 °C for 3 h. Then, the membrane was washed in 2×SSC, 0.1% SDS and the signal was detected on BioMax MS film.

siRNA

siRNAs for target gene silencing of β-actin, K-Ras and N-Ras were obtained from Ambion. Transfection optimization was performed according to the manufacturer's protocol.

Real-time PCR

Total RNA was isolated using the QIAGEN RNeasy kit according to the manufacturer's protocol; 2 μg of RNA was used for synthesis of cDNA by SuperScript RT system (Invitrogen). Samples were treated with RNase A and an equal amount of sample was used for real-time PCR. Reactions were performed using IQ™ SYBR® Green supermix (Bio-Rad laboratories, Hercules, CA) according to the manufacturer's protocol. For comparative analysis of Northern blotting and real-time PCR results, data were normalized using 18 S rRNA levels. The extent of target gene knockdown is expressed as a percentage of mRNA remaining in cells treated with gene-specific siRNA or miRNA compared to cells treated with negative control, random miRNA. Primer sequences are located in the Supplementary Data.

Western and immuno-fluorescence analysis

The concentration of protein in each sample was measured with a Micro BCA (MB) protein assay reagent kit (Pierce). A sample of protein (5 μg) was separated by electrophoresis on a NuPAGE 10% Bis-Tris gel (Invitrogen). Proteins were transferred to nitrocellulose membrane by a semi-dry transfer technique. Membrane was blocked with TBE, 3% (v/v) non-fat milk with 0.05%

(v/v) Tween 20 for 1 h at room temperature. After that, the membrane was incubated with mouse-anti-β-actin and GAPDH antibodies (Ambion) diluted 1/20,000, for 30 min at room temperature. To detect K-Ras and N-Ras proteins, expression membranes were incubated with mouse-anti-K-Ras or mouse-anti-N-Ras antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1/1000, overnight at 4 °C. Membranes were washed and incubated with secondary, goat-anti-mouse-HRP antibodies (Pierce) diluted 1/20,000, for 30 min at room temperature. They were washed five times more and incubated in SuperSignal West Pico (Pierce). The signal was detected on BioMax MS film (Kodak).

Immuno-fluorescence staining was performed as follows. Live cells were washed with PBS and fixed at room temperature for 20 min with 4% (v/v) *para*-formaldehyde prepared in PBS. After that, cells were permeabilized by incubation for 20 min at room temperature with PBS, 0.1% (v/v) Triton X-100. Cells were then incubated with mouse-anti-β-actin antibodies (Ambion) diluted 1/1000, for 30 min at room temperature or with mouse-anti-K-Ras or mouse-anti-N-Ras antibodies (Santa Cruz Biotechnology Inc.) diluted 1/100, overnight at 4 °C. Cells were washed and incubated with secondary, goat-anti-mouse-FITC antibodies diluted 1/5000, for 30 min at room temperature, washed once more with PBS, placed in anti-bleach/4′,6-diamidino-2-phenylindole solution, covered with a coverslip, and analyzed.

Luciferase reporter assay

The sequences of miRNAs target sites (see Supplementary Data) were cloned into the pMIR-REPORT miRNA expression vector (Ambion), which contains the firefly luciferase expression vector, according to the manufacturer's protocol. The final products were sequenced and confirmed prior to transfection. Transfection of plasmids into the cells was performed using Fugene 6 reagent (Roche) according to the manufacturer's protocol. In brief, cells were placed in 24-well tissue-culture plate (50,000 cells per well) and the next day cells were transfected with 0.1 μg of control plasmid pRL-TK (Promega, Madison, WI) expressing *Rr*-luciferase along with 0.1 μg of pMIR-REPORT carrying the corresponding miRNA binding site and with or without 0.1 μg of pSilencer 4.1 vector expressing pre-miRNA to be tested. Firefly and renilla luciferase activities were quantified simultaneously using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's protocol; assays were performed in triplicate.

Two-dimensional gel electrophoresis

Electrophoresis in the first dimension was performed on a Protean IEF system (Bio-Rad, Hercules, CA) using 11 cm immobilized pH gradient (IPG) strips (pH 3–10). The IPG strips were rehydrated with 200 μg of protein in rehydration buffer (1% Chaps, 7 M urea, 2 M thiourea, 1% DTT, 1% (v/v) ampholytes, 1% Triton X-100) for 14 h at 50 V. Isoelectric focusing was conducted using a rapid voltage ramp; 250 V for 15 min, 6000 V for 3 h, 6000 V for 5.45 h and maintained at 500 V, as needed. Following isoelectric focusing, the IPG strips were equilibrated for 15 min in 10 ml of reducing reagent (50 mM Tris–HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 1% DTT) followed by a second 15 min equilibration in 10 ml of alkylating reagent (50 mM Tris–HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 4% (v/v) iodoacetamide). Electrophoresis in the second dimension was performed using 12.5% (w/v) Criterion pre-cast polyacrylamide gels (Bio-Rad) at 200 V for 80 min. The gels were stained with silver nitrate.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2006.02.063

References

1. Lee, R. C., Feinbaum, R. L. & Ambros, V. (1993). The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, **75**, 843–854.
2. Wightman, B., Ha, I. & Ruvkun, G. (1993). Post-transcriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell*, **75**, 855–862.
3. Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, **116**, 281–297.
4. Ambros, V. (2001). MicroRNAs: tiny regulators with great potential. *Cell*, **107**, 823–826.
5. Ambros, V. (2004). The functions of animal microRNAs. *Nature*, **431**, 350–355.
6. Cullen, B. R. (2004). Derivation and function of small interfering RNAs and microRNAs. *Virus Res.* **102**, 3–9.
7. de Moor, C. H., Meijer, H. & Lissenden, S. (2005). Mechanisms of translational control by the 3′ UTR in development and differentiation. *Semin. Cell Dev. Biol.* **16**, 49–58.
8. Dennis, C. (2002). The brave new world of RNA. *Nature*, **418**, 122–124.
9. McManus, M. T. & Sharp, P. A. (2002). Gene silencing in mammals by small interfering RNAs. *Nature Rev. Genet.* **3**, 737–747.
10. McManus, M. T. (2003). MicroRNAs and cancer. *Semin. Cancer Biol.* **13**, 253–258.
11. Lu, J., Getz, G., Miska, E. A., varez-Saavedra, E., Lamb, J., Peck, D. *et al*. (2005). MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834–838.
12. Gregory, R. I. & Shiekhattar, R. (2005). MicroRNA biogenesis and cancer. *Cancer Res.* **65**, 3509–3512.
13. Gallant, P. (2005). Protein degradation, signaling, microRNAs and cancer. *Genome Biol.* **6**, 316.
14. Zhao, Y., Samal, E. & Srivastava, D. (2005). Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature*, **436**, 181.
15. Hatfield, S. D., Shcherbata, H. R., Fischer, K. A., Nakahara, K., Carthew, R. W. & Ruohola-Baker, H. (2005). Stem cell division is regulated by the microRNA pathway. *Nature*, **435**, 974.

16. O'Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V. & Mendell, J. T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. *Nature*, **435**, 839–843.
17. Sullivan, C. S., Grundhoff, A. T., Tevethia, S., Pipas, J. M. & Ganem, D. (2005). SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature*, **435**, 682–686.
18. Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J. *et al*. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, **433**, 769–773.
19. John, B., Enright, A. J., Aravin, A., Tuschl, T., Sander, C. & Marks, D. S. (2004). Human microRNA targets. *PLoS. Biol.* **2**, e363.
20. Gregory, R. I. & Shiekhattar, R. (2005). MicroRNA biogenesis and cancer. *Cancer Res.* **65**, 3509–3512.
21. Denli, A. M., Tops, B. B., Plasterk, R. H., Ketting, R. F. & Hannon, G. J. (2004). Processing of primary microRNAs by the microprocessor complex. *Nature*, **432**, 231–235.
22. Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. & Shiekhattar, R. (2004). The microprocessor complex mediates the genesis of microRNAs. *Nature*, **432**, 235–240.
23. Mattick, J. S. & Makunin, I. V. (2005). Small regulatory RNAs in mammals. *Hum. Mol. Genet.* **14**, R121–R132.
24. Baskerville, S. & Bartel, D. P. (2005). Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA*, **11**, 241–247.
25. Ying, S. Y. & Lin, S. L. (2005). Intronic microRNAs. *Biochem. Biophys. Res. Commun.* **326**, 515–520.
26. Ying, S. Y. & Lin, S. L. (2004). Intron-derived microRNAs—fine tuning of gene functions. *Gene*, **342**, 25–28.
27. Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W. & Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* **20**, 6877–6888.
28. Hutvagner, G. & Zamore, P. D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science*, **297**, 2056–2060.
29. Doench, J. G. & Sharp, P. A. (2004). Specificity of microRNA target selection in translational repression. *Genes Dev.* **18**, 504–511.
30. Stark, A., Brennecke, J., Russell, R. B. & Cohen, S. M. (2003). Identification of Drosophila microRNA targets. *PLoS. Biol.* **1**, e60.
31. Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P. & Burge, C. B. (2003). Prediction of mammalian microRNA targets. *Cell*, **115**, 787–798.
32. Kiriakidou, M., Nelson, P. T., Kouranov, A., Fitziev, P., Bouyioukos, C., Mourelatos, Z. & Hatzigeorgiou, A. (2004). A combined computational-experimental approach predicts human microRNA targets. *Genes Dev.* **18**, 1165–1178.
33. Bonnet, E., Wuyts, J., Rouze, P. & Van de, P. Y. (2004). Detection of 91 potential conserved plant microRNAs in *Arabidopsis thaliana* and *Oryza sativa* identifies important target genes. *Proc. Natl Acad. Sci. USA*, **101**, 11511–11516.
34. Grad, Y., Aach, J., Hayes, G. D., Reinhart, B. J., Church, G. M., Ruvkun, G. & Kim, J. (2003). Computational and experimental identification of *C. elegans* microRNAs. *Mol. Cell*, **11**, 1253–1263.
35. Rehmsmeier, M., Steffen, P., Hochsmann, M. & Giegerich, R. (2004). Fast and effective prediction of microRNA/target duplexes. *RNA*, **10**, 1507–1517.
36. Burgler, C. & Macdonald, P. M. (2005). Prediction and verification of microRNA targets by MovingTargets, a highly adaptable prediction method. *BMC. Genomics*, **6**, 88.
37. Saetrom, O., Snove, O., Jr & Saetrom, P. (2005). Weighted sequence motifs as an improved seeding step in microRNA target prediction algorithms. *RNA*, **11**, 995.
38. Adai, A., Johnson, C., Mlotshwa, S., rcher-Evans, S., Manocha, V., Vance, V. & Sundaresan, V. (2005). Computational prediction of miRNAs in *Arabidopsis thaliana*. *Genome Res.* **15**, 78–91.
39. Robins, H., Li, Y. & Padgett, R. W. (2005). Incorporating structure to predict microRNA targets. *Proc. Natl Acad. Sci. USA*, **102**, 4006–4009.
40. Lewis, B. P., Burge, C. B. & Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, **120**, 15–20.
41. Moss, E. G. & Tang, L. (2003). Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites. *Dev. Biol.* **258**, 432–442.
42. Vella, M. C., Reinert, K. & Slack, F. J. (2004). Architecture of a validated microRNA::target interaction. *Chem. Biol.* **11**, 1619–1623.
43. Xie, X., Lu, J., Kulbokas, E. J., Golub, T. R., Mootha, V., Lindblad-Toh, K. *et al*. (2005). Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. *Nature*, **434**, 338–345.
44. Harada, F., Sawyer, R. C. & Dahlberg, J. E. (1975). A primer ribonucleic acid for initiation of in vitro Rous sarcarcoma virus deoxyribonucleic acid synthesis. *J. Biol. Chem.* **250**, 3487–3497.
45. Abrahante, J. E., Daul, A. L., Li, M., Volk, M. L., Tennessen, J. M., Miller, E. A. & Rougvie, A. E. (2003). The *Caenorhabditis elegans* hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. *Dev. Cell*, **4**, 625–637.
46. Lin, S. Y., Johnson, S. M., Abraham, M., Vella, M. C., Pasquinelli, A., Gamberi, C. *et al*. (2003). The *C elegans* hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. *Dev. Cell*, **4**, 639–650.
47. Cai, X., Hagedorn, C. H. & Cullen, B. R. (2004). Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA*, **10**, 1957–1966.
48. Nykanen, A., Haley, B. & Zamore, P. D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell*, **107**, 309–321.
49. Shaffer, A. L., Wojnar, W. & Nelson, W. (1990). Amplification, detection, and automated sequencing of gibbon interleukin-2 mRNA by *Thermus aquaticus* DNA polymerase reverse transcription and polymerase chain reaction. *Anal. Biochem.* **190**, 292–296.
50. Johnson, S. M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A. *et al*. (2005). RAS is regulated by the let-7 microRNA family. *Cell*, **120**, 635–647.
51. Bentwich, I., Avniel, A., Karov, Y., Aharonov, R., Gilad, S., Barad, O. *et al*. (2005). Identification of hundreds of conserved and nonconserved human microRNAs. *Nature Genet.* **37**, 766.
52. Iwai, N. & Naraba, H. (2005). Polymorphisms in human pre-miRNAs. *Biochem. Biophys. Res. Commun.* **331**, 1439–1444.

53. Boese, Q., Leake, D., Reynolds, A., Read, S., Scaringe, S. A., Marshall, W. S. & Khvorova, A. (2005). Mechanistic insights aid computational short interfering RNA design. *Methods Enzymol.* **392**, 73–96.
54. Scherr, M. & Eder, M. (2004). RNAi in functional genomics. *Curr. Opin. Mol. Ther.* **6**, 129–135.
55. Cheng, A. M., Byrom, M. W., Shelton, J. & Ford, L. P. (2005). Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucl. Acids Res.* **33**, 1290–1297.
56. Bengert, P. & Dandekar, T. (2005). Current efforts in the analysis of RNAi and RNAi target genes. *Brief. Bioinform.* **6**, 72–85.
57. Doench, J. G., Petersen, C. P. & Sharp, P. A. (2003). siRNAs can function as miRNAs. *Genes Dev.* **17**, 438–442.
58. Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R. & Pasquinelli, A. E. (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell*, **122**, 553–563.

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