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Systematic identification of microRNA functions by combining target prediction and expression profiling

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ABSTRACT

Target predictions and validations are major obstacles facing microRNA (miRNA) researchers. Animal miRNA target prediction is challenging because of limited miRNA sequence complementarity to the targets. In addition, only a small number of predicted targets have been experimentally validated and the miRNA mechanism is poorly understood. Here we present a novel algorithm for animal miRNA target prediction. The algorithm combines relevant parameters for miRNA target recognition and heuristically assigns different weights to these parameters according to their relative importance. A score calculation scheme is introduced to reflect the strength of each parameter. We also performed microarray time course experiments to identify downregulated genes due to miRNA overexpression. The computational target prediction is combined with the miRNA transfection experiment to systematically identify the gene targets of human miR-124. miR-124 overexpression led to a significant downregulation of many cell cycle related genes. This may be the result of direct suppression of a few cell growth inhibitors at the early stage of miRNA overexpression, and these targeted genes were continuously suppressed over a long period of time. Our high-throughput approach can be generalized to globally identify the targets and functions of other miRNAs.

INTRODUCTION

MicroRNAs (miRNAs) are a new family of small RNA molecules (~22 nt) that control the expression levels of their target genes (1,2). Hundreds of miRNA have been identified in recent years and miRNA functional identification has become one of the most active research fields in biology. miRNAs function through suppressing the expression of their targeted genes. They are known to be involved in many diverse functions such as regulation of cell proliferation, differentiation, apoptosis, carcinogenesis and viral infection (1–8).

Unfortunately most of the hundreds of newly identified animal miRNAs have unknown functions (9). One major obstacle is to identify the targets regulated by miRNAs. Target prediction in plants is relatively straightforward because of the near-perfect alignment between a miRNA and its target sequence (10). In contrast, prediction of animal miRNA targets is very challenging because there are only partial miRNA pairing to the targets. In addition, only a limited number of predicted targets have been experimentally validated and the miRNA mechanism is poorly understood. All these factors make it difficult to develop rational target prediction algorithms based on experimental results. Although multiple approaches have been suggested from bioinformatics (11–18), accurate target prediction and validation are still major obstacles facing miRNA researchers.

In plants, most miRNAs regulate target gene expression via mRNA degradation (19,20). In animals, a general model has been that miRNAs suppress target gene functions by translational repression (2). The mRNA level has generally not been altered if the target-binding sites have only partial sequence complementarity to the miRNA. However, recent studies have convincingly demonstrated that animal miRNAs can also reduce mRNA expression level via mRNA degradation (21); another study has demonstrated that miR-16 can mediate mRNA degradation although the binding site has only partial sequence match (22); a few miRNAs in *Drosophila* have led to the downregulation of their predicted target mRNAs (23,24); more recent analyses suggested the mutually exclusive tissue expression of miRNAs and their predicted targets (25,26). These findings indicate that expression regulation at the mRNA level may be a common mechanism for miRNA function (20). This has broad implications for miRNA target validation because, comparing to protein expression changes, it is more convenient to monitor

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transcriptional changes using a high-throughput experimental approach. Overexpression of miRNA in cells has been reported to lead to the downregulation of a large number of transcripts as revealed by microarrays (27). There is a significant enrichment of miRNA complementary sequences in these downregulated genes, implying they could be directly regulated by miRNA. Thus, it might be possible to use microarrays to simultaneously identify a large number of miRNA targets.

Here we present a novel algorithm for animal miRNA target prediction. The algorithm combines relevant parameters for miRNA target recognition and heuristically assigns different weights to these parameters according to their relative importance. A raw score calculation scheme is introduced to reflect the strength of each parameter. We have also performed microarray time course experiments to identify downregulated genes due to miRNA overexpression. The computational target prediction is combined with the miRNA overexpression experiments to systematically identify the gene targets and functions of human miR-124.

MATERIALS AND METHODS

Sequence retrieval

The 3′-untranslated region (3′-UTR) sequences were downloaded from ENSEMBL website http://www.ensembl.org (28) for five organisms: human, mouse, rat, dog and chicken. The orthologous gene clusters were built using ENSEMBL ortholog gene index to map UTR sequences across different organisms. NCBI Gene IDs were used to map ENSEMBL IDs and GenBank accessions. miRNA mature sequences were retrieved from Sanger miRBase version 7.0 (29).

Gene Ontology (GO) annotations

The annotations were downloaded from http://geneontology.org/. The index file mapping GO IDs to NCBI Gene IDs were downloaded from ftp://ftp.ncbi.nlm.nih.gov/gene/. Since only the lowest level in the GO tree were specified for a Gene ID, a recursive search for all the parent GO IDs based on the GO hierarchical data structure was performed to identify all the parent GO IDs associated with each Gene ID.

miRNA transfections

miR-124 RNA duplex (Pre-miR) molecule and negative control miRNA duplex were transfected into HepG2 cell line using the Reverse Transfection protocol recommended by Ambion. In brief, siPORT NeoFX Transfection Agent (Ambion) was diluted in optiMEM medium (Invitrogen). miR-124 RNA duplex, Pre-miR-124, was also diluted in optiMEM medium for a final concentration of 30 nM. The diluted transfection reagent was combined with the diluted miRNA duplex followed by incubation at room temperature for 10 min. The mixture was dispensed into an empty 6-well dish and then seeded at 2.3×10^5 cells per well. The same amount of negative control RNA duplex and GAPDH siRNA (Ambion) were also transfected. Total RNAs were extracted at different time points (0, 4, 8, 16, 24, 32, 72 and 120 h post transfection) and were used for microarray experiments. Additional transfections were performed to generate RNA samples for real-time RT–PCR validations.

Microarrays

Total RNA was amplified with MessageAmp II (Ambion), labeled and hybridized to Affymetrix human U133Plus2 chips following the manufacturer's protocols (30,31) (http://www.affymetrix.com/support/technical/technotes/hgu133_p2_technote.pdf). Array signals were normalized using the RMA method from the BioConductor package (http://www.bioconductor.org/). A gene was considered to be downregulated if the expression reduction was at least 50% when compared with both negative control and the 4 h miR-124 reference time points.

Real-time RT–PCR

All PCR primer sequences were retrieved from PrimerBank website http://pga.mgh.harvard.edu/primerbank (32). The primers were synthesized at the Integrated DNA Technologies.

RT reaction was carried out with RETROscript System under conditions suggested by the manufacturer (Ambion). A 20 µl RT reaction contained 0.5 µg of total RNA, 2 µl of 50 µM random hexamers, 2 µl of 10× RT, 4 µl of dNTP mix (25 mM each dNTP), 1 µl of MMLV-RT (100 U/µl), 1 µl of RNase Inhibitor (10 U/µl) and Nuclease-free water. After incubation at 25°C for 30 min and 37°C for 1 h, the reaction mixture was incubated at 92°C for 10 min.

Real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems) with the following conditions: 50°C for 2 min and then at 95°C for 10 min followed by 35 cycles of amplification (95°C for 15 s; 60°C for 30 s; 72°C for 30 s). PCR specificity was checked by melting curves and agarose gel electrophoresis.

RESULTS

miRNA target prediction algorithm

Our miRNA target prediction algorithm is summarized in Figure 1. The algorithm was implemented as a Perl program, MirTarget, running on a Linux platform. As the first step of target prediction, the miRNA seed sequence (positions 2–8) was scanned against all human 3′-UTR sequences to quickly identify perfect base pairing using a computer hashing technique. Then the level of cross-species conservation of seed pairing was examined. MirTarget evaluated orthologous sequences from five organisms and a gene candidate was rejected if the perfect seed pairing was not found in the orthologs from at least three organisms. The miRNA/target site duplex stability was evaluated by binding free energy (ΔG). ΔG values were computed using RNAFold (33). A candidate target site was rejected if the ΔG value was higher than -13 kcal/mol. If a candidate site passed these screening filters, local sequence alignment was performed to extend the alignment between miRNA and 22 bases downstream of the seed-binding site in 3′-UTR. Bases surrounding the seed sequences are important for target recognition (14). Thus limited seed extension was evaluated for pairing to miRNA positions 1, 9 and 10. The longest stretch of perfect matches (including positions 2–8) was considered as an extended seed for raw score calculation.

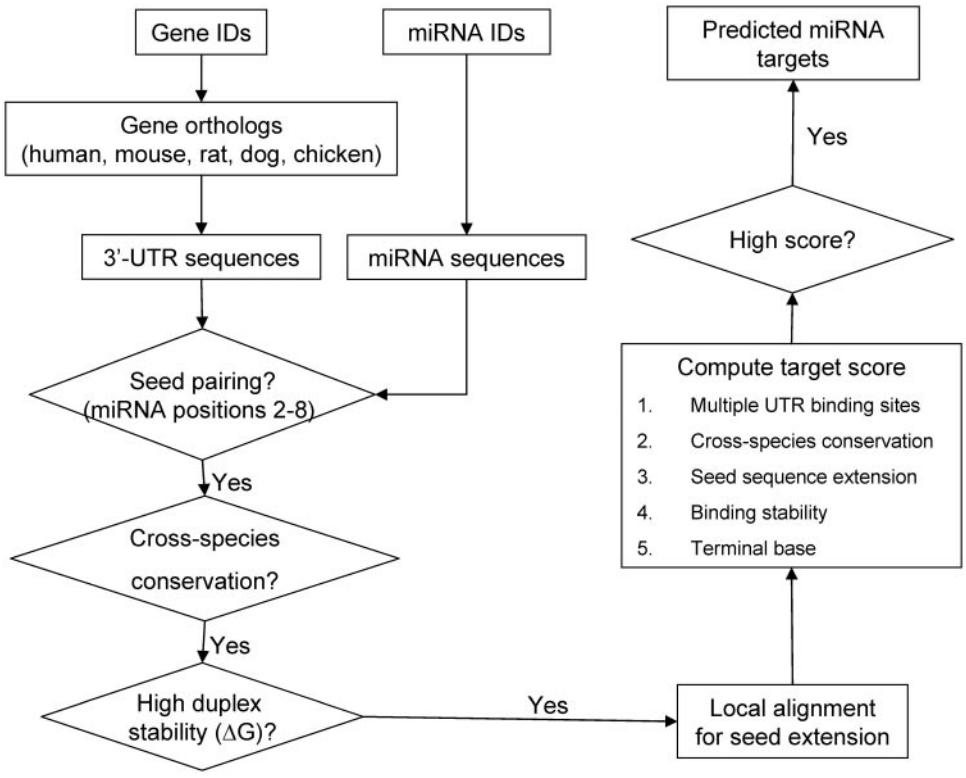

Figure 1. A simple flowchart for miRNA target prediction algorithm.

Raw scores were calculated separately for the screening filters. One example is given in Supplementary Figure 1. The cross-species conservation filter raw score was calculated with the following equation, where n is the number of species with miRNA seed pairing.

$$S_i = \frac{9 + 2^{n-1}}{10},$$

where $n > 2$ to pass the cutoff threshold. Raw score value increases exponentially as n gets larger. In this way, high degree of seed pairing conservation was exponentially rewarded. Raw scores for limited seed extension (positions 1–10) and binding free energy were calculated in a similar way by first converting the parametric values into relative ranks. Based on literature mining of validated targets, A/T terminal match is often observed in miRNA target sites (14). Thus MirTarget assigns a positive raw score 1 to A/T terminal match.

The target prediction score is computed based on the raw scores from individual filters as well as the total number of binding sites. The presence of multiple binding sites may enhance miRNA regulation (24).

$$S = \sum_{j=1}^{n} \sum_{i=1}^{4} W_i * S_i,$$

where i represents four screening filters; S_i represents the raw score for each filter and W_i represents the raw score weight; j represents the number of binding sites in one UTR sequence. Different weights were assigned to the filters in the following order to differentiate their relative importance: seed conservation > limited seed extension > duplex binding stability > terminal base match. A score is recorded if it is no less than the threshold value 30.

MirTarget was used to predict the potential gene targets of all known human miRNAs. Overall, 8810 gene targets were predicted for 319 miRNAs (Supplementary Table 2). The prediction result and more details about the MirTarget algorithm will be available at http://www.ambion.com. To estimate the level of false positives, the miRNA sequences were shuffled 100 times and the total numbers of predicted targets were recorded for these shuffled sequences. On average one round of shuffling only produced 317 ± 72 predicted targets for 319 human miRNAs, indicating the false positive rates of the target prediction are well contained ($P = 0$ by Z-test). As to miR-124 target prediction, 131 candidate genes received prediction scores and 85 gene targets were predicted (score $\geqslant$30, Supplementary Table 1). In contrast, one shuffled miR-124 sequence has only 1.7 predicted targets on average.

miR-124 transfection microarrays

miR-124 RNA duplex (Pre-miR) was transfected into HepG2 cell line. The Pre-miR RNA duplex mimics the miR-124 precursor. miR-124 is highly expressed in brain and kidney (34), and it does not express in the cell line we studied. miR-124 overexpression profiles were examined at 4, 8, 16, 24, 32, 72 and 120 h post transfections. A negative control RNA duplex was also transfected as reference for each of these time points.

Expression profiles were first examined at 72 h post transfection, and hundreds of genes were shown to be down-regulated by miR-124 overexpression. These genes were

Table 1. The most affected GO categories after miR-124 transfection for 72 h

GO ID	GO description	Enrichment *P*-value[a]
GO:0000278	Mitotic cell cycle	3.1E−14
GO:0000075	Cell cycle checkpoint	1.5E−12
GO:0051301	Cell division	5.2E−08
GO:0051325	Interphase	9.9E−08
GO:0005819	Spindle	1.3E−06
GO:0006260	DNA replication	3.5E−06

[a]*P*-values were used to assess whether there was a significant enrichment of genes in each GO category. The *P*-values were calculated by hypergeometric test using all 14 485 genes represented by U133Plus2 arrays as background.

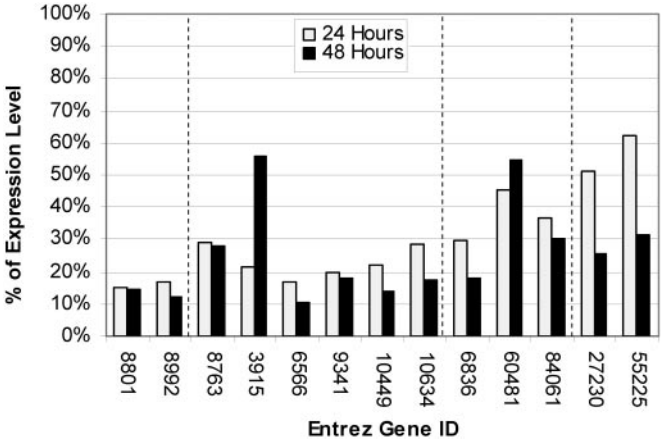

Figure 2. Real-time RT–PCR validation of the downregulated predicted miR-124 targets. The mRNA levels for downregulated targets at 4, 24 and 48 h were examined. The expression levels at 24 and 48 h are presented as percentages of that at 4 h. Among the validated targets, the microarray results indicated that genes 8801 and 8992 were first downregulated at 8 h; genes 8763, 3915, 6566, 9341, 10 449 and 10 634 were first downregulated at 16 h; genes 6836, 60 481 and 84 061 were first downregulated at 24 h; genes 27 230 and 55 225 were first downregulated at 32 h (downregulation is defined as at least 50% reduction of gene expression).

analyzed in the context of GO annotations to identify significantly affected GO functional categories. As shown in Table 1, the most significant changes were observed in categories related to cell cycle/proliferation. K-means clustering for downregulated genes also resulted in a cluster highly enriched in genes involved in cell cycle control (data not shown). This suggested miR-124 may be involved in cell growth control.

Potential miR-124 targets were computationally predicted by MirTarget. Of the 85 predicted targets 76 were represented on the arrays. The downregulated genes at different time points were examined to count how many genes were predicted to be miR-124 targets. We also performed real-time RT–PCR to validate all predicted targets downregulated at early stages of transfections. Figure 2 is a summary of the real-time RT–PCR validation results. The expression levels at 24 and 48 h were presented as percentages of the expression levels at 4 h. Among the validated targets, the microarray data indicated that genes 8801 and 8992 were first downregulated at 8 h; genes 8763, 3915, 6566, 9341, 10 449 and 10 634 were first downregulated at 16 h; genes 6836, 60 481 and 84 061 were first downregulated at 24 h; genes 27 230 and 55 225 were first downregulated at 32 h (downregulation is defined as at least 50% reduction of gene expression by array measurement). Thus, the real-time RT–PCR results agreed well with the microarray results.

Table 2. Downregulation of predicted miRNA targets after miR-124 transfection

Transfection time (h)	Downregulated genes	Predicted miRNA targets	Enrichment *P*-value[a]
8	6	2	4.0E−04
16	37	8	1.3E−11
24	134	11	9.2E−11
32	159	13	1.6E−12
72	583	20	1.1E−11
120	46	8	8.6E−11
Array	Represented genes	Represented targets	
U133Plus2	14 485	76	

[a]*P*-values were used to assess whether there was a significant enrichment of predicted miR-124 targets in total downregulated genes at each time point. The *P*-values were calculated by hypergeometric test using all genes represented by U133Plus2 arrays as background.

Characterization of the downregulated miR-124 targets

As shown in Table 2, there were very significant enrichments of predicted targets at all the time points we examined. There was a rapid accumulation of downregulated targets at early stages of miR-124 overexpression. However, the rate of accumulation slowed down dramatically at later time points. The rate showed a good fit to a logarithmic relationship (Figure 3A). Because the number of downregulated genes increased dramatically at later transfection stages, the percentages of predicted targets among downregulated genes decreased rapidly (Figure 3B), following a power law distribution.

The score distribution of the downregulated predicted targets at 24 h was compared with that of all predicted miR-124 targets in human. The two target lists are significantly different ($P = 0.002$ by Wilcoxon rank-sum test). The median score of all predicted targets is 33 while the downregulated predicted targets have median score of 54 (Figure 4). Of all predicted targets 28% have scores higher than 35. In contrast, 80% of downregulated targets have scores in the same range. There are nine predicted targets with two target sites in their 3′-UTR sequences among all human genes, and six of them were downregulated 24 h after transfection ($P = 7.3\text{E}{-5}$ by hypergeometric test).

Eight miR-124 targets were downregulated at 16 h, which represents 22% of all downregulated genes (Figure 3). These genes can be classified into four functional categories (Table 3). Three of the genes are associated with cell growth arrest: (i) CD164 is a potent signaling molecule and functions to suppress hematopoietic cell proliferation (35); (ii) the mouse homolog of GAS2L1 is an actin-associated protein highly abundant in growth-arrested cells. GAS2L1 mouse homologue is negatively regulated by serum and growth factors (36); (iii) SLC16A1 mediates the transport of butyrate across the colonocyte luminal membrane. SLC16A1 is able to induce cell cycle arrest and differentiation and its expression is significantly downregulated during colon carcinogenesis (37). Therefore, the downregulation of these gene

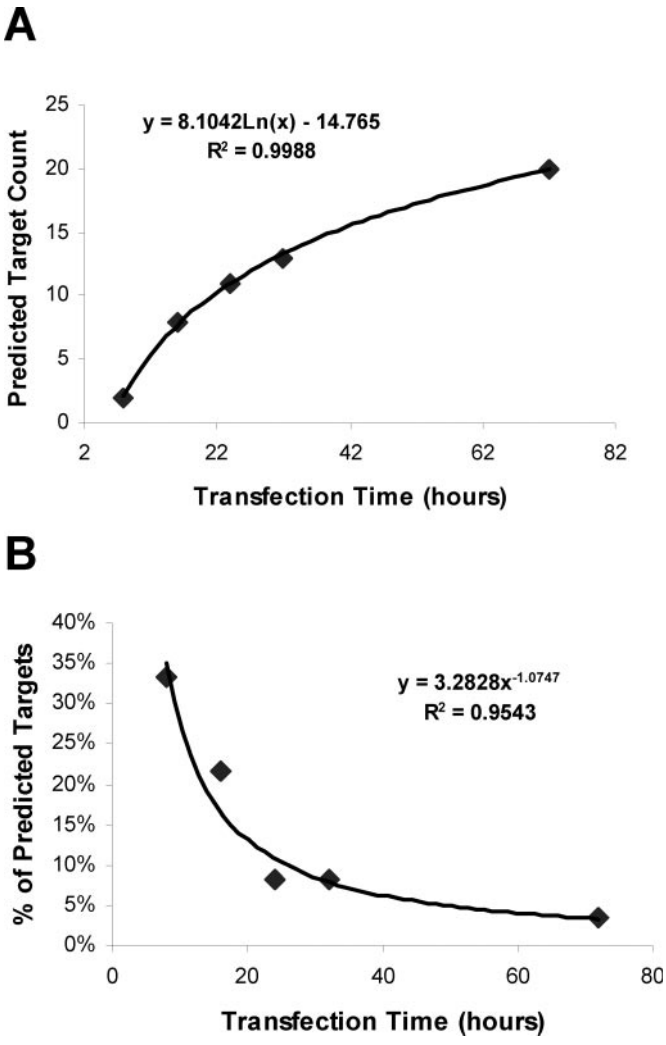

Figure 3. miR-124 overexpression time course analysis to identify predicted targets in total downregulated genes. (**A**) Counts of downregulated predicted targets at different time points after miR-124 transfections. (**B**) The percentages of predicted targets in total downregulated genes.

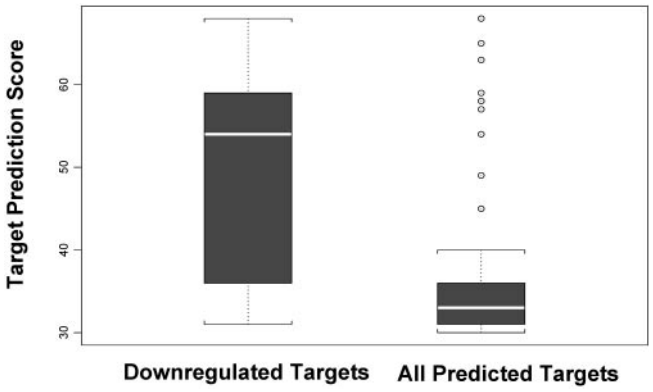

Figure 4. A boxplot to compare the prediction scores from all predicted miR-124 targets and from downregulated targets 24 h after transfection. The box in each panel represents the 50% range of each target list. The median scores were 33 and 54 for all human predicted targets and the downregulated targets, respectively.

targets by miR-124 can profoundly affect the regulation of cell proliferation.

After 5 days of miR-124 transfection, the number of downregulated genes was significantly reduced compared with that at 72 h (Table 2). When the data at 16 and 120 h post transfection were compared, the numbers of downregulated genes were similar; however, only 13 genes were found to be downregulated at both stages. It is interesting to notice that most predicted targets downregulated at 16 h were among these overlapping genes (Figure 5). Thus, gene targets downregulated at the early stage of transfection were more likely to be continuously suppressed over a long period of time.

DISCUSSION

miRNA target prediction

Because of the scarcity of experimentally validated miRNA targets, target prediction rules have not been clearly defined to date. Recent analyses have suggested the importance of perfect seed (miRNA positions 2–8) pairing to target-binding sites (13,38). To exemplify this, one popular prediction algorithm considers only seed pairing and its evolutionary conservation in target prediction (14). Other studies have also indicated that most target sites align perfectly to miRNA seed sequences. Therefore, perfect seed pairing is likely to be the most important filter for target recognition in our algorithm. In other settings, this strategy has been shown to greatly reduce the false positive rate (14). In the case of target prediction for miR-124, 92% of all gene candidates would have been excluded if the seed pairing filter were applied alone. It has been known that a small number of validated targets have imperfect matches to miRNA seed sequences (5). Thus, our algorithm will miss these targets because perfect seed pairing is required.

Cross-species conservation of seed pairing is another important parameter for target prediction (14,39). However, genome annotations are still a work in progress and the risk of omitting valid target candidates will increase if we place too much emphasis on incompletely annotated genomic information (16). For example, many gene 3′-UTR sequences have not been identified in non-human genomes. In our algorithm, a balanced approach is adopted to require seed pairing conservation in at least three out of five orthologs. This strategy was chosen to minimize the effect of insufficient cross-species conservation as well as poor genome quality on target prediction.

Unlike most existing prediction algorithms, MirTarget uses a combinatorial heuristic approach to consider the relative importance of each filter for score computation. The contribution of each filter to the prediction score may vary depending on its filter weight and strength. For example, the seed pairing conservation filter has a higher weight than the terminal base pairing filter. And seed pairing across five species will contribute more than seed pairing conserved in only three species. In this way, a 'weak' filter may be compensated by another 'strong' filter so that the score can pass the cutoff threshold.

Target validation by mRNA profiling

Recent studies have indicated it is common for animal miRNAs to downregulate target mRNA expression. This

Table 3. Downregulated predicted targets after miR-124 transfection for 16 h

Entrez gene ID	Gene symbol	Proliferation arrest	Cytoskeleton/cell morphology	Metabolism	Transport	Gene description
8763	CD164	Y (35)	–	–	–	CD164 antigen, sialomucin
10634	GAS2L1	Y (36)	Y (36)	–	–	growth arrest-specific 2 like 1
6566	SLC16A1	Y (37)	–	Y (37)	Y (37)	monocarboxylic acid transporters, member 1
3915	LAMC1	–	Y	–	–	laminin, gamma 1
10449	ACAA2	–	–	Y	–	acetyl-Coenzyme A acyltransferase 2
8801	SUCLG2	–	–	Y	–	succinate–coa ligase, gdp-forming, beta subunit
8992	ATP6V0E	–	–		Y	Lysosomal ATPase, H+ transporting, V0 subunit e
9341	VAMP3	–	–	–	Y	vesicle-associated membrane protein 3

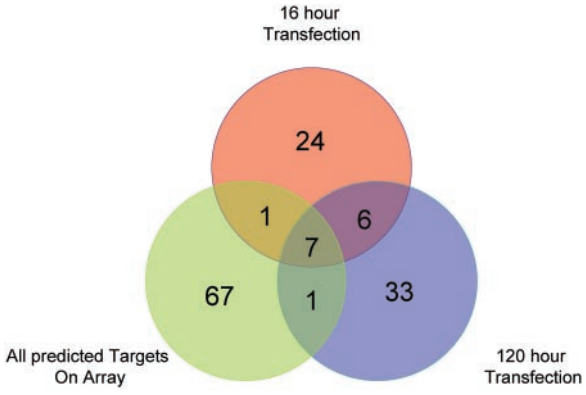

Figure 5. Overlapping downregulated genes after 16 and 120 h of miR-124 overexpression. Thirteen genes were in both lists and seven of them were miR-124 targets. Overall, 76 predicted targets were represented on the arrays.

opens a new door to experimental target validation by mRNA expression profiling. Our approach may miss some miRNA targets that are principally downregulated at the protein level; on the other hand, it could also produce some false positive targets because the transfection overexpression of miR-124 may not properly reflect the physiological conditions *in vivo*. Despite these pitfalls, the high-throughput capacity still makes it a convenient way to quickly identify a large number of promising miRNA targets.

We performed miR-124 transfection experiments and analyzed the transcript abundance in conjunction with computational target prediction. As shown in Table 2, hundreds of genes were downregulated at 72 h and GO functional categories for cell cycle/proliferation control were significantly affected (Table 1). We discovered a rapid accumulation of downregulated targets at early stages of miR-124 overexpression. The rate of accumulation decreased significantly at later stages. Thus most mRNA downregulations at 72 h are likely to be indirect effects from miR-124, as the percentage of predicted targets is much smaller comparing to that seen at earlier stages of miR-124 overexpression (Figure 3).

In general, the downregulated targets were found to have prediction scores significantly higher than those from all predicted miR-124 targets (Figure 4). This suggested that the prediction scores may be used as a confidence measurement of the bioinformatics prediction. Six out of nine predicted targets with multiple 3′-UTR binding sites were found to be downregulated when miR124 was overexpressed. Thus the presence of multiple binding sites is likely to be an indicator of strong miRNA regulation.

The downregulated targets at 16 h post transfection were examined for their functions. Three of them are known to be negative regulators of cell proliferation and/or abundantly expressed in growth-arrested cells (Table 3). Therefore, we hypothesize that miR-124 may be a master positive regulator of cell proliferation by suppressing the expression of cell growth inhibitors.

In summary, we have presented a novel algorithm for miRNA target prediction using a combination of weighted selection filters. The computational predictions were combined with microarray expression profiles to identify downregulated miR-124 gene targets. miR-124 overexpression led to a significant downregulation of many cell cycle related genes. This may be the result of direct suppression of a few cell growth inhibitors at the early stage of miR-124 overexpression, and these targeted genes were continuously suppressed over a long period of time. Our high-throughput approach can be generalized to globally identify the targets and functions of other miRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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Conflict of interest statement. None declared.

REFERENCES

1. Bartel,D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, **116**, 281–297.
2. He,L. and Hannon,G.J. (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nature Rev. Genet*., **5**, 522–531.
3. Miska,E.A. (2005) How microRNAs control cell division, differentiation and death. *Curr. Opin. Genet. Dev*., **15**, 563–568.
4. O'Donnell,K.A., Wentzel,E.A., Zeller,K.I., Dang,C.V. and Mendell,J.T. (2005) c-Myc-regulated microRNAs modulate E2F1 expression. *Nature*, **435**, 839–843.
5. Johnson,S.M., Grosshans,H., Shingara,J., Byrom,M., Jarvis,R., Cheng,A., Labourier,E., Reinert,K.L., Brown,D. and Slack,F.J. (2005) RAS is regulated by the let-7 microRNA family. *Cell*, **120**, 635–647.

6. He,L., Thomson,J.M., Hemann,M.T., Hernando-Monge,E., Mu,D., Goodson,S., Powers,S., Cordon-Cardo,C., Lowe,S.W., Hannon,G.J. *et al*. (2005) A microRNA polycistron as a potential human oncogene. *Nature*, **435**, 828–833.
7. Lecellier,C.H., Dunoyer,P., Arar,K., Lehmann-Che,J., Eyquem,S., Himber,C., Saib,A. and Voinnet,O. (2005) A cellular microRNA mediates antiviral defense in human cells. *Science*, **308**, 557–560.
8. Sullivan,C.S., Grundhoff,A.T., Tevethia,S., Pipas,J.M. and Ganem,D. (2005) SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature*, **435**, 682–686.
9. Ambros,V. (2004) The functions of animal microRNAs. *Nature*, **431**, 350–355.
10. Wang,X.J., Reyes,J.L., Chua,N.H. and Gaasterland,T. (2004) Prediction and identification of *Arabidopsis thaliana* microRNAs and their mRNA targets. *Genome Biol*., **5**, R65.
11. Enright,A.J., John,B., Gaul,U., Tuschl,T., Sander,C. and Marks,D.S. (2003) MicroRNA targets in Drosophila. *Genome Biol*., **5**, R1.
12. Krek,A., Grun,D., Poy,M.N., Wolf,R., Rosenberg,L., Epstein,E.J., MacMenamin,P., da,P., I, Gunsalus,K.C., Stoffel,M. *et al*. (2005) Combinatorial microRNA target predictions. *Nature Genet*., **37**, 495–500.
13. Lewis,B.P., Shih,I.H., Jones-Rhoades,M.W., Bartel,D.P. and Burge,C.B. (2003) Prediction of mammalian microRNA targets. *Cell*, **115**, 787–798.
14. Lewis,B.P., Burge,C.B. and Bartel,D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, **120**, 15–20.
15. Lim,L.P., Lau,N.C., Weinstein,E.G., Abdelhakim,A., Yekta,S., Rhoades,M.W., Burge,C.B. and Bartel,D.P. (2003) The microRNAs of *Caenorhabditis elegans*. *Genes Dev*., **17**, 991–1008.
16. Robins,H., Li,Y. and Padgett,R.W. (2005) Incorporating structure to predict microRNA targets. *Proc. Natl Acad. Sci. USA*, **102**, 4006–4009.
17. Stark,A., Brennecke,J., Russell,R.B. and Cohen,S.M. (2003) Identification of *Drosophila* microRNA targets. *PLoS. Biol*., **1**, E60.
18. Kiriakidou,M., Nelson,P.T., Kouranov,A., Fitziev,P., Bouyioukos,C., Mourelatos,Z. and Hatzigeorgiou,A. (2004) A combined computational-experimental approach predicts human microRNA targets. *Genes Dev*., **18**, 1165–1178.
19. Dugas,D.V. and Bartel,B. (2004) MicroRNA regulation of gene expression in plants. *Curr. Opin. Plant Biol*., **7**, 512–520.
20. Sontheimer,E.J. and Carthew,R.W. (2005) Silence from within: endogenous siRNAs and miRNAs. *Cell*, **122**, 9–12.
21. Bagga,S., Bracht,J., Hunter,S., Massirer,K., Holtz,J., Eachus,R. and Pasquinelli,A.E. (2005) Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell*, **122**, 553–563.
22. Jing,Q., Huang,S., Guth,S., Zarubin,T., Motoyama,A., Chen,J., Di Padova,F., Lin,S.C., Gram,H. and Han,J. (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell*, **120**, 623–634.
23. Lai,E.C. (2002) Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. *Nature Genet*., **30**, 363–364.
24. Lai,E.C., Tam,B. and Rubin,G.M. (2005) Pervasive regulation of *Drosophila* notch target genes by GY-box-, Brd-box-, and K-box-class microRNAs. *Genes Dev*., **19**, 1067–1080.
25. Farh,K.K., Grimson,A., Jan,C., Lewis,B.P., Johnston,W.K., Lim,L.P., Burge,C.B. and Bartel,D.P. (2005) The widespread impact of mammalian microRNAs on mRNA repression and evolution. *Science*, **310**, 1817–1821.
26. Stark,A., Brennecke,J., Bushati,N., Russell,R.B. and Cohen,S.M. (2005) Animal microRNAs confer robustness to gene expression and have a significant impact on 3′UTR evolution. *Cell*, **123**, 1133–1146.
27. Lim,L.P., Lau,N.C., Garrett-Engele,P., Grimson,A., Schelter,J.M., Castle,J., Bartel,D.P., Linsley,P.S. and Johnson,J.M. (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, **433**, 769–773.
28. Hubbard,T., Andrews,D., Caccamo,M., Cameron,G., Chen,Y., Clamp,M., Clarke,L., Coates,G., Cox,T., Cunningham,F. *et al*. (2005) Ensembl 2005. *Nucleic Acids Res*., **33**, D447–D453.
29. Griffiths-Jones,S. (2004) The microRNA registry. *Nucleic Acids Res*., **32**, D109–D111.
30. Affymetrix Inc. (2002) *GeneChip Expression Analysis Technical Manual*.
31. Affymetrix Inc. (2005) *Design and Performance of the GeneChip Human Genome U133 Plus 2.0 and Human Genome U133A 2.0 Arrays. Technical Note*.
32. Wang,X. and Seed,B. (2003) A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res*., **31**, e154.
33. Hofacker,I.L. (2003) Vienna RNA secondary structure server. *Nucleic Acids Res*., **31**, 3429–3431.
34. Lu,J., Getz,G., Miska,E.A., Alvarez-Saavedra,E., Lamb,J., Peck,D., Sweet-Cordero,A., Ebert,B.L., Mak,R.H., Ferrando,A.A. *et al*. (2005) MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834–838.
35. Zannettino,A.C., Buhring,H.J., Niutta,S., Watt,S.M., Benton,M.A. and Simmons,P.J. (1998) The sialomucin CD164 (MGC-24v) is an adhesive glycoprotein expressed by human hematopoietic progenitors and bone marrow stromal cells that serves as a potent negative regulator of hematopoiesis. *Blood*, **92**, 2613–2628.
36. Goriounov,D., Leung,C.L. and Liem,R.K. (2003) Protein products of human Gas2-related genes on chromosomes 17 and 22 (hGAR17 and hGAR22) associate with both microfilaments iand microtubules. *J. Cell Sci*., **116**, 1045–1058.
37. Cuff,M., Dyer,J., Jones,M. and Shirazi-Beechey,S. (2005) The human colonic monocarboxylate transporter Isoform 1: its potential importance to colonic tissue homeostasis. *Gastroenterology*, **128**, 676–686.
38. Doench,J.G. and Sharp,P.A. (2004) Specificity of microRNA target selection in translational repression. *Genes Dev*., **18**, 504–511.
39. Grun,D., Wang,Y.L., Langenberger,D., Gunsalus,K.C. and Rajewsky,N. (2005) microRNA target predictions across seven *Drosophila* species and comparison to mammalian targets. *PLoS Comput. Biol*., **1**, e13.