Physiological identification of human transcripts translationally regulated by a specific microRNA

Mika Nakamoto¹, Peng Jin¹, William T. O’Donnell¹,† and Stephen T. Warren¹,2,3,∗

¹Department of Human Genetics, ²Department of Biochemistry and ³Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322, USA

Received August 30, 2005; Revised and Accepted October 13, 2005

One mechanism by which endogenous microRNAs (miRNAs) function is to suppress translation of target mRNAs. Computational identification of target mRNAs is hampered by the partial complementarity between miRNAs and their targets and the lack of in vivo approaches to identify targets. Here, we identify mRNAs that are regulated by specific endogenous miRNA by detecting shifts in individual mRNA abundance in polyribosome profiles following miRNA knockdown via siRNA. We have identified human genes whose mRNAs were found at significantly increased levels in the heavy polyribosome fractions following miRNA \textit{miR-30a-3p} knockdown. If antibody was available, targets showed an increase in protein levels following the miRNA knockdown and a decrease following the miRNA overexpression. Although all identified transcripts have sequences that partially complement \textit{miR-30a-3p}, none was identified by commonly used computational means. These data suggest that the functional interaction between miRNAs and mRNA targets is more complex than previously realized and describe an approach to refine predictive algorithms.

INTRODUCTION

It has been recently appreciated that small non-coding miRNAs play critical roles in metazoan development, influencing cell proliferation, differentiation and death by the post-transcriptional regulation of their target transcripts (1–6). Although siRNAs cause cleavage of their target transcripts via perfect pairing, translation suppression occurs without transcript degradation when complementarity between the transcript recognition sequences and their cognate miRNA is partial (7–9).

This imperfect complementarity occurs at variable positions and presents challenges for traditional homology based search algorithms to identify miRNA target transcripts. Although several bioinformatic methods have been developed, the consistency among different methods is low (10–14). The chief reason is that the rules that govern the pairing between an miRNA and its target transcripts have not been fully delineated. This is especially true in humans, where only a few experimentally validated targets are known (6,15). Furthermore, as many miRNAs are known to be differentially expressed during the course of development and differentiation (16–20), genome-wide predictions may identify an miRNA and target transcripts that are not co-expressed in the same cells. Therefore, establishing a general physiological system to identify, in a variety of cell types, target transcripts that are functionally regulated by specific miRNAs is critical to investigate miRNA-mediated translational regulation in vivo. Lim et al. (21) developed an in vivo approach examining transcript abundance following miRNA overexpression. Their results suggested that, similar to siRNAs, endogenous miRNAs could act on cleavage of transcripts. However, no study has reported the in vivo, genome-wide identification of target transcripts based on translational regulation by specific endogenous miRNAs, a canonical but poorly understood mechanism of the miRNA pathway.

RESULTS AND DISCUSSION

To address this need, we have developed a system that can identify, on a genome-wide scale, co-expressed target transcripts of a specific miRNA and indirectly quantify the degree of translational suppression. Assuming a transcript’s position in a profile of polyribosomes reflects, in part, the
degree of its translation, then shifts into heavier polyribosomes should reflect enhanced translation. Pools of mRNA from polyribosome fractions of cultured cells before and after siRNA knockdown of an endogenous miRNA are collected. Using microarray analysis, we should identify transcripts moving toward heavier polyribosomes, reflecting the relief of miRNA-mediated translational suppression. We validated this hypothesis using a reporter construct with bona fide miRNA target sequences and then proceeded to identify endogenous target transcripts of the miRNA miR-30a-3p in the human cell line HepG2.

A firefly luciferase reporter (Luc-3'-UTR) was constructed, driven by the SV40 promoter, containing the 3'-UTR of the mouse fragile X-mental retardation (mFmr1) gene and a polyA signal (Fig. 1A). Another version of this construct (Luc-T30) had embedded within the 3'-UTR, four 21 nt artificial target sequences partially complementing the sequence of human miRNA miR-30a-3p (Fig. 1B), a widely expressed and comparatively well-studied miRNA (22). As a control, Luc-AT30, was the same except the miR-30a-3p target sequences were in the antisense orientation. When all three constructs were individually transfected into human HepG2 cells (along with a Renilla luciferase transfection control), the levels of reporter mRNA were equivalent (blue bars, Fig. 1C). However, the luciferase activity (orange bar) produced from the Luc-T30 construct was reduced ~50% relative to either the Luc-3'-UTR or the Luc-AT30 constructs, whose activities were not significantly different from each other. These data would be consistent with the post-transcriptional down regulation of Luc-T30 by the endogenous miR-30a-3p.

To confirm that the post-transcriptional suppression of luciferase activity of Luc-T30 was indeed due to the endogenous miR-30a-3p, we designed a siRNA duplex (siRNA-p) in which one strand completely complemented the loop region of the miR-30a-3p precursor (Fig. 2A) (27). As would be expected, if miR-30a-3p was indeed knocked down by siRNA-p, the post-transcriptional suppression of Luc-T30 was reversed when siRNA-p was included in the transfection mix, whereas a control siRNA duplex (siRNA-c), which has been designed not to target the miRNA precursor, had no effect (Fig. 2B). The reversal of the suppression of Luc-T30 activity was also dependent upon the concentration of siRNA-p (Fig. 2C). By solution hybridization with a probe complementary to mature miR-30a-3p, we have verified that the amount of the miRNA was reduced to undetectable level by knocking down with siRNA-p (Fig. 2D). Taken together, these data suggest that Luc-T30 activity was likely subject to miR-30a-3p-mediated translational suppression.

Because the translation efficiency is reliably determined by the association of a specific mRNA with polyribosomes (23), we hypothesized that by knocking down a specific endogenous miRNA, target transcripts would be relieved from translation suppression and shift to a heavier polyribosome fraction on sucrose gradients, reflecting more active translation. Indeed, when endogenous miR-30a-3p was knocked down by siRNA-p, we detected a striking shift of the reporter...
transcript to heavier polyribosome fraction relative to cells transfected with the control siRNA-c (Fig. 3). Quantitative RT–PCR showed no change in the total Luc-T30 transcript between the two groups (Fig. 3B). These data, therefore, validate our assumption that knocking down endogenous specific miRNA can shift the distribution of its target transcripts to heavier polyribosomal fractions.

Assuming that endogenous target transcripts against miR-30a-3p behave similar to the reporter Luc-T30 when miR-30a-3p is knocked down, we now could interrogate microarrays to identify transcripts sensitive to this miRNA in HepG2 cells. We conducted three independent experiments using Affymetrix U133 Plus 2.0 human GeneChips on total cellular mRNA and mRNA derived from heavy polyribosomal fractions (Supplementary Material, Table S1). Those data were normalized using Robust Multiarray Analysis (24) and subjected to both two-way analysis of variance (ANOVA) and log ratio analyses. As shown in Figure 4, there was little change in substantial magnitude noted in the total input mRNA (Fig. 4A) between control cells or those in which miR-30a-3p was knocked down. However, there appeared to be a shift in transcripts, particularly toward the heavy polyribosomal fraction, in miR-30a-3p knocked down cells (Fig. 4B).

Analysis of the GeneChip data using the criterion of significance of $P < 0.02$ and log ratio change of $>0.425$ in miR-30a-3p knocked down cells showed 51 probe sets (out of 54 675), as shifting toward the heavy polyribosome fraction (indicated in green in Fig. 4). Of these 51 probe sets, 34 were associated with gene symbols, whereas the remaining 17 represented expressed sequence tags (ESTs). As is common to Affymetrix GeneChips, multiple probe sets ascertained the transcript levels of the same genes. Of the 34 gene symbol probe sets, eight genes were identified where multiple probe sets for each gene reflected similar values (tmem2: transmembrane protein 2, thbs1: thrombospondin 1, slc7a6: solute carrier family 7, member 6, cyr61: cysteine-rich, angiogenic inducer, 61, vezatin: transmembrane protein vezatin, tuba3: tubulin, alpha 3, pro2730: hypothetical protein pro2730 and cdk6: cyclin-dependent kinase 6). For four genes (slc7a6, cyr61, tuba3 and pro2730), all the ascertained probe sets met the statistical criteria set earlier, whereas the other four genes had one or multiple probe sets meeting this criteria and additional probe sets with values not meeting this level of significance but showing expression changes in similar directions. We selected these eight transcripts to characterize further.

Real-time PCR analyses verified, in independent experiments, the increase of the selected eight transcripts in the heavy polyribosome fraction of miR-30a-3p knocked down cells (Fig. 5). For some, there was a substantial increase in transcript abundance in the heavy polyribosomal fractions (e.g.


Figure 4. Distribution of all present probe sets comparing miR-30a-3p knockdown cells with control cells expressed as ANOVA P-value versus log ratio of transcript abundance change. Three independent experiments, each measuring total input mRNA and mRNA recovered from the heavy polyribosome fraction, were performed on each knockdown and control. (A) Total input (non-fractionated) mRNA. (B) Statistically significant increase ($P < 0.02$) of mRNAs in the heavy polyribosome fraction was detected following miR-30a-3p knockdown. The 51 probe sets that fulfill the statistical threshold in heavy polyribosome fraction are indicated by green in both figures.

tmem2 and thbs1) relative to the other genes, although all showed a significant shift to the heavy polyribosome fraction with miR-30a-3p knocked down replicating the GeneChip data. Some transcripts showed a modest increase in the total input mRNA when miR-30a-3p was knocked down (Fig. 5). This is consistent with a recent report suggesting that miRNAs overexpression could also reduce target mRNA abundance (21).

Of the proteins encoded by the selected eight transcripts, only antibodies against CYR61 and CDK6 were available. However, by western blot, we verified that, as predicted by their shift toward the heavy polyribosome fraction, both CYR61 and CDK6 abundance were increased in miR-30a-3p knocked down cells (Fig. 6, lane 2). Moreover, overexpression of miR-30a-3p by transfection results in a significant decrease in the abundance of both CYR61 and CDK6 (Fig. 6, lane 4). These data support the premise that polyribosome shifts can reveal transcripts sensitive to specific miRNAs and can be used as a general screen for such transcripts.

Perfect complementarity between target transcripts and the nt 2 to 6 or 7 from the 5' of miRNA (the ‘seed’ sequence) has been shown to be important for effective suppression of translation in vitro (24). We therefore searched the complementary sequence between this region of miR-30a-3p and the eight transcripts we identified. We found that all eight transcripts had at least one perfectly matched seed sequence in either the coding region or the 3'-UTRs, except for cyr61, tmem2 and tuba3, each of which had a perfect match if one G:U wobble is permitted (Table 1). Moreover, all eight transcripts had seed matches with ΔGs low enough to allow effective suppression (25).

A computational program, miRanda (12), developed for predicting pairing sites for miRNA found possible target sequences in six out of the eight transcripts using the default threshold (Table 2). However, the predicted pairings in those six transcripts did not score high and were not listed among the top 1143 transcripts predicted as miR-30a-3p targets (12). PicTar, one of the most recently developed programs to identify miRNA targets, is based on an established algorithm for transcription factor binding sites (13) and predicted 258 human target transcripts for miR-30a-3p, although not the eight genes we functionally identified earlier. Because neither program identified the target mRNAs identified earlier, we conversely determined the outcome in our GeneChip experiments for transcripts predicted as target mRNAs by both programs. Between the miRanda and the PicTar predictions, eight genes were in common. Three were not expressed in HepG2 cells and the remaining five expressed transcripts did not show any statistically significant polyribosomal shift ($P = 0.02$) in miR-30a-3p knocked down cells (Table 3). These data indicate a significant difficulty with current computational predictions of human miRNA targets.

It is unclear why the target transcripts identified here were not predicted by these computational methods. Such programs score known target transcripts in Caenorhabditis elegans and Drosophila melanogaster with reasonable accuracy. Indeed, in some instances, it has been verified that the levels of predicted target proteins are altered by overexpression of the miRNA. With PicTar, seven out of 13 predicted targets have been experimentally verified, although mRNA levels were not examined (13). Basal mRNA levels are important, as it has been shown in vitro that the extent of base pairing determines whether a target transcript is either degraded or silenced without cleavage (26). As computational prediction methods often place substantial importance on the extent of base pairing, changes in protein levels rather than reflecting translational regulation may reflect transcript degradation caused by highly matched base pairing. This discrepancy between computational prediction and data acquired in vivo suggests
that other factors besides the typical seed pairing and species conservation, on which many computational prediction methods are based, are used by the miRNA pathway in humans. One factor possibly affecting this is that we frequently observed seed matches in the coding region of genes, not just in the 3'-UTR within which many computational methods are limited. Another factor may be the allowance of a seed G:U wobble, as seen in cyr61, tuba3 and, in particular, tmem2 which showed a substantial shift in the polyribosome profile (Fig. 5). We also observed an atypical seed match in cyr61, which has three bulged nucleotides in the seed but had a markedly low minimum free energy (ΔG = −8.4 kcal/mol) between the transcript and the first 8 nt of miR-30a-3p (Table 1). Similar pairing had also been found in one of the let-7 binding sites in lin-41 in C. elegans (2), although in this instance, the cyr61 seed match that is functional remains to be determined. We propose that a systematic screen for functional miRNA targets, using the approach described earlier, should provide adequate data to more fully illuminate rules governing miRNA-mediated translational suppression and lead to improved computational predictions.

MATERIALS AND METHODS

Construction of reporter plasmids

For the plasmid Luc-3'-UTR, a 2.3 kb fragment that includes the last 120 bp of the mouse Fmr1 coding sequence and the entire 2.2 kb mouse Fmr1 3'-UTR was cloned into the XbaI site of the pGL3-control vector (Promega). The plasmid Luc-T30 was constructed by inserting a 113 bp fragment bearing the artificial target sequence against miR-30a-3p, 5'-AAATTCAGCTGGTCAACCGTCAAACAAAGACTGAA AGTCGCTGCAAAAAGACTGAAAGTGAAGCTGCAA ACAAAAGACTGAAAGCTGCAAAACAAAGACTGAA AGCTTG-3' into EcoRI site of the Fmr1 3'-UTR. The plasmid Luc-AT30 was constructed similarly, except that the artificial target sequence was inverted. All reporter plasmids were confirmed by sequencing.

siRNA preparation

siRNA duplex against human miR-30a precursor was designed to target the loop region of the precursor (27) as follows: 5'-UGUGGCGCUUACGUUGCACCTT-3', 5'-UGUGGCUUACGUUGCACCTT-3' and 5'-UGUGGCGCUUACGUUGCACCTT-3'. All duplexes were synthesized using Silencer™ siRNA Construction Kit (Ambion).

Solution hybridization

Small RNAs from HepG2 transfected with or without siRNA-p were fractionated using mirVana miRNA Isolation Kit (Ambion). Thirty-three microgram each of the small RNAs was subjected to solution hybridization, according to manufacturer’s protocol (mirVana miRNA Detection Kit, Ambion). Riboprobe against miR-30a-3p was made with mirVana miRNA Probe Construction Kit (Ambion).
HepG2 cells were transiently transfected with each plasmid and siRNA duplex at the same concentration as above, 24 h after seeding at 5.5 × 10⁵/150 cm dish. Three dishes were used for each sample. Another 24 h later, cells were treated with cycloheximide at 37°C for 15 min, harvested and lysed in the lysis buffer (200 mM Tris–HCl pH 9.0, 130 mM KCl, 36 mM MgCl₂, 1% each of Triton X-100, NP-40 and sodium deoxycholate, 0.8% beta-mercaptoethanol) at 4°C for 10 min. After centrifugation at 20,800 g at 4°C for 10 min, the supernatant was loaded on linear gradient of 15–45% sucrose bed and subjected to ultracentrifugation at 260,809 g at 4°C for 75 min, followed with fractionation by 1.1 ml each while monitoring OD at 254 nm with the UA-6 recording spectrophotometer (ISCO).

Total RNA purification and GeneChip experiments

Total RNAs were purified from sucrose gradient fractions of each sample (conducted at 24 h after transfection as described earlier) using modified AGPC (28) followed by column purification (RNeasy, Promega). We performed three independent GeneChip analyses using three independent total RNAs purified from heavy polyribosome fractions of miR-30a-3p knocked down cells and control cells. Hybridization to GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) was carried out according to manufacturer’s manual.

Statistical analysis

Raw data obtained via the GCOS software (Affymetrix) were subjected to background adjustment and normalization with RMAExpress (24) to all experiments at once. Log₂ scaled results were then used for two-way ANOVA on Spotfire (Spotfire) to calculate two-sided P-values. Natural scaled results of RMAExpress were provided for the calculation of log ratio after filtering out absent calls. To combine the results of P-value and log ratio by weighting the same importance on each analysis, criterion was determined as follows: first, we ascertained the number probe sets that met the threshold P-value (α = 0.02). Secondly, the absolute values of log ratio that gives the same number of the probe sets were provided.
Table 2. Presumable pairing between miR-30a-3p and each transcript highly scored with miRanda

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Predicted Pairing by miRanda</th>
<th>Location</th>
<th>ΔG (kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyr61</td>
<td>3' CGAC---GUUUUGGCGUGAC---UC 5'</td>
<td>coding</td>
<td>-5.0</td>
</tr>
<tr>
<td></td>
<td>5' GCUGUGCCCAACCGUGGCUGUGCAAG 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3' CGACGUUGUA------G-GC-UAGCUUUC 5'</td>
<td></td>
<td>-2.3</td>
</tr>
<tr>
<td></td>
<td>5' ACUGUAAACAUACGUACAGUAGUAGUGG 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tmem2</td>
<td>3' CGACGUUGUA6GCGCUGACUUUC 5'</td>
<td>coding</td>
<td>-7.6</td>
</tr>
<tr>
<td></td>
<td>5' CCUGU--GCAUUC--ACUGCAAG 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>thbs1</td>
<td>3' CGACGUUGUA6GCGCUGACUUUC 5'</td>
<td>3'UTR</td>
<td>-8.1</td>
</tr>
<tr>
<td></td>
<td>5' UCUG--GAAGAGUCGUGCAAG 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>slc7a6</td>
<td>3' CGACGUUGUA--AGCCUGACUUC 5'</td>
<td>3'UTR</td>
<td>-4.8</td>
</tr>
<tr>
<td></td>
<td>5' ACCGCAUGAUGUCCUGCA 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vezatin</td>
<td>3' CGACGUUGUA6GCGCUGACUUUC 5'</td>
<td>coding</td>
<td>-7.1</td>
</tr>
<tr>
<td></td>
<td>5' CUUGC--AGC--UCCAUCUGAAG 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdk6</td>
<td>3' CGACGUUGUA6GCGCUGACUUUC 5'</td>
<td>3'UTR</td>
<td>-6.5</td>
</tr>
<tr>
<td></td>
<td>5' UUUCGCGACGUGCGAGGA 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pro2730</td>
<td>none</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tuba3</td>
<td>none</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The sequence of miR-30a-3p is indicated by bold green letters. Typical seed match is indicated by red and atypical seed match by blue. ΔGs between the first 8 nt of miR-30a-3p and predicted target sequence were calculated using mFold v3.2. G:U wobble is indicated by ':'.

Table 3. Comparison on changes in the amount of transcripts predicted by computational programs in polyribosome fraction

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Predicted target sites P.PicTar, M:miRanda</th>
<th>Affy probe set ID</th>
<th>ANOVA/ P-value</th>
<th>Log ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>apc</td>
<td>P: TTATTGCTGTATGTTAACTGTAGCTGAAT</td>
<td>203525</td>
<td>0.453</td>
<td>0.204</td>
</tr>
<tr>
<td></td>
<td>M: ACTGTAAAAACATGTAATGTTAACTGTGTTACGGAAT</td>
<td>203526</td>
<td>0.743</td>
<td>-0.034</td>
</tr>
<tr>
<td>tol1</td>
<td>P: AGCTTCTAGTGTTTTTTTTTACTGAAATAGTGGTTCTCATGTTAGAGGGACTGAAT</td>
<td>202704</td>
<td>0.392</td>
<td>0.220</td>
</tr>
<tr>
<td></td>
<td>M: CATGTTAAGGGAGCTGAAT</td>
<td>228834</td>
<td>0.088</td>
<td>-0.339</td>
</tr>
<tr>
<td>sui1</td>
<td>P: GTGTAACCTCCTCATGCAATATACCTGAAAA</td>
<td>202021</td>
<td>0.104</td>
<td>0.117</td>
</tr>
<tr>
<td></td>
<td>M: CCTCATGCAATAAACCTGAAAA</td>
<td>211956</td>
<td>0.836</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>212130</td>
<td>0.022</td>
<td>-0.139</td>
</tr>
<tr>
<td></td>
<td></td>
<td>212227</td>
<td>0.043</td>
<td>-0.114</td>
</tr>
<tr>
<td>pmp22</td>
<td>P: CATCTGCACCTTTCCCTCTACTGGAAGA</td>
<td>210139</td>
<td>0.384</td>
<td>0.122</td>
</tr>
<tr>
<td></td>
<td>M: CATCTAACAACACTCTGGAAGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sp3</td>
<td>P: CATGAAACCTTGTGAACACGACTGAAT</td>
<td>213168</td>
<td>0.233</td>
<td>-0.305</td>
</tr>
<tr>
<td></td>
<td>M: CTGTTATACGACGACTGAAT</td>
<td>238035</td>
<td>0.436</td>
<td>0.112</td>
</tr>
</tbody>
</table>

Five transcripts, that were expressed in HepG2, out of eight overlapping transcripts between PicTar and miRanda are listed with corresponding Affymetrix probe set IDs in addition to the ANOVA/P-value and log ratio comparing miR-30a-3p knock down cells with control cells. apc, adenomatosis polyposis coli; tob1, transducer of ERBB2, 1; sui1, putative translation initiation factor; pmp22, peripheral myelin protein 22; sp3, Sp3 transcription factor. n = 3.
determined as 0.425. Finally, the probe sets that fulfill both thresholds of $P$-value and log ratio were obtained and subjected to further investigation.

**Real-time PCR analyses**

One microgram each of total RNA from either the heavy polyribosome fraction or the input RNA was used for a cDNA reaction in final volume of 20 μl using GeneAmp RNA PCR kit (Roche). An aliquot of 0.5–2 μl of the products was used for subsequent PCR reactions with Light Cycler (Roche) with SYBR Green I dye as a monitor. On the basis of the sequences obtained from GeneBank (accession nos: cyr61, NM_001554; tmem2, NM_013390; thbs1, NM_003246; scl7a6, NM_003983; vezatin, AF277625; cdk6, NM_001259.5; pro2730, CR604741 and tuba3, NM_006009), the primer sequences are as follows: cyr61: 5′-GCATCTATAACACCTTTAC-3′, TCTTCA CACTCAAAACACCCAGTAC-3′, tmem2: 5′-ACACTGGCAAT GAGT ACAGT-3′, 5′-CTTGGATTAAGACATGTGCCC-3′, thbs1-s1: ACTCTGAGAAGATTTCC-3′, 5′-ACTCTAC AGGACATTTC-3′, scl7a6: 5′-CCCTCCACATGTGA GCTAGTG-3′, 5′-GCTAGTCTAGGAAATACCACT-3′, vezatin: 5′-CCTGGTTGTTAATAACTGAGTCTG-3′, 5′-AGGT AGCAAAATTCTGCAAG-3′, cdk6: 5′-TAAGAATGTGG CAGGTTGAC-3′, 5′-GCTATGCTCTATACCATACCTG-3′, pro2730: 5′-CAGTGGCCACCTTAAGAATTG-3′, 5′-TTGGG TGGCAGAATGATCC-3′ and tuba3: 5′-AAACGTCACAA AGGTGTGCTG-3′, 5′-AGCTTTGGCTGTGAACAAAG-3′.

**Western blotting**

HepG2 cells transiently transfected with each siRNA duplex were harvested at 24 h after transfection, then added to lysis buffer (50 mM Tris–HCl, 300 mM NaCl, 30 mM EDTA, 0.5% Triton X-100, pH 7.6) for 15 min at 4°C. Total protein concentration was measured with Bradford, and 40 μg each of cell lysate was separated on 4–15% gradient polyacrylamide gel, followed with blotting to PVDF membrane at 45 V overnight. Antibodies against CYR61 (H-78, Santa Cruz) and CDK6 (B10010, Stratagene) or eIF-4E (610269, BD Biosciences) were hybridized at 25°C overnight, and horseradish peroxidase-conjugated secondary antibodies specific to the host species of those antibodies were used for detection by ECL Western Blotting Detection System (Amersham Bioscience).

**miRNA computational predictions**

For miRanda prediction of an miR-30a-3p target mRNA, miRanda v1.0b was used with the settings of 50 as a score threshold and −20 kcal/mol as an energy threshold (12). For mFold predictions, free energy of the first 8 nt of miR-30a-3p binding to the predicted target sequences was calculated, using the mFold server 3.2 (29) as described in the literature (25).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank members of the Warren laboratory for helpful discussion, Michael Epstein and Mario Caceres for statistical assistance and Jerry Boss and Kate Garber for comments on the manuscript. This work was supported in part by NIH grants HD20521 and HD35576 (to S.T.W.).

Conflict of Interest statement. None declared.

**REFERENCES**


