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Identification of miRNA targets with stable isotope labeling by amino acids in cell culture

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ABSTRACT

miRNAs are small noncoding RNAs that regulate gene expression. We have used stable isotope labeling by amino acids in cell culture (SILAC) to investigate the effect of miRNA-1 on the HeLa cell proteome. Expression of 12 out of 504 investigated proteins was repressed by miRNA-1 transfection. This repressed set of genes significantly overlaps with miRNA-1 regulated genes that have been identified with DNA array technology and are predicted by computational methods. Moreover, we find that the 3′-untranslated region for the repressed set are enriched in miRNA-1 complementary sites. Our findings demonstrate that SILAC can be used for miRNA target identification and that one highly expressed miRNA can regulate the levels of many different proteins.

INTRODUCTION

MicroRNAs (miRNAs) arise from ~21 bp RNA duplexes that are generated by the processing of ~70 bp hairpin precursors by the RNAse III enzyme Dicer (1). One of the duplex strands is incorporated into the RISC complex and mediates specific binding to mRNAs with complementary sequence (1). If the miRNA has a perfect match to the target mRNA, RISC induces cleavage of the mRNA (1). Otherwise, if the miRNA is partially complementary to the target mRNA initiation of translation is inhibited (1,2). Moreover, translational repression is associated with relocalisation of the repressed mRNA to P-bodies (3,4), which may induce decapping dependent degradation of the mRNA (5).

Computational studies based on conservation of miRNA complementary sequences in the 3′-untranslated regions (3′-UTRs) of mRNAs (6–8) as well as experimental studies (9) have shown that the 5′ end of the miRNA (also called the seed) is important for recognition of target sequences. These studies also estimate that between 10 and 30% of all human protein coding genes are regulated by miRNAs and that each miRNA has many conserved targets (6–8).

DNA microarrays have previously been used for miRNA target identification. After miR-1 duplex transfection into HeLa cells the level of 96 mRNAs were found to be significantly reduced after both 12 and 24 h (10). Interestingly, the 3′-UTR of these mRNAs were enriched for miR-1 complementary sequences, indicating that the regulation occurs via the 3′-UTR. The mechanism responsible for regulation on the mRNA level remains unclear, but may be a secondary effect of translational repression, relocalisation to P-bodies and decapping (5).

The primary regulatory effect of miRNAs is believed to occur at the translational level, but compared to mRNA profiling techniques changes in protein levels are difficult to assess in a high through-put fashion. We have used one of the best available quantitative proteomics method to investigate the effect of a miRNA on protein levels. Using this method we find that expression of 12 out of 504 investigated proteins was repressed by miRNA-1 transfection. This repressed set of genes significantly overlaps with miR-1 regulated genes that have been identified with DNA array technology and are predicted by computational methods. Moreover, we find that the 3′-UTRs for the repressed set are enriched in miRNA-1 complementary sites.

METHODS

SILAC experiment

HeLa cells (ACTT# CCL-2) were cultured for 9 days in DMEM (Invitrogen) without lysine and arginine supplemented with dialysed fetal calf serum and isotopically labeled Arginine ($^{13}C_6$-$^{15}N_4$-Arg) and Lysine ($^{13}C_6$-$^{15}N_2$-Lys) or normal Arginine and Lysine. Three 15 cm dishes of ~40% confluent labeled HeLa cells were transfected with Oligofectamine transfection reagent (Invitrogen) and a miR-1 duplex (5′-UGGAAUGUAAAGAAGUAUGUAA-3′, 5′-ACAUACUUCUUUACAUUCAAUA-3′) (Ambion, annealed

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and HPLC purified custom siRNA), whereas the control cells were transfected with oligofectamine only. Oligofectamine was used according to the recommendations of the manufacturer, except that DMEM without methionine, lysine and arginine were used for dilution instead of Opti-MEM. The transfection volume was 20 ml and the final concentration of miR-1 duplex in the transfected dishes was 100 nM. 22 h post transfection the cells were trypsinised and equal numbers of the miR-1 and control transfected cells were mixed, spun down and resuspended in hypertonic buffer [10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and Complete protease inhibitor (Roche)]. The cells were allowed to swell 5 min on ice and homogenized with 12 upward strokes in a Dounce homogenizer. The nuclei were pelleted by centrifugation 5 min at 218 *g*. The supernatant (cytoplasmic fraction) were removed and stored at −20°C and the nuclear pellet were resuspended in 3 ml of 0.25 M sucrose containing 10 mM $MgCl_2$ and layered on the top of a 3 ml 0.35 M sucrose cushion containing 0.5 mM $MgCl_2$. After centrifugation (1430 *g*, 5 min) the supernatant was removed and the nuclear pellet resuspended in 0.5 ml of sucrose solution and stored at −20°C. The nuclear and cytoplasmic factions were fractionated on a 4–10% NuPage (Invitrogen) SDS-gel that were stained with Novex colloidal Coomassie (Invitrogen). The lanes were divided into 10 gel pieces that were in gel reduced and alkylated and digested with trypsin over night. The resulting peptide mixtures were purified on C18 columns and analysed in 20 runs of nanoscale liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a linear ion trap-Fourier Transform mass spectrometer (LTQ-FT, Thermo Finnigan) using data dependent acquisition as previously described (11).

RAW data files from the mass spectrometer were searched against the Mascot database to identify peptides and map them to proteins. The MSQuant open source software package (http://msquant.sourceforge.net/) was used for quantification of SILAC ratios for individual peptides.

Data analysis

A total 6311 unique peptides were identified and quantified in the MS experiments. These were mapped to proteins and protein ratios were calculated by averaging the individual peptide ratios. To increase accuracy of the protein ratios we demanded four or more quantified peptides for proteins to be included in the analysis. If proteins were identified and quantified with four or more peptides in more than one run of the LC-MS/MS the mean of the protein ratios was used. This resulted in a set of 504 proteins (unique Ensembl protein IDs) that were identified and quantified (Supplementary Table 1 online). The obtained protein ratios were log2 transformed and plotted in a histogram (Figure 1B). The distribution of log transformed protein ratios is skewed to the left, but if the 20 most negative ratios are removed the resulting distribution is normal [Lilliefors (Kolmogorov–Smirnov) normality test, $n = 484$, $P = 0.1202$]. Comparing to this distribution 12 proteins are repressed by miR-1 ($P < 0.001$, one-sided parametric bootstrap test) (Supplementary Table 1 online).

The set of 504 identified and quantified proteins map to 496 unique Ensembl gene IDs (Supplementary Table 1 online). These IDs were used for the comparisons in Figures 1C, 1D and 2B. The RNA ratios for miR-1 transfected HeLa cells at 24 h (10) were downloaded from http://www.ncbi.nlm.nih.gov/geo/ accession number: GSE2075. Out of the 496 Ensembl gene IDs identified in this study 398 could be mapped to a corresponding mRNA ratio from the study by Lim *et al*. (10). A total of 78 genes had failed the quality control of the DNA array and the remaining 20 genes could not be identified on the array. For calculation of the overlap between the miR-1 downregulated genes at the RNA and protein level 96 genes from the Supplementary Table 1 from Lim *et al*. was used. Out of the 96 genes, 94 could be mapped to an Ensembl gene ID and 7 of these were also identified in this study. The *P*-value for the overlap between the miR-1 was calculated with Fisher's exact test for count data.

We also compared our data to miR-1 target predictions from computational studies (6–8). TargetScanS predictions were obtained from http://genes.mit.edu/tscan/targetscanS2005.html, MiRanda predictions from http://www.microrna.org/ and PicTar predictions from http://pictar.bio.nyu.edu/cgi-bin/new_PicTar_vertebrate.cgi. The significance of the overlaps with the different predictions were calculated with the Fisher's exact test for count data.

For analysis of miR-1 seedmatch (CAUUCC) occurrence 3′-UTR sequences were obtained from the PACdb (12) database and coding sequences were obtained from BioMart (http://www.ensembl.org/Multi/martview). The occurrence of the miR-1 seedmatch (CATTCC) per kb was calculated for the 12 miR-1 downregulated genes, the remaining 484 genes identified in our study and for the entire database. *P*-values were calculated by comparing the observed miR-1 seedmatch per kb value with a distribution of miR-1 seedmatch per kb values obtained by repeated sampling of 12 sequences from the set of 496 identified genes (Supplementary Figure 1).

Luciferase assays

The HSV TK promoter was PCR amplified (Advantage PCR Kit, Clontech) from pRL-TK (Promega) with the following primers: 5′-ATCGAGGTACCAAATGAGTCTTCGGACC-TCGC-3′ and 5′-AGAAGAATCAAGCTTTTAAGCGG-3′. The PCR fragment was digested with KpnI and HindIII and cloned into a KpnI/HindIII digested pGL4.12[luc2CP] vector (Promega) to give pGL4.12-TK. A multiple cloning linker: 5′-CTAGAGATCTGAATTCGGTACCACGCGTCTGCAG-CTCGAGT-3′ and 5′-CTAGACTCGAGCTGCAGACGCG-TGGTACCGAATTCAGATCT-3′ was inserted into the XbaI site of pGL4.12-TK to give pGL4.12-TK+. For the 16 genes found to be repressed by miR-1 the 3′-UTR sequences (Supplementary Table 2 online) were amplified from HeLa genomic DNA using a primary and a nested PCR (Advantage PCR Kit, Clontech) with two gene specific primersets (Supplementary Table 2 online). The second primerset included BglII and XhoI restriction sites (HAND2, ACTR3, XRCC6, HNRPU, VARS2, DHX15, CAP1 and TPM3), BamHI and XhoI restriction sites (ADAR and XRCC5) and BglII and SalI (G6PD and CALR) for directional cloning into pGL4.12-TK+. For 11 out of the 12 genes a nested PCR product was obtained and TOPO cloned into pCR4-TOPO (Invitrogen) and subsequently subcloned into pGL4.12-TK+ using

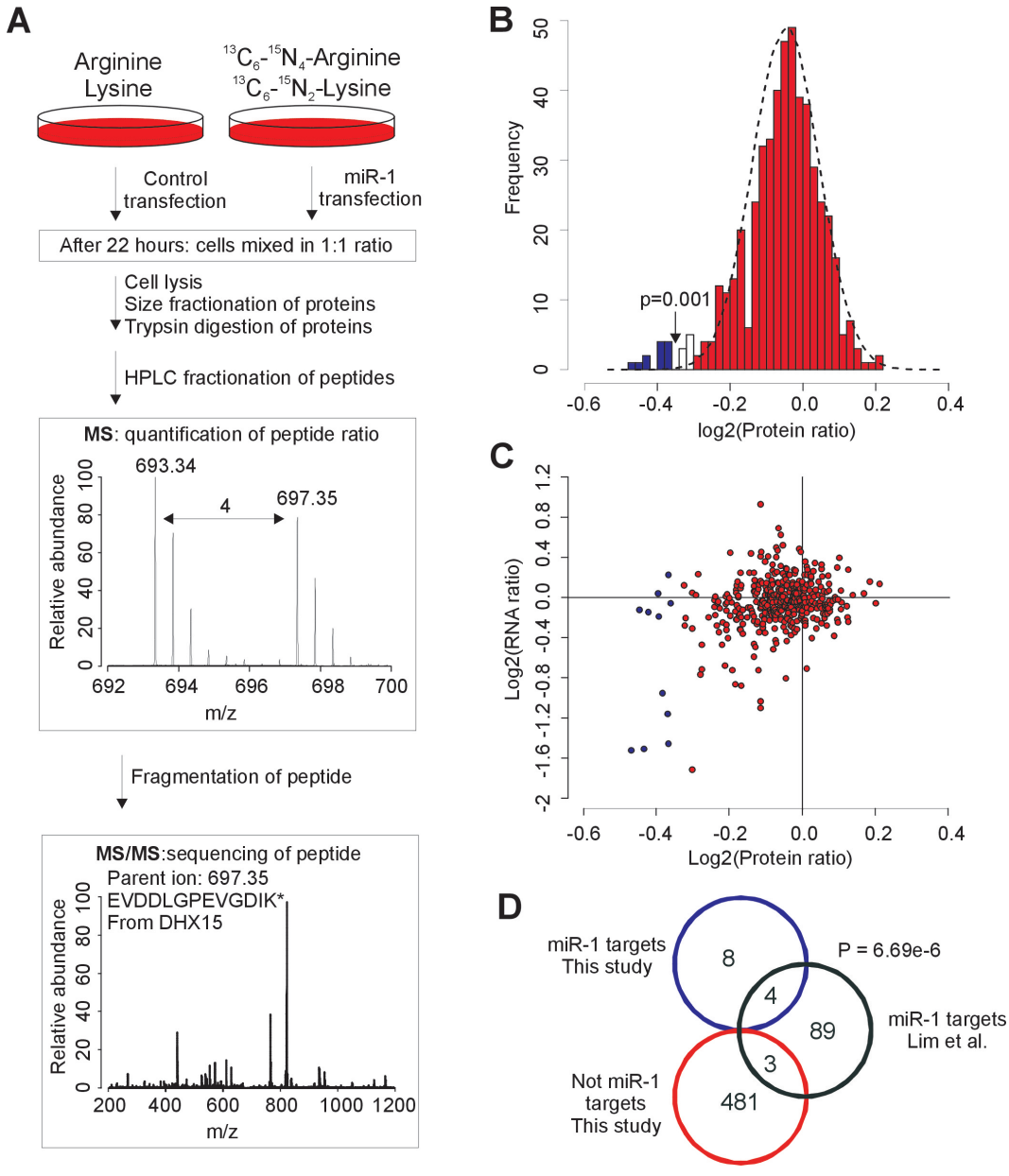

Figure 1. Identification of miR-1 targets with quantitative MS. (**A**) Schematic representation of the experimental set-up and the SILAC method. (**B**) Histogram of the 504 log2 transformed protein ratios determined in this study. The distribution is skewed to the left, but if the 20 most negative ratios are removed the resulting distribution (colored in red) is normal (Lilliefors 'Kolmogorov–Smirnov' normality test, $n = 484$, $P = 0.1202$). Comparing to this distribution 12 proteins (colored in blue) are repressed by miR-1 ($P < 0.001$, one-sided parametric bootstrap test) (Supplementary Table 1 online). (**C**) 398 of the protein ratios obtained in this study plotted against corresponding mRNA ratios (10) obtained 24 h after miR-1 transfection. (**D**) Venn diagram of genes regulated on the mRNA level by miR-1 after 12 and 24 h (10) and the miR-1 repressed set of proteins identified in this study. *P*-value for the overlap is calculated with Fisher's exact test for count data.

the primer encoded restriction sites. In addition the 3′-UTR from Hand2 was cloned as a positive control (13). All 3′-UTR sequences were verified by sequencing. For the luciferase assays, 80 000 HeLa cells/well in 12 well plates were cotransfected with 0.9 µg pGL4.12-TK+ 3′-UTR constructs, 0.1 µg pRL-TK (Promega) and 50 pmol miR-1 duplex (5′-UGGAAUGUAAAGAAGUAUGUAA-3′, 5′-ACAUACUUCUUUACAUUCAAUA-3′) or miR-1 shuffled control duplex (5′-UUGUAUAGAAUAGAUGAGGAU-3′, 5′-CCUCAUCUAUUCUAUACAAUU-3′) (Ambion, annealed and HPCL purified custom siRNA) per well. Transfections were carried out with 3 µl of Lipofectamine 2000 (Invitrogen) per well according to the recommendations of the manufacturer. The empty pGL4.12-TK+ vector was used as control. Twenty-two hours after the transfection, firefly and renilla luciferase activities were measured with the Dual-luciferase kit (Promega) on a Turner Biosystems 20/20n Luminometer using the DLR-2-INJ Promega protocol according to the recommendations of the manufacturer. Transfections were carried out in 10 replications and the error bars indicate

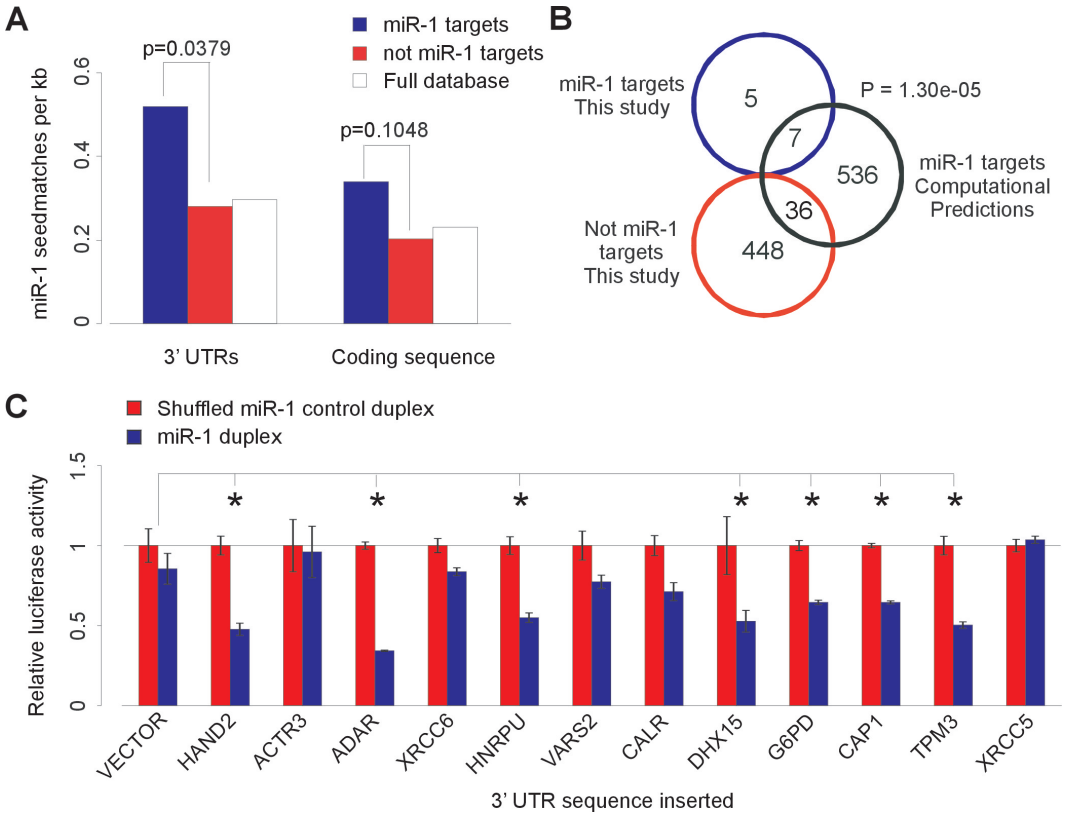

Figure 2. miR-1 complementary sites in 3′-UTR of repressed genes (**A**) The occurrence of the miR-1 seedmatch (CATTCC) per kb of 3′-UTR and coding sequence was calculated for the 12 miR-1 regulated genes, the remaining identified genes and for the entire 3′-UTR and coding sequence databases (Supplementary Table 1 online). *P*-values were calculated using a parametric bootstrap test comparing the observed value to a distribution of values obtained by repeated calculation miR-1 seedmatches per kb for 12 of 3′-UTRs or coding sequences randomly chosen from the set of identified genes (see Supplementary Figure 1). (**B**) Overlap between our data and the combined computational predictions of miR-1 targets (Supplementary Table 1 online). The *P*-value for the overlap was calculated with Fisher's exact test for count data. (**C**) Relative luciferase activities for reporter constructs containing the indicated 3′-UTR sequences (Supplementary Table 2 online). Error bars indicate the SD ($n = 10$). For each transfection firefly luciferase activity was normalized to renilla luciferase activity. For each construct the values are normalized to control miR transfection. *P*-values was calculated by comparing the ratio between the miR-1 and miR-control transfections to the same ratio for the empty vector with the Student's *t*-test ($n = 18$) and correcting for multiple testing with the Bonferroni correction. Standard deviations for the ratios were calculated using standard error propagation. *P*-values <0.05 are indicated. Data and calculations are included in Supplementary Table 2 online.

the standard deviation. For each transfection the firefly luciferase activity was normalized to the renilla luciferase activity. For each construct the values are normalized to control miR transfection and a *P*-value was calculated with the Student's *t*-test ($n = 18$) by comparing to the ratio between the miR-1 and miR-control transfections to the same ratio for the empty vector. Standard deviations for the ratios were calculated using standard error propagation.

RESULTS

To investigate the regulatory effect of miRNA on the proteome, we have used stable isotope labeling by amino acids in cell culture (SILAC) (14). This method allows quantitation of the relative protein abundance between two cell populations (Figure 1A). In this study we compared control-transfected HeLa cells with SILAC labeled HeLa cells transfected with the muscle specific miR-1. Cells from the two cell pools were mixed in a 1:1 ratio and nuclear and cytoplasmic lysates were prepared. The two lysates were size fractionated on a regular SDS-gel into 2 × 10 fractions, in-gel digested with trypsin and the resulting peptide mixtures were analysed in 20 runs of nanoscale LC-MS/MS on an ion trap-Fourier Transform Mass Spectrometer, capable of very high mass accuracy and of sequencing several peptides per second (11). A total of 6311 unique peptide sequences were matched to human genes with an average mass accuracy of 1 p.p.m. For each protein the corresponding peptide ratios were averaged to give a representative protein ratio. To increase accuracy of the measured ratios, we only included the 504 proteins that had at least four unique quantified peptides in our analysis, which also effectively removes any risk of misidentification of the proteins (Supplementary Table 1 online). These 504 proteins map to 496 unique Ensembl gene IDs. The distribution of log2 transformed protein ratios (Figure 1B) is skewed to the left as would be expected if some proteins were present at a lower levels in the miR-1 transfected than the control transfected cells. A set of 12 proteins have protein ratios that are significantly smaller than the remaining protein ratios, which demonstrates that the level of these proteins have been repressed by miR-1 transfection.

We were able to map 398 of our 496 identified genes to the mRNA data obtained 24 h after miR-1 transfection (10)

(Figure 1C). The remaining 98 genes (including one from our repressed set) either could not be identified on the array or had failed the array quality control (see Supplementary Table 1 online). For five of the proteins in our regulated set we find a clear correlation with the effects observed on the mRNA level, but for the remaining six there is no correlation (Figure 1C). Lim *et al*. (10) identify a set of 96 miR-1 targets that are down regulated both at 12 and 24 h. We have protein data for 7 of these 96 genes and find that 4 are repressed on the protein level as well as the RNA level. The overlap between the two dataset is much larger than what would be expected by chance (Figure 1D), indicating that the repression observed on the protein and RNA levels are related.

Generally miRNA target sites are found in the 3′-UTRs of genes. We find that the 3′-UTRs from 8 out of the 12 genes in our miR-1 repressed set contain miR-1 seed sequences, which is a significant enrichment for miR-1 seedmatches compared to the non-regulated set and the full 3′-UTR data sequences (12) (Figure 2A). This indicates that the repression we observe is mediated via the 3′-UTR. Moreover, we find a highly significant overlap with the combined MiRanda (6), PicTar (7) and TScanS (8) miR-1 target predictions, showing that many of the miR-1 target that are detected by the SILAC method also are predicted by computational methods (Figure 2B). The overlap with individual prediction methods is also highly significant (see Supplementary Table 1 and Supplementary Figure 2 online). To experimentally verify that miR-1 regulation occurred via the 3′-UTRs, we tested the 3′-UTRs from 11 of our regulated proteins in a reporter system. As a positive control we used the 3′-UTR from Hand2, which has previously been shown to be a miR-1 target (13). Compared to the empty vector 6 out of the 11 UTRs were found to repress expression in an miR-1 dependent fashion (Figure 2C).

DISCUSSION

In this study we have transfected miR-1 into HeLa cells to investigate the effect of miRNAs on protein levels. Of the 504 identified and quantified proteins 12 (2.4%) were found to be significantly repressed by miR-1 (Supplementary Table 1 online). In comparison, 96 out of ∼22 000 mRNAs (0.44%) were found to be repressed by miR-1 using DNA microarrays (10). Our findings therefore suggest that single highly expressed miRNA, such as miR-1, can regulate a substantial percentage of the proteome and have a profound effect on expression in that tissue.

In human cells miRNAs are believed to predominantly affect the translation of target mRNAs (1), although regulation of mRNA levels has also been reported (10). We find a significant overlap between the genes repressed by miR-1 on the mRNA and protein levels. For some genes repression is observed both at the mRNA and the protein level, while others are regulated only on the protein or the RNA level (Figure 1C). Recently, it has been suggested that miRNAs decrease mRNA levels by relocalization of translational repressed mRNAs to P-bodies and increasing decapping (5). It is possible that some mRNAs are more prone to decapping and degradation in P-bodies than others, which would explain the partial overlap between the effects observed on the mRNA (10) and protein level. However, some miR-1 target genes may have a very long mRNA or protein half-life and this could also be an explanation for our findings. In all cases our results suggests that the regulation occurring on the mRNA and protein levels are related.

It is now well documented that most siRNAs will have off-target effects that are dependent on the seed region and can detected at the RNA level (10,15,16). Our findings suggest that some siRNA off-target effects could be present only at the protein level and therefore further emphasize that results from siRNA experiments should be interpreted with caution and proper controls should be performed.

In vivo, miR-1 is expressed at very high levels (more than 100 000 copies per cell) in skeletal muscle and heart tissue, but only at very low levels in other tissues (10). Several of the proteins that we identify as miR-1 targets have muscle related functions and could potentially be important miR-1 targets *in vivo*. ACTR3 (ARP3 actin-related protein 3 homolog) is part of the Arp2/3 complex, which is involved in nucleating the formation of actin filaments (17). TPM3 (tropomyosin 3) is a component of the sarcomere's thin filaments and is involved in calcium mediated regulation of the myosin–actin interaction (18). CALR (calreticulin) is a calcium-binding protein localized to the lumen of the endoplasmic and sarcoplasmic reticulum that may be involved in calcium storage (19).

For 6 out 11 miR-1 target genes identified in our SILAC experiment, we demonstrate that the miR-1 mediated repression occurs via the 3′-UTR. For the remaining five we see little or no effect of the 3′-UTR in our reporter experiments. It is possible that these genes are regulated *in vivo* via miR-1 complementary sites in the coding sequence or that the 3′-UTRs were incorrectly annotated. One example of this is probably ACTR3. For the reporter experiments we have used the 1135 bases long ACTR3 3′-UTR sequence from the NCBI refseq mRNA that does not have any miR-1 complementary sites. However, the 3′-UTR annotated in the PACdb 3′-UTR database (12) is considerable longer and contains two miR-1 seed sequences. Obviously, it is also possible that some of the miR-1 target genes that we identify are repressed by miR-1 indirectly as a result of secondary effects. XRCC5 (Ku autoantigen, 80 kDa) and XRCC6 (Ku autoantigen, 70 kDa) make up the regulatory subunit of the nuclear serine/threonine protein kinase DNA-PK that is crucial for repair of double stranded DNA breaks. Interestingly, we find that the nuclear and cytoplasmic protein levels of both these proteins are repressed by miR-1 transfection. However, the 3′-UTR from the mRNAs encoding these two proteins does not have any recognizable miR-1 complementary sites and do not inhibit expression in our reporter experiment, indicating that the regulation of XRCC5 and XRCC6 may be secondary to some miR-1 or transfection induced regulation of the DNA-PK protein complex.

In our experiments we have tested the effect of transfection of the muscle specific miR-1 into HeLa cells that are derived from the cervix. It is possible that some of the miR-1 target genes that we and Lim *et al*. (10) identify are physiologically irrelevant in the sense that they may be expressed in HeLa, but not together with miR-1 *in vivo*. The 3′-UTR of mRNAs that are not expressed in muscle cells has a tendency to contain nonconserved miR-1 target sites and many of these are

still functional for miR-1 repression (20). To avoid detecting such physiologically irrelevant miR target genes it could be advantageous to use miRNA inhibition rather than miRNA transfection for future miRNA target genes identification experiments.

The capacity of the quantitative MS/MS method for miRNA target gene identification that we present here could be further improved by more extensive fractionation of proteins before MS analysis. Also addition of an affinity purification step could be combined with our method to allow for detection of specific classes of proteins and less abundant proteins, such as transcription factors. Compared to strategies based on DNA microarray detection of mRNAs, proteomic strategies to miRNA target detection will remain low through-put. Nevertheless, we believe that methods such as ours could complement DNA microarray experiments. First, all methods for detection of miRNA target genes are susceptible to detection of indirect targets that are repressed through secondary effects. It is therefore necessary to use short time frames for the experiments. For some genes the half life of the protein may be much shorter than the mRNA allowing for detection of miRNA mediated repression only at the protein level. Second, the exact mechanism of miRNA mediated repression is still debated and it is not known how miRNA mediated mRNA destabilization and translational repression relate to each other. DNA microarray experiments only detect a fraction of the potential miRNA targets that can be predicted *in silico* (10,15,16). Our results indicate that some genes may be repressed on the protein level without being affected on the mRNA level and for such miRNA target genes a proteomic strategy will be necessary for detection.

In conclusion, we demonstrate that many proteins can be directly repressed by a single highly expressed miRNA and that SILAC can be used for miRNA target identification.

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. Pillai,R.S., Bhattacharyya,S.N., Artus,C.G., Zoller,T., Cougot,N., Basyuk,E., Bertrand,E. and Filipowicz,W. (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science*, **309**, 1573–1576.
3. Liu,J., Valencia-Sanchez,M.A., Hannon,G.J. and Parker,R. (2005) MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nature Cell Biol*., **7**, 719–723.
4. Sen,G.L. and Blau,H.M. (2005) Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nature Cell Biol*., **7**, 633–636.
5. Valencia-Sanchez,M.A., Liu,J., Hannon,G.J. and Parker,R. (2006) Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev*., **20**, 515–524.
6. John,B., Enright,A.J., Aravin,A., Tuschl,T., Sander,C. and Marks,D.S. (2004) Human MicroRNA targets. *PLoS Biol*., **2**, e363.
7. Krek,A., Grun,D., Poy,M.N., Wolf,R., Rosenberg,L., Epstein,E.J., Macmenamin,P., da Piedade,I., Gunsalus,K.C., Stoffel,M. *et al*. (2005) Combinatorial microRNA target predictions. *Nature Genet*., **37**, 495–500.
8. 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.
9. Brennecke,J., Stark,A., Russell,R.B. and Cohen,S.M. (2005) Principles of microRNA-target recognition. *PLoS Biol*., **3**, e85.
10. 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.
11. Ong,S.E., Blagoev,B., Kratchmarova,I., Kristensen,D.B., Steen,H., Pandey,A. and Mann,M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell Proteomics*, **1**, 376–386.
12. Olsen,J.V. and Mann,M. (2004) Improved peptide identification in proteomics by two consecutive stages of mass spectrometric fragmentation. *Proc. Natl Acad. Sci. USA*, **101**, 13417–13422.
13. Brockman,J.M., Singh,P., Liu,D., Quinlan,S., Salisbury,J. and Graber,J.H. (2005) PACdb: PolyA Cleavage Site and 3′-UTR Database. *Bioinformatics*, **21**, 3691–3693.
14. Zhao,Y., Samal,E. and Srivastava,D. (2005) Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature*, **436**, 214–220.
15. Birmingham,A., Anderson,E.M., Reynolds,A., Ilsley-Tyree,D., Leake,D., Fedorov,Y., Baskerville,S., Maksimova,E., Robinson,K., Karpilow,J. *et al*. (2006) 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nature Methods*, **3**, 199–204.
16. Jackson,A.L., Burchard,J., Schelter,J., Chau,B.N., Cleary,M., Lim,L. and Linsley,P.S. (2006) Widespread siRNA 'off-target' transcript silencing mediated by seed region sequence complementarity. *RNA*, **12**, 1179–1187.
17. Higgs,H.N. and Pollard,T.D. (2001) Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annu. Rev. Biochem*., **70**, 649–676.
18. Gunning,P.W., Schevzov,G., Kee,A.J. and Hardeman,E.C. (2005) Tropomyosin isoforms: divining rods for actin cytoskeleton function. *Trends Cell Biol*., **15**, 333–341.
19. Michalak,M., Guo,L., Robertson,M., Lozak,M. and Opas,M. (2004) Calreticulin in the heart. *Mol. Cell. Biochem*., **263**, 137–142.
20. 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.