ARTICLES

Widespread changes in protein synthesis induced by microRNAs

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Animal microRNAs (miRNAs) regulate gene expression by inhibiting translation and/or by inducing degradation of target messenger RNAs. It is unknown how much translational control is exerted by miRNAs on a genome-wide scale. We used a new proteomic approach to measure changes in synthesis of several thousand proteins in response to miRNA transfection or endogenous miRNA knockdown. In parallel, we quantified mRNA levels using microarrays. Here we show that a single miRNA can repress the production of hundreds of proteins, but that this repression is typically relatively mild. A number of known features of the miRNA-binding site such as the seed sequence also govern repression of human protein synthesis, and we report additional target sequence characteristics. We demonstrate that, in addition to downregulating mRNA levels, miRNAs also directly repress translation of hundreds of genes. Finally, our data suggest that a miRNA can, by direct or indirect effects, tune protein synthesis from thousands of genes.

MicroRNAs are key *trans*-acting factors that post-transcriptionally regulate metazoan gene expression, and identifying miRNA targets as well as the effect that miRNAs exert on them is a fundamental question for understanding life, health and disease[1–5]. The first identified miRNA targets in *Caenorhabditis elegans* were found to be translationally repressed whereas target mRNA levels were only mildly downregulated. Subsequently, similar cases were reported in mammalian systems[6,7]. Reporter constructs provided experimental evidence that miRNAs can directly repress translation initiation[8–10]. Furthermore, it has been shown that different mechanisms exist by which miRNAs repress protein synthesis or induce mRNA degradation[6,11]. Overexpressing a miRNA in human cell lines causes mostly mild (less than twofold) downregulation of hundreds of mRNAs, of which many are direct targets[12]. Nonetheless, these results do not reveal how much control miRNAs exert on protein synthesis. Because protein synthesis is one of the most important quantities for the phenotype, a fundamental question about gene regulation has therefore remained unanswered.

Identifying miRNA targets has been the subject of a steeply growing number of computational[13–16] and experimental[17–20] approaches. Although certain features of the miRNA-binding site such as seed sites (Watson–Crick consecutive base pairing between mRNAs and the miRNA at position 2–7 counted from its 5′ end) located in the 3′ untranslated regions (3′ UTRs) of mRNAs are important, it is unknown how relevant they are for changes in protein production. Several rules regarding the architecture of miRNA-binding sites have been proposed to explain differences in their efficacy in mRNA degradation versus translational repression[6,21]. However, these rules were based on a few target sites that were studied mostly in reporter assays with non-endogenous proteins. Another study about the effects of miRNA on the proteome was limited by the small number (12) of detected downregulated proteins[22]. Furthermore, different proteins have different turnover times. For example, if a miRNA completely shuts off protein production, steady-state levels of high-turnover proteins will change rapidly whereas stable proteins will be affected later. Therefore, changes in protein concentrations as measured by standard techniques cannot quantify changes in protein synthesis if protein levels are not stationary. In fundamental biological processes such as differentiation, the expression of miRNAs is strongly induced (or switched off) in a relatively small time window[23]. Thus, to assess endogenous regulation of mRNA translation by miRNAs, a technique is needed to measure directly genome-wide changes in protein synthesis shortly after changes in miRNA expression.

pSILAC measures changes in protein production

To overcome these problems, we devised a new variant of SILAC (stable isotope labelling with amino acids in cell culture). In SILAC, proteins are metabolically labelled by cultivating cells in growth medium containing heavy isotope versions of essential amino acids[24,25]. Mass spectrometry can distinguish peptides derived from SILAC-labelled proteins. The ratio of peptide peak intensities reflects differences in corresponding protein abundance. We reasoned that by pulse-labelling with two different heavy stable isotope labels we could measure changes in protein production between two samples. In our pulsed SILAC (pSILAC) method, cells in the two samples are pulse-labelled with two different heavy versions of amino acids. During labelling, all newly synthesized proteins will be 'heavy' or 'medium-heavy' (Fig. 1a). Pre-existing proteins present before labelling remain in the light form and are ignored. Only intensity differences between newly synthesized proteins (medium-heavy and heavy) are considered. Hence, pSILAC quantifies differences in protein production between both samples integrated over the measurement time after the pulse[26]. This is fundamentally different from pulse-labelling with a single label to determine protein turnover or transport[27–29]. We combined pSILAC with state-of-the-art mass-spectrometry-based proteomics[30–32] to measure changes in production of ~5,000 proteins altogether.

We performed transfections to individually overexpress five human miRNAs in HeLa cells. These miRNAs are tissue-specific and virtually absent in HeLa cells (miR-1, miR-155) or expressed in many tissues (miR-16, miR-30a, let-7b) including HeLa cells[33]. At least 90% of all cells could be efficiently transfected (Supplementary Fig. 1), and miRNAs were overexpressed for at least 32 h post-transfection (not shown). Changes in protein production

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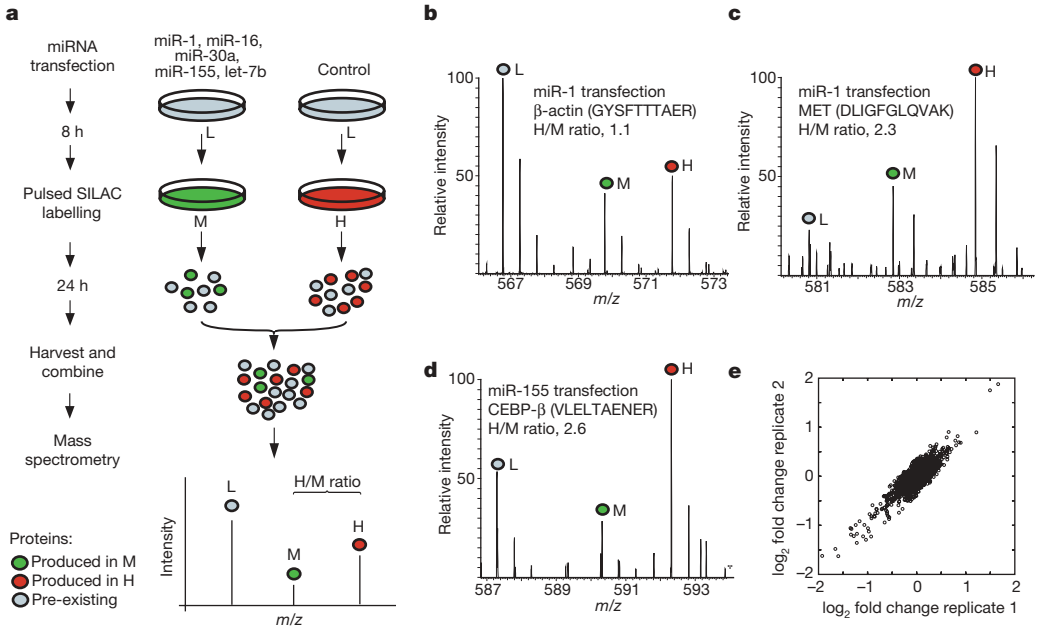
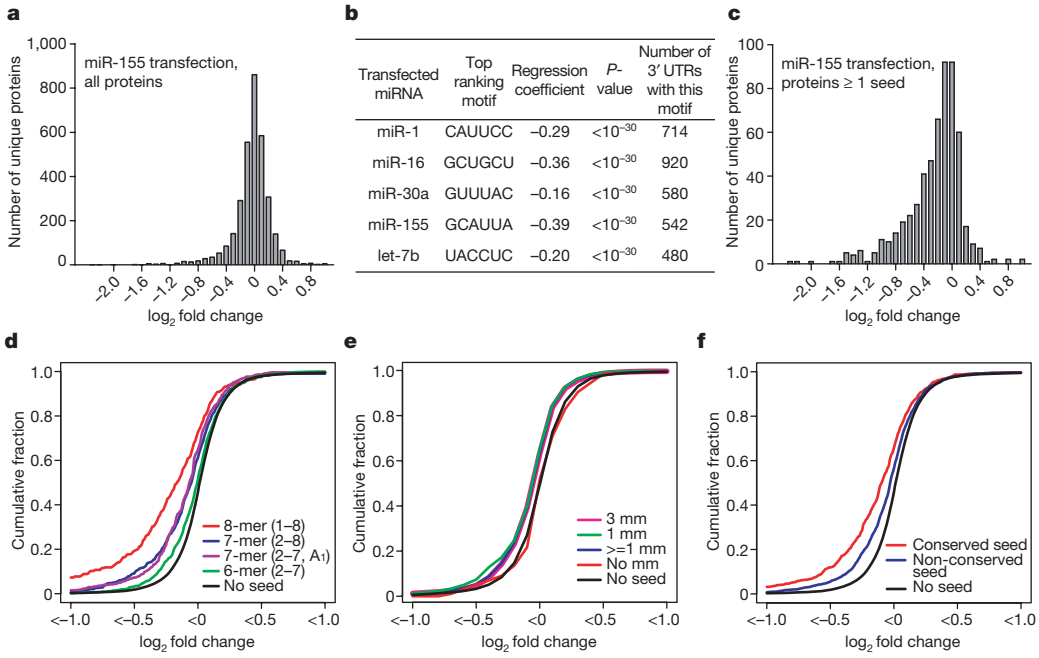


Figure 1 | Global analysis of changes in protein production induced by microRNAs. a, HeLa cells cultivated in normal light (L) medium were either transfected with a miRNA or mock transfected. After 8 h, transfected and control cells were pulse-labelled by transferring them to culture medium containing medium-heavy (M) or heavy (H) isotope-labelled amino acids, respectively (pSILAC). All newly synthesized proteins will appear in the H or M form. Samples were combined after 24 h and analysed by mass spectrometry. Intensity peak ratios between heavy and medium-heavy peptides (H/M ratio) reflect changes in protein production. RNA from the same samples was analysed by microarrays. **b**–**d**, Exemplary peptide mass spectra (sequences are in parentheses). The production of most proteins is unaltered, as shown for a β-actin peptide. In contrast, synthesis of MET and CEBPβ is reduced by miR-1 or miR-155 overexpression. **e**, Reproducibility of pSILAC (biological replicate, see Supplementary Methods).

were measured by pulse-labelling at 8 h post-transfection over a time period of 24 h. Representative mass spectra are shown in Fig. 1b–d. In total, we identified 4,961 proteins in HeLa cells with high confidence (false discovery rate <1%, see Supplementary Methods). Although mass spectrometry is biased to detect more highly expressed genes, this bias was mild and did not affect the detection range (Supplementary Fig. 2). We validated 16 out of 16 selected pSILAC measurements by western blotting (Supplementary Fig. 3). Analysis of biological replicates showed high correlation (Pearson correlation coefficient ~0.9) over the entire dynamic range (Fig. 1e).



Transfected miRNA	Top ranking motif	Regression coefficient	*P*-value	Number of 3′ UTRs with this motif
miR-1	CAUUCC	−0.29	$<10^{-30}$	714
miR-16	GCUGCU	−0.36	$<10^{-30}$	920
miR-30a	GUUUAC	−0.16	$<10^{-30}$	580
miR-155	GCAUUA	−0.39	$<10^{-30}$	542
let-7b	UACCUC	−0.20	$<10^{-30}$	480

Figure 2 | miRNAs downregulate protein synthesis of hundreds of genes. a, Histogram of changes in production of 3,299 proteins in HeLa cells after miR-155 overexpression. **b**, An unbiased search for 3′ UTR motifs that correlate with pSILAC fold changes yielded precisely the miRNA seed sequences. **c**, Proteins with miR-155 seeds tend to be downregulated by miR-155 overexpression. **d**, Cumulative distributions of different seed classes (matches to positions 1–8 (8-mer), 2–8 (7-mers), 2–7 with adenosine in position 1 (2–7, A_1) and 2–7 (6-mer)). **e**, Mismatches (mm) between positions 9 and 11 of the miRNA and target mRNAs with a seed correlate with downregulation. Protein synthesis from mRNAs with perfect complementarity at positions 9–11 (red) and synthesis from mRNAs without seeds (black) is indistinguishable. **f**, Conserved seeds mediate more downregulation than non-conserved seeds. Results are shown for pooled data based on messages with one seed only (**d**–**f**).

mRNA sequence features of repressed proteins

Perhaps surprisingly, pSILAC revealed that miRNA overexpression had, overall, mild effects on the synthesis of most of the 3,000–3,500 proteins quantified in each transfection (shown for miR-155 in Fig. 2a). Because miRNAs are thought to target mRNAs primarily by binding *cis*-regulatory sites in 3′ UTRs, we used a linear-regression-based analysis[34] to identify 3′ UTR sequence motifs that correlated best with changes in protein production. This method performs an unbiased screen for all nucleotide motifs of one to six nucleotides in length. For each miRNA, the most significant motif of all possible 5,460 motifs was precisely the seed of the respective miRNA (Fig. 2b), and correlated with downregulation. The same motif search in 5′ UTRs had no significant results. Searching coding sequences yielded the seed in only two experiments (let-7b, miR-16), and further analyses showed that 3′ UTRs exert the strongest effect (Supplementary Fig. 4). Taking miR-155 as an example, the seed enrichment in downregulated proteins is illustrated by the histogram of fold changes for proteins that contain at least one seed in their mRNA 3′ UTRs (Fig. 2c). Thus, proteins with reduced synthesis are enriched in direct miRNA targets, and a primary motif to mediate this reduction is the 3′ UTR seed. Certain characteristics such as seed-flanking nucleotides have been reported to affect the degree of mRNA degradation by miRNAs[35,36], and we show that these effects are also involved in repressing protein production (Fig. 2d).

When small interfering RNAs (siRNAs) are perfectly complementary to their targets, mRNA cleavage occurs between nucleotides 10 and 11 opposite the siRNA guide strand; in contrast, mismatches in this region strongly reduce cleavage[37–39]. A small-scale study with reporter constructs suggested that siRNA–mRNA pairs with mismatches between nucleotides 9–11 of the siRNA are mainly repressed at the protein level with little effect on the transcript[21]. We found that only seed-containing mRNAs with at least one mismatch were, overall, repressed at the protein level (Fig. 2e). In contrast, protein production from seed-containing mRNAs with perfect base pairing from nucleotides 9 to 11 and mRNAs lacking seeds was indistinguishable. Hence, although mismatches are deleterious to siRNA-mediated cleavage of mRNAs, they correlate with increased repression of protein production by miRNAs. We also found that, on average, repression is more pronounced for conserved than for non-conserved seed sites (Fig. 2f), indicating that our experiments reflect biological relevance and that there are determinants in addition to the seed that mediate efficient downregulation of protein synthesis.

We next quantified how many of the downregulated proteins can be explained by the seed. We recorded how many proteins with at least one 3′ UTR seed site were downregulated by at least *c*-fold as a function of *c* (Fig. 3a). For example, the production of more than 300 proteins with seeds was downregulated by at least 30% ($\log_2$-fold change <-0.5). These proteins amounted to roughly 60–70% of all measured proteins downregulated by at least this much (Fig. 3b). Because the background seed frequency is 10–30% (Fig. 3b, dashed horizontal lines), we can explain up to 60% of the ~300 proteins by the presence of seeds. It remains an open question how many proteins without a seed are direct targets. Nevertheless, pSILAC clearly generates lists of proteins enriched in direct targets. We independently validated the 3′ UTR-dependence of protein production by dual luciferase reporters for eight 3′ UTRs with a seed for either miR-1 or let-7b (see Supplementary Methods). The correlation with the corresponding pSILAC data was high (Fig. 3c).

pSILAC data and target predictions

Having shown that pSILAC data are enriched in direct miRNA targets, we tested how miRNA target predictions correlate with our data. We calculated the fraction of predicted mRNA targets for which protein production was downregulated by at least *c*-fold. The results were consistent for all values of *c* and all miRNAs individually (data not shown). For example, roughly 27% of all 24,238 mRNAs present in the pSILAC data were downregulated more strongly than -0.1 $\log_2$-fold change (Fig. 3d and Supplementary Table 1). A completely random selection would therefore have 27% overlap with pSILAC data. This background accuracy was exceeded by all methods except one based on 5′ UTRs. Simply considering seed sites boosts the accuracy to 44%. This accuracy was only topped by three methods that use evolutionary conservation of seed sites as an additional filter. Almost all other methods, in part based on site-accessibility evaluation, made fewer predictions with less accuracy.

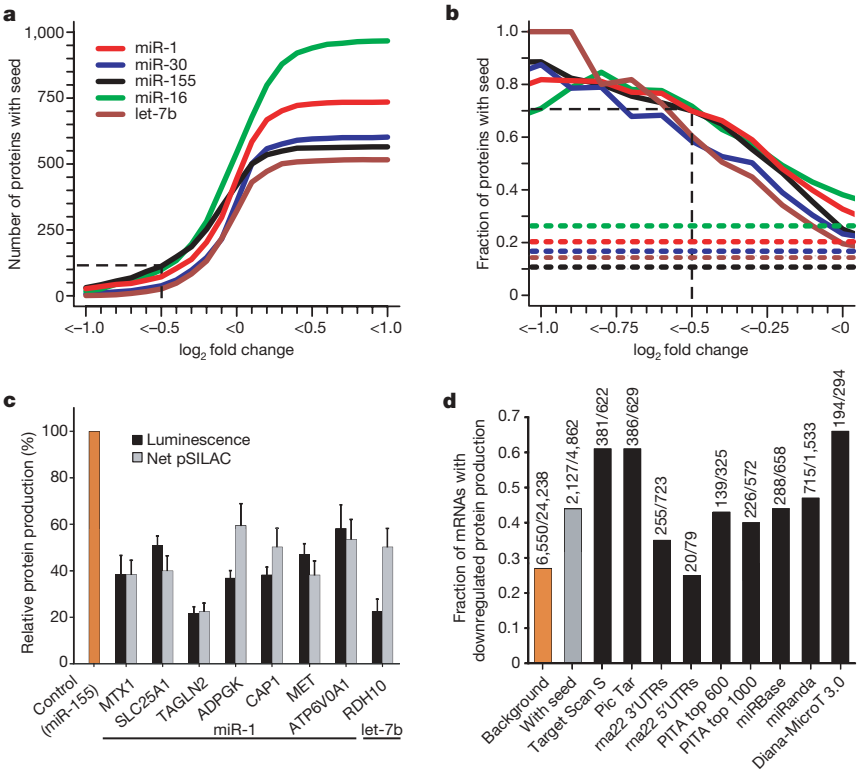


Figure 3 | The miRNA seed explains a large fraction of downregulated protein synthesis. **a**, Cumulative number of proteins with seeds as a function of changes in their production. For a given cutoff, this indicates the number of downregulated seed-containing proteins (shown for -0.5 $\log_2$-fold change). **b**, Fraction of proteins with a seed as a function of repression. Background seed frequency of unchanged proteins (absolute $\log_2$-fold change <0.1) ranges from 10–30% (dashed lines). **c**, Dual luciferase reporter assays for 3′ UTR-mediated regulation by miRNAs (± s.d., $n = 3$). 'Net pSILAC' refers to the difference of pSILAC $\log_2$-fold changes for the miRNA and the control (error bars show 95% confidence interval, see Supplementary Methods). **d**, The fraction of computationally predicted target mRNAs with reduced protein production ($\log_2$-fold change <-0.1) is calculated for all five miRNA data sets pooled.

Translational repression by miRNAs

pSILAC measures changes in the amount of newly synthesized proteins between two samples. This depends on changes of mRNA levels and, in addition, on translational regulation. To discern these two mechanisms, in all pSILAC experiments we measured the mRNA fold changes between the miRNA-transfected sample and the control by Affymetrix microarrays at the beginning of the pulse labelling ($t_1 = 8$ h) and at the end ($t_2 = 32$ h). A total of 69 quantitative polymerase chain reactions with reverse transcription (qRT–PCRs) demonstrated that our microarray data have little compression or other distortion effects in the range where most mRNA fold changes were observed (Supplementary Fig. 5).

For miR-1 as an example, we present the relationship between miRNA-induced fold changes in protein production (pSILAC) and mRNA fold changes (Fig. 4a, b) separately for t_1 and t_2. Very few genes had fold changes of unequal sign and reasonable magnitude ($\geq$1.3-fold). The correlation between mRNA fold changes and pSILAC fold changes became better at t_2. In particular, many genes with downregulated protein production but little mRNA fold changes at t_1 shifted towards greater mRNA fold changes at t_2. Similar overall effects could be seen for the other miRNAs. Nevertheless, the considerable scatter indicates substantial and widespread post-transcriptional regulation of gene expression.

The distribution of fold changes measured by microarray and pSILAC was similar (Fig. 4c, histograms). However, the average number (s) of seeds per gene was higher for more highly downregulated genes. Seed enrichment was not observed for upregulated genes, indicating that the recently reported miRNA-mediated activation of gene expression did not occur under our experimental conditions[40]. For downregulated genes, log-fold changes were linearly correlated with s. Thus, if a target has two seeds, the repressive effect is multiplicative, as has been observed in small-scale studies[15,41]. pSILAC data also support earlier findings[36] that synergistic effects are higher for two nearby seeds (<40 nucleotides) compared to larger spacings (>40 nucleotides; P-value 0.003, one-sided Wilcoxon test). Intriguingly, the slope of s in Fig. 4c is steeper for pSILAC fold changes, suggesting that the multiplicity of a miRNA-binding site in the same 3′ UTR exerts a stronger direct effect on protein production than on mRNA levels. To assess miRNA-mediated changes in translation rates for each gene, we subtracted the $\log_2$ mRNA from the $\log_2$ pSILAC fold changes, and plotted s as a function of these differences (Fig. 4d). The linear decay of s towards the regime of equal fold changes indicates that, in addition to mediating mRNA downregulation[12], the seed also mediates direct repression of translation rates for hundreds of genes.

Endogenous miRNA knockdown

It could be argued that the overexpression of miRNAs can lead to largely non-physiological effects. We therefore used a locked nucleic acid (LNA) approach[42,43] to knockdown let-7b in HeLa cells (Fig. 5a), and measured changes in protein production and mRNA levels as before. Luciferase reporter experiments demonstrated that our knockdown functionally derepressed a known *let-7* target[44] mediated by seed sites (Supplementary Fig. 6). As in the overexpression experiments, an unbiased search for 3′ UTR motifs identified the let-7b seed as the best match. Coding sequences and 5′ UTRs did not yield significant results. Further analyses showed that all effects for seed-mediated targets that we report for the overexpression experiments hold true for the let-7b knockdown after flipping the sign of pSILAC and microarray fold changes, including correlation of target-finding algorithms with pSILAC data (Supplementary Fig. 7). Together, these data suggest that the miRNA overexpression experiments are also physiologically relevant.

let-7b tunes production of thousands of proteins

When we compared the cellular response to let-7b overexpression and knockdown we observed a marked anti-correlation, not only for seed-mediated let-7b targets but also for most of the ~2,700 proteins quantified in both experiments (that is, for both direct and indirect effects; Fig. 5b). For example, when considering all ~130 proteins with a fold change of at least 15% in both the overexpression and knockdown experiments, most were up in one of the experiments but down in the other, irrespective of seeds (Fig. 5c). In contrast, almost all proteins with seeds were down in the overexpression experiment

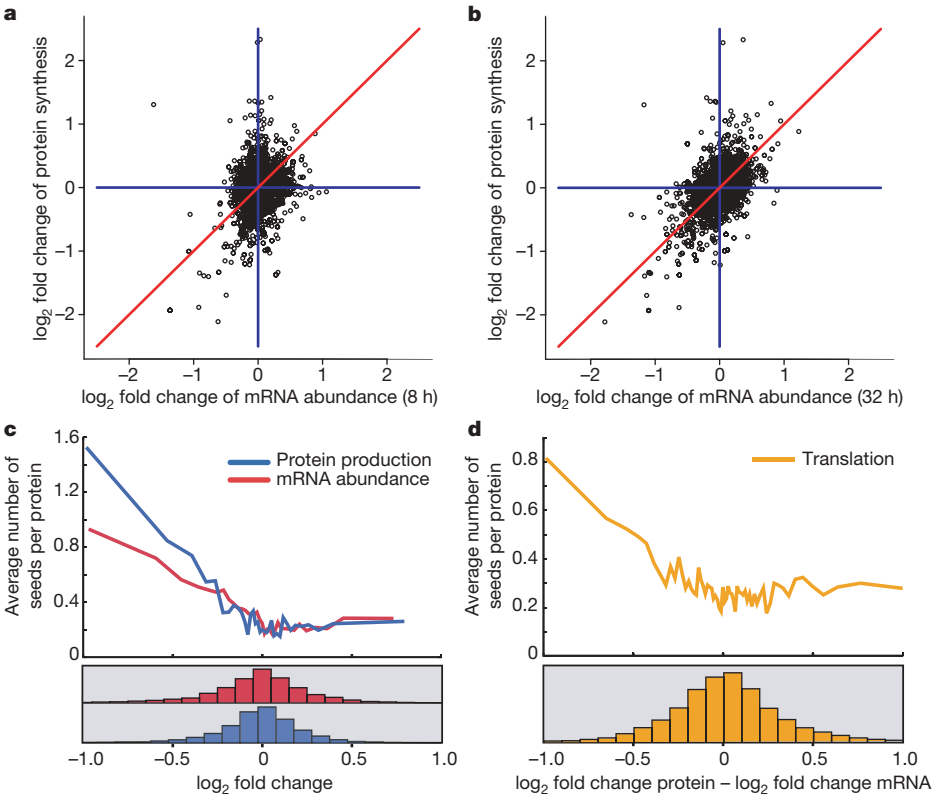


Figure 4 | miRNAs inhibit translation on a genome-wide scale. **a**, Changes in protein production between 8 h and 32 h after miR-1 transfection with mRNA fold changes at 8 h reveal poor overall correlation. **b**, mRNA levels at 32 h correlate remarkably well with changes in protein synthesis. **c**, Overall fold changes of mRNA and protein synthesis are similar (histograms). Reduced protein production and mRNA levels correlate with seed frequency (curves represent proteins ranked by fold change and grouped into bins of 250). **d**, Translational repression by miRNAs is revealed by subtracting mRNA log changes from log changes in protein production. Increased seed frequency, averaged as in **c**, correlates with translational repression. Results are shown for pooled data (**c**, **d**) after discarding genes with mRNA and pSILAC changes of unequal sign.

and up in the knockdown. When averaging the data, we found a linear response of the entire proteome to miRNA misexpression with a slope of −0.3 (Fig. 5b, inset), demonstrating that, on average, let-7b overexpression induced roughly threefold higher $\log_2$-fold changes than let-7b knockdown. Together, these data indicate that upregulation and downregulation of stationary let-7b levels has largely complementary effects on the proteome; that is, let-7b levels can tune protein production from thousands of genes.

Discussion

Here we have measured for the first time changes in cellular protein synthesis in response to miRNA induction or knockdown on a proteome-wide scale. Our results show that a single miRNA can directly downregulate production of hundreds of proteins. In addition to the known effect on global mRNA levels[12], our data strongly indicate that miRNAs translationally repress hundreds of direct target genes. Using an unbiased approach, we identified the seed sequence in the 3′ UTR as a primary motif of miRNA-mediated regulation of protein production. The seed correlated with both mRNA degradation and translational repression (Fig. 4c, d).

Perhaps surprisingly, the repressive effect on individual proteins was relatively small and rarely exceeded fourfold. Because we performed pulsed labelling, this result cannot be explained by persistence of stable proteins. Other investigators observed much higher fold changes (up to 30-fold) in a similar system (double-stranded RNA (dsRNA) transfection in HeLa cells) with artificial reporter constructs[41]. One explanation for this apparent discrepancy is that very few (<0.5%) 3′ UTRs in our data set have more than three seed sites for a given miRNA (and this value is representative for the whole genome) whereas artificial reporter constructs are designed to contain up to six closely spaced miRNA binding sites.

Identifying functionally important miRNA targets is crucial for understanding miRNA functions. By directly measuring changes in protein production, pSILAC data are likely to be more relevant to the phenotypes than microarray data. We also note that a number of targets are almost exclusively repressed at the level of translation and hence missed by microarrays. pSILAC allows assessment of the early effects of miRNAs on translation. This is a considerable advantage over techniques that assay changes in steady-state protein levels and are therefore almost certainly confounded by indirect effects. Although not all changes in peptide peak intensities reflect true differences in protein synthesis, a direct comparison of pSILAC and luciferase measurements yields very similar results over two orders of magnitude[26]. Catalogues of proteotypic peptides will further improve this accuracy and help to achieve full-proteome coverage[45]. pSILAC and microarray data can be queried at http://psilac.mdc-berlin.de.

Although artificially overexpressing miRNAs might cause non-physiological effects, we found that overexpression and knockdown of let-7b inversely modulates protein production, suggesting that such effects do not dominate. Nevertheless, transfecting miRNAs that are not endogenously expressed will probably expose many mRNAs to miRNAs that are never coexpressed in the same cell type. Therefore, it could be argued that a number of target for miR-1 and miR-155 identified by pSILAC are irrelevant *in vivo*. However, transfecting a tissue-specific miRNA into HeLa cells shifts the entire gene expression profile towards that tissue[12]. Furthermore, we show that evolutionarily conserved target sites cause stronger effects than non-conserved sites. Altogether, our data probably contain many physiologically relevant direct targets. These arguments are strengthened by the highly significant correlation of pSILAC data with a number of published miRNA target predictions. Seed-based methods had the highest overlap with pSILAC data. Consistently, many downregulated genes could be explained by seed sites. A number of repressed proteins without seeds are nevertheless probably direct targets of the respective miRNAs. However, although some algorithms include searches for such sites, it seems that they could not identify these non-canonical sites with high success.

Our data indicate that most targets are repressed at both the mRNA and the translational level. As revealed by Fig. 4d, how much both processes contribute to downregulation depends on the individual miRNA–mRNA pair. To test whether targets with strong translational repression share functional properties, we performed gene ontology analysis for proteins with large protein and mRNA fold-change differences ($\log_2$-fold change pSILAC − mRNA < −0.3). Intriguingly, we found over-representation of proteins synthesized at endoplasmic-reticulum-associated ribosomes (gene ontology categories 'intrinsic to membrane' and 'endoplasmic reticulum', corrected *P*-values <0.0001 and <0.005, respectively; Supplementary Table 2). Hence, translational repression seems stronger for mRNAs translated at endoplasmic-reticulum-associated ribosomes compared to free cytosolic ribosomes. Thus, endoplasmic-reticulum-associated ribosomes might be more sensitive to miRNA-mediated translational repression. It is tempting to speculate that mRNAs from free ribosomes but not from endoplasmic-reticulum-associated ribosomes are targeted to processing bodies (P-bodies) for degradation[46]. Because the endoplasmic reticulum is considered to lack proteolytic activity, this finding also suggests that co-translational degradation of nascent peptides is not the predominant mechanism of miRNA-mediated translational repression for this subset of targets[47].

Finally, we showed that overexpression and knockdown of let-7b had largely inverse effects on the protein production of thousands of genes, indicating that altering stationary levels of an endogenously expressed miRNA can tune synthesis levels of a major fraction of the proteome. We noticed that Dicer, which has several *let-7* 3′ UTR seeds, is one of the most strongly upregulated genes in the let-7b knockdown pSILAC (>4-fold) but not in the microarray data

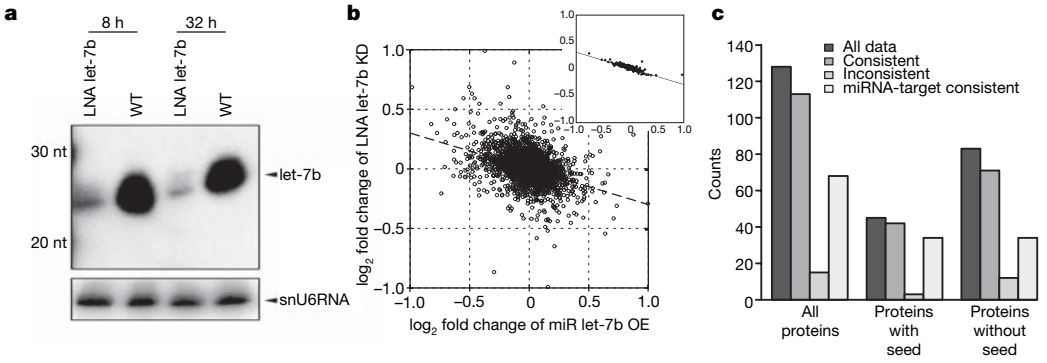


Figure 5 | Endogenous miRNA knockdown. **a**, Northern blotting demonstrates specific and stable let-7b knockdown by means of LNA. nt, nucleotide; WT, wild type. **b**, Scatter plot of changes in protein production in the let-7b overexpression (OE) versus the let-7b knockdown (KD) experiments. The inset shows the same data, averaged over bins of 20 genes. **c**, 'Consistent' refers to proteins with pSILAC fold changes that were upregulated in one experiment but down in the other, and 'inconsistent' refers to all other cases. 'miRNA-target consistent' is the subset of 'consistent' proteins that were downregulated in the overexpression experiment but upregulated in the knockdown.

(<1.3-fold). Therefore, Dicer is likely to be a direct translational target of let-7b. This raises the interesting possibility that let-7b regulates mature miRNA levels, which may in part explain our findings.

METHODS SUMMARY

HeLa cells were transfected with 100 nM synthetic dsRNAs designed to mimic mature endogenous miRNAs using DharmaFECT1 (Dharmacon) at 60–70% confluence, or with LNA-anti-let-7b (BioTez). Mock transfections were performed in the same way but without miRNAs. Eight hours post-transfection, cells were split into new dishes containing medium-heavy and heavy SILAC medium prepared as described[48] and incubated for 24 h until harvest. Corresponding protein and mRNA samples were always derived from the same transfection experiment. For the proteome analysis, miRNA/LNA-transfected cells and corresponding control cells were combined, lysed, and separated by SDS–PAGE. Gel lanes were cut into 15 slices, reduced, alkylated and trypsin-digested. Peptides were extracted and analysed by liquid chromatography–tandem mass spectrometry on a LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher). All samples were analysed in triplicate resulting in 45 mass spectrometry runs (5 days measurement time) per sample. Raw data files were processed with MaxQuant developed by J. Cox and M. Mann at the Max Planck Institute of Biochemistry (personal communication). False discovery rates were estimated using the target-decoy strategy[49] against an in-house-curated version of the IPI human protein database (version 3.37). In total, we identified 3,097,418 peptides (66,989 unique sequences) with average absolute mass accuracy of 0.65 p.p.m. We identified 4,961 unique proteins with at least two peptides each at a maximum false discovery rate of 1%. In individual experiments we only considered protein quantifications based on at least three peptide quantifications. Microarray analyses were performed with Human Genome U133 Plus 2.0 chips (Affymetrix), normalized by the standard rma()function (http://www.bioconductor.org) and annotated with the current NetAffx annotation file (http://www.affymetrix.com).

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