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Proteomics for understanding miRNA biology

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Abstract

MicroRNAs (miRNAs) are small noncoding RNAs that play important roles in posttranscriptional regulation of gene expression. Mature miRNAs associate with the RNA interference silencing complex to repress mRNA translation and/or degrade mRNA transcripts. Mass spectrometry-based proteomics has enabled identification of several core components of the canonical miRNA processing pathway and their posttranslational modifications which are pivotal in miRNA regulatory mechanisms. The use of quantitative proteomic strategies has also emerged as a key technique for experimental identification of miRNA targets by allowing direct determination of proteins whose levels are altered because of translational suppression. This review focuses on the role of proteomics and labeling strategies to understand miRNA biology.

Keywords

Cell biology; iTRAQ; miRNA; Multiple reaction monitoring; Noncoding RNA; SILAC

1 Background

MicroRNAs (miRNAs) are small noncoding RNAs of about 22 nucleotides that regulate various cellular functions such as differentiation [1], metabolism [2], senescence [3], autophagy [4], proliferation, and apoptosis [5]. miRNAs impose this layer of posttranscriptional gene regulation on a wide spectrum of biological processes ranging from gametogenesis [6], development [7], and tissue repair [8] to aging [9]. This widespread regulation reflected in the fact that up to ~60% of the human protein-coding genes are potentially modulated by miRNAs [10]. Given the extensive involvement of miRNA in physiology, dysregulation of miRNA expression can be associated with cancer pathobiology including oncogenesis [11], proliferation [12], epithelial-mesenchymal transition [13], metastasis [14], aberrations in metabolism [15], and angiogenesis [16], among others. To date, the online miRNA repository database, miRBase (Release 18), has documented over 2000 human mature miRNA sequences and over 1200 mouse mature miRNA sequences along with miRNAs reported in other organisms [17]. With the availability of next generation sequencing technologies, this list will undoubtedly continue to expand further [18].

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In general, genes encoding miRNAs are initially transcribed as primary miRNAs (pri-miRNAs) by RNA polymerase II, and then pri-miRNAs are processed to be precursor miRNAs (pre-miRNAs) by the microprocessor complex comprising of Drosha and DGCR8 [19]. The pre-miRNAs, hairpin-like structures of ~60–80 nucleotides, are transported to the cytosol by exportin-5 [20], where they are processed by Dicer in association with TRBP to form ~22-nucleotide double stranded mature miRNAs (Fig. 1) [21]. In parallel, the mature miRNAs are assembled with the RNA interference silencing complex (RISC) comprising of Argonaute and TNRC6 (GW182 protein) [22]. The assembly process also involves the chaperones Hsc70/Hsp90 and ATP [23]. The mature miRNA consists of two strands – a passenger strand which is degraded and a functional guide strand which pairs with its cognate mRNA (target) mostly in the 3′ untranslated region (3′UTR) by complementary-base pairing [24]. Binding of miRNAs to their cognate target mRNAs results in translation repression and/or mRNA destabilization by either decapping target mRNAs or by deadenylation followed by mRNA degradation [25].

The vast majority of studies on miRNA biology thus far have primarily focused on two aspects: elucidating mechanisms of miRNA-mediated posttranscriptional gene regulation and identifying targets of miRNAs. This has been made possible by the use of established methodologies such as gene expression microarrays, next generation sequencing, computational prediction of miRNA targets, and mass spectrometry-based proteomic approaches [26]. Each of these high-throughput technologies has its own advantages, thus complementing one another. In this article, we will highlight the importance and advantages of proteomics to decipher the role of miRNA in regulating biological processes.

2 Approaches for computational analysis and proteomics

2.1 Computational approaches to predict miRNA targets

A large majority of reports describing identification of miRNA targets are based on computational approaches or detection of altered mRNA levels. However, the mechanism of target recognition is still not fully understood and as a result the algorithms that are employed for target gene prediction are not accurate and often overpredict miRNA targets. Numerous web-based miRNA target prediction tools such as TargetScan (www.targetscan.org), miRanda (www.microrna.org), TarBase (diana.cslab.ece.ntua.gr), PicTar (pictar.mdcberlin.de), RNA22 (cbcsrv.watson.ibm.com/rna22.html), mirWIP (ambroslab.org), PITA (genie.weizmann.ac.il/pubs/mir07/mir07_dyn_data.html), and miRDB (mirdb.org) predict miRNA targets based on selection criteria such as sequence pairing (which involves perfect or nearly perfect complementarity between nucleotides 2 and 8 at the 5′ end of miRNA (seed sequence) to the mRNA target site), secondary structure modeling, free energy calculation, and comparative genomic sequence conservation. However, the field is greatly expanding with significant improvements in technologies resulting in the generation of a vast amount of data. Furthermore, most prediction tools overlook the potentiality of miRNA binding in 5′UTR region as well as the coding sequence, even though there is substantial evidence provided by several groups [27–29]. Thus, it should be noted that miRNA target prediction by computational methods alone is not sufficient and it becomes essential to experimentally identify and validate targets of miRNAs.

2.2 Importance of proteomics in understanding miRNA biology

One of the mechanisms by which miRNA regulates gene expression is by repressing translation without mRNA degradation [30,31]. Determining the mRNA expression levels is not ideal for identifying targets as mRNA levels do not necessarily correlate with the levels of protein expression [32–34]. In addition, the discordance between half-lives of mRNAs

and proteins adds another layer of complexity in drawing conclusions about the proteome from analysis of the transcriptome. Furthermore, a recent study revealed that the efficiency of miRNA-mediated gene silencing can be downregulated by poly (ADP-ribosyl)ation of Argonaute/RISC, suggesting that the mere presence of miRNA does not necessarily reduce the protein production [35]. Therefore, the abundance of mRNA/miRNA species does not serve as the best surrogate marker for biological activity of these proteins. This problem can be alleviated by the use of mass-spectrometry-based proteomic approaches that enable studying the protein complement of cells more directly.

2.3 Mass spectrometry as a discovery tool

The dynamic nature and complexity of the proteome present enormous technological challenges. Mass spectrometry is a powerful analytical technology that enables elucidation of protein sequence as well as abundance. Recent advances in mass spectrometry have significantly enhanced the throughput of protein identification and quantification by improved sensitivity, high resolving power, reproducibility, and the dynamic range of proteomic analyses. A typical proteomic experiment involves isolation and extraction of proteins from cells or tissues, protease digestion to generate peptides generally followed by sample fractionation to reduce proteome complexity. The fractionated samples are then introduced into a mass spectrometer where the peptides are ionized and their masses measured. The peptide ions are then picked for fragmentation in a data-dependent fashion leading to generation of MS/MS spectra. Improved separation methods are essential for increasing the comprehensiveness and throughput of proteome analyses and to address the complexity of proteomes.

2.4 Proteomic strategies in miRNA research

Over the past decade, various strategies have been developed and optimized to characterize the proteome. A large majority of proteomic experiments in miRNA research have employed mass spectrometry-based quantitative proteomic approaches. Quantitative proteomics has emerged as a promising tool to identify proteins differentially expressed in various biological processes [36]. To enable protein quantitation, two main strategies are currently employed by the community in combination with mass spectrometry (Fig. 1). These are referred as “label” and “label free” strategies. Labeling techniques utilize isotopic labels as a reference for either relative or absolute quantitation. These labels can be introduced in vivo by labeling methods such as SILAC [37] or by in vitro labeling strategies such as iTRAQ [38], ^{18}O labeling [39] or TMT tags [40], among others. Apart from labeling-based methods, a semiquantitative strategy, less frequently used, is based on differential spot analysis by two-dimensional electrophoresis [41–43].

In recent years, there has been significant progress in the development and application of technologies for targeted analysis of peptides in complex protein samples. Selected reaction monitoring (SRM) or multiple reaction monitoring is a robust tandem mass spectrometry method employed to monitor these protein-specific peptides and thus provide quantitative measurements [44,45]. SRM assays allow identification and quantitation of peptides with very low limits of detection, high reproducibility, specificity, and sensitivity. In contrast to discovery-type proteomic experiments, SRM assays allow one to perform repeat measurements with high-throughput and sensitivity. Several groups have employed labeling strategies to identify targets of miRNAs. However, to date there are only two studies (both by the same group) where SRM-based targeted studies were performed to identify and validate predicted targets in *C. elegans* [46, 47].

Non-MS-based proteomic approaches such as reverse phase protein microarray have also been employed to study various biological processes. These include profiling immune

response to infection, protein–protein interactions, protein–nucleic acid interactions and signaling pathways and have also been utilized to understand the mechanism of miRNA regulation. In a recent study, Iliopoulos et al. carried out microRNA profiling and reverse-phase protein microarray analysis on the same patient-derived osteoarthritic cartilage to identify genes involved in osteoarthritic pathogenesis. Integrating the data from both these approaches together with the use of miRNA prediction algorithms, they created an interactome network and uniquely identified 17 miRNA-gene target pairs implicated in pathogenesis of osteoarthritis [48].

3 Application of proteomics in miRNA research

The power of MS-based proteomics has been exploited to identify several critical components of the miRNA biogenesis pathway and their posttranslational modifications. Additionally, quantitative proteomic strategies have facilitated quantifying proteomic changes secondary to the perturbation of certain miRNAs. This confers a powerful high throughput platform to predict miRNA targets that are not fully appreciated by bioinformatics tools. These aspects will be covered in the following sections.

3.1 Identification of key components in miRNA biogenesis

MS-based proteomics has enabled identification of several associating factors such as DGCR8, TRBP, and TNRC6 that are all indispensable for generating miRNAs. Gregory et al. immunoprecipitated Drosha in HEK293T cells, identifying DGCR8 as the co-eluate in the complex that decisively retained the pri-miRNA processing activity [19]. Analyzing Dicer affinity eluates through mass spectrometry, Chendrimada et al. identified TRBP, a protein necessary for the functional interaction between Dicer and Argonaute. They observed diminished generation of mature miRNA upon TRBP knockdown [21]. Using shotgun proteomic analysis, Duchaine et al. identified Dicer (DCR-1)-associated proteins in *C. elegans* using immunoaffinity purification approach. They identified a high confidence set of 20 interactors, many of which were previously not known to interact with DCR-1. Interaction between Dicer and the translation initiation factor, EIF2C2, has partially unveiled the mechanism of miRNA-mediated translation suppression [49]. Association between Argonaute and TNRC6 was initially revealed through multidimensional protein identification technology by Liu et al. and supported by several other groups' finding [50–53]. Identification of these core factors of miRNA processing pathways has initiated further functional and quantitative studies that will be discussed below. Wang et al. employed a quantitative proteomic approach to compare functions across different Argonaute proteins in mouse skin and human melanoma cells [54]. They employed spectral counting, a label-free quantitative method, to measure changes in the abundance of Ago1–4 in mouse skin cells. Their study showed that the abundance of Ago3/4 is negligible and the ratio between Ago1 and Ago2 is close to their associated miRNAs, an implication that miRNAs are loaded randomly onto Ago1 and Ago2.

3.2 Mapping posttranslational modifications on key components of miRNA biogenesis

The miRNA processing pathway is now known to be regulated by posttranslational modifications on key components such as Drosha, TRBP, and Argonaute. Importantly, these modifications such as phosphorylation and hydroxylation result in alteration of their activity that, in turn, influences miRNA biogenesis. To delineate the regulation of pri-miRNA processing, Tang et al. examined the mechanism by which Drosha, one of the key enzymes in the miRNA processing machinery, localizes to the nucleus. Mass spectrometric analysis revealed phosphorylation at Ser-300 or Ser-302 and with additional functional studies they proved that phosphorylation at either site is critical for nuclear localization [55]. Serine phosphorylation is also important for functions of TRBP and Argonaute. Paroo et al. have

shown that Dicer complexes with phosphorylated TRBP to generate mature miRNAs in HeLa cells. They identified four serine residues (Ser-142, Ser-152, Ser-283, and Ser-286) phosphorylated by MAPK/Erk and unequivocally demonstrated that phosphorylated TRBP confers stability to the Dicer-TRBP complex [56]. Additionally, this study also revealed phosphorylation of Argonaute by MAPK-activated protein kinase 2. A study by Zeng et al. showed that phosphorylation of Ago-2 at Ser-387 mediates localization of Argonaute to processing bodies which are the site for the process of miRNA-mediated gene silencing [57]. Further, studies have shown that apart from phosphorylation at key residues, other modifications also play important roles in the functioning of Argonaute. Qi et al. studied the interactome of Argonaute with MS-based proteomics and have shown that it physically interacts with the subunits of prolyl-4-hydroxylase. They further demonstrated that hydroxylation at Pro-700 of Ago-2 is essential for its own stability and for miRNA-mediated gene silencing [58]. Overall, the regulation of the miRNA processing pathway by posttranslational modifications has been relatively less investigated and additional studies are required to reveal the intricate mechanism of miRNA processing.

3.3 Proteome analysis for suppression of miRNA processing pathways

Perturbation of miRNA processing pathway such as Dicer and Argonaute results in phenotypic changes; thus functional studies regarding these components are of prime interest. Dicer is one of the key enzymes of the miRNA processing machinery and is also known to play important roles in cell differentiation and apoptosis. Knockout of Dicer gene has been reported to cause various defects [59–62]. To investigate the functions of Dicer in vivo, our group generated SILAC mice to study the effects of inducible deletion of Dicer [63]. Our study showed abnormal lipid accumulation in small intestine. Evaluation of the proteomic changes in the small intestine revealed a critical role of Dicer in lipid transport [63]. In an episomal shRNA expression system, Drosha knockdown experiments in MCF-7 and HCT116 showed size-biased proteome modifications [42]. Similar quantitative proteomics strategies need to be employed in the future to increase our depth of understanding the components involved in the canonical and noncanonical miRNA processing pathways.

3.4 Identification of miRNA targets using quantitative proteomics

A complete delineation of the miRNA targeting mechanism entails concurrent measurement of mRNAs and their protein products. In the past few years, several groups have made significant efforts to integrate high-throughput techniques to enable identification of *bona fide* miRNA targets (Table 1). Most studies thus far have employed overexpression of the miRNAs of interest in a transient or stable fashion to identify potential targets. Other studies have employed anti-miRNA oligonucleotides and miRNA knockout mouse model. SILAC-based strategy has been widely used in most studies to identify miRNA targets [64–67]. Baek et al. measured the effects of addition of miRNAs such as miR-124, a brain specific miRNA, miR-1, and miR-181 on the expression levels of proteins in HeLa cells. Based on the identification of the repressed proteins, this study revealed that these miRNAs recognize seed sequences located within the 3′ UTRs. In addition, they also studied the effects of miRNA knockout to identify endogenous targets since ectopic addition of miRNAs would only provide insights into miRNA target recognition and potential targets [64]. Our group has also adopted SILAC-based strategy and knocked down expression of endogenous miR-21 to identify its potential targets [68]. This miRNA is known to play important roles in tumorigenesis. To enable identification and quantitation of proteins synthesis on a global scale, Ebner et al. devised a new strategy which is a variation of SILAC technology and designated it pulsed SILAC (pSILAC). pSILAC measures the differences in the amount of protein synthesized over a period of time depending upon the incorporation rate of heavy amino acids [66]. They applied this strategy to study the protein regulatory mechanisms by

miRNAs. Kaller et al. employed pSILAC and microarray analysis to study the effects of miR-34a, an important mediator of p53-mediated tumor suppressor activities on mRNA and proteome expression [69]. Employing iTRAQ-based quantitative strategy coupled with computational prediction, Taguchi et al., identified hypoxia inducible factor-1α as a novel target of miR17–92 cluster in lung cancer cells. Overexpression of miR17–92 cluster has been shown to play important roles in lung cancer and in B-cell lymphoma development. Using an SRM-based approach, Jovanovic et al. quantified and validated predicted targets of let-7 and miR-58 in *C. elegans*. Their results revealed ztf-7 as a *bona fide* target of let-7 which was also supported by another independent study [46].

In addition to identifying individual miRNA targets, the application of quantitative proteomics also reveals several signaling networks of cancer biology. Schliekelman et al. use SILAC to determine which proteins are regulated by miR-200 family to cause the epithelial-mesenchymal transition in lung cancer. Many of them are associated with TGFβ-1, a finding corroborating with the current understanding [70]. To decode the intricate network of Wnt signaling in carcinogenesis, Schepeler et al. took a two-step approach [71]. They first adopted a conditional suppression system of Wnt signaling and identified miR-145, miR-126, miR-30e-3p, and miR-139–5p as critical regulators for their ectopic expression causing growth inhibition. Subsequently, they used spectral counting to characterize proteomic changes and luciferase assays to confirm that PIK3C2A is related to aberrant Wnt signaling. Another network-revealing study about cancer biology was conducted by Cheng et al., who transfected miR-34a in a hepatocellular carcinoma cell line. In the two-dimensional gel electrophoresis analysis coupled with mass spectrometry, half of the differentially regulated proteins were found to be miR-34a targets and shown belong to p53 signaling pathway [43].

Although these studies have led to identification of many novel miRNA candidate targets, a clear distinction between direct and indirect targets is lacking. Studies have shown that identification of direct miRNA targets is possible by co immunoprecipitation of miRISCs with the associated mRNAs, which is then coupled to microarrays (RIP-Chip) or mRNA sequencing (CLIP-Seq). However, these approaches do not measure the protein expression level of the direct targets. Recently, Jovanovic et al. developed a targeted, quantitative proteomic approach called RIP-chip-SRM to identify direct targets of miRNAs. This combinatorial approach involves RIP-chip analysis of miRISC complexes isolated from wild-type and miRNA deletion mutants followed by SRM analysis to determine the abundance of the protein products of the candidate transcripts [47]. One noteworthy caveat of the immunoprecipitation-based study was recently observed by Riley et al., who showed that the association between Argonaute proteins and miRNAs can happen after cell lysis [72]. This points out the possibility of false positive results from this type of study.

3.5 Future perspective

The use of quantitative proteomic strategies to characterize targets of miRNAs has opened new avenues to study miRNA biology. More and more targets of important miRNAs that relate to cancers are being identified and helping us unravel the intricacies of cancers and potentially even develop novel anticancer approaches. Meanwhile, our knowledge of post-translational modifications on components of the miRNA processing pathway and their associated factors is also expanding, paving the road for more functional analysis of miRNA-mediated gene regulation. Much of this progress has been enabled by quantitative proteomics, and we envision that this trend will continue in miRNA research and accelerate in the years to come.

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Abbreviations

miRNA	microRNA
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
pSILAC	pulsed SILAC
RISC	RNA interference silencing complex
SRM	selected reaction monitoring

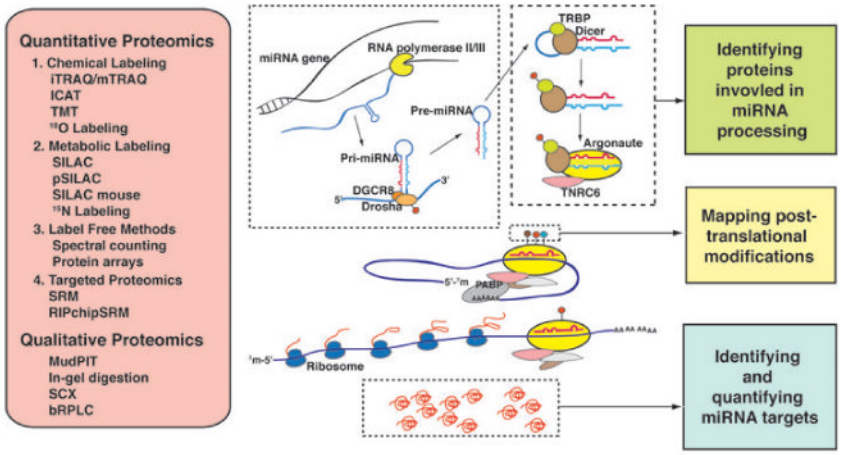

Figure 1.

Proteomic strategies for studying the mechanism of miRNA processing and miRNA-mediated translation repression. Genes encoding miRNAs are transcribed as pri-miRNAs by RNA polymerase II and processed by the microprocessor complex consisting of Drosha and DGCR8. This complex cleaves pri-miRNAs into ~60–80 nt hairpin structures, called pre-miRNAs, which are transported out of nucleus by exportin-5. In the cytoplasm, Dicer associates with TRBP and excises the loop portion of pre-miRNAs generating double stranded mature miRNAs. These are loaded onto RNA interference silencing complex (RISC) consisting of Argonaute and TNRC6. Translation inhibition is brought about by base-pairing of guide strand miRNA to the 3′UTR of mRNA and interfering with translation initiation. Quantitative proteomics analyses encompass different categories of strategies including chemical labeling, metabolic labeling, label-free methods and targeted proteomics. Among them, iTRAQ and SILAC are the most frequently used. Along with various techniques in qualitative proteomics, these methods can enable not only characterization of the miRNA processing pathway components and their posttranslational modifications but also discovery of miRNA targets.

Table 1

A list of publications that have employed quantitative proteomics to identify miRNA targets

No.	miRNA	Type of perturbation	Cell line	Transcriptomics analysis	Quantitative proteomics	Total protein	Reference
1	let-7a	Stable lentiviral vector overexpression	SGC-7901 (human gastric carcinoma cells)	NA	2DE	10	Zhu et al. [73]
2	let-7b	LNA-anti-let-7b knockdown	HeLa (ovarian cancer)	Microarray	pSILAC	2700	Selbach et al. [65]
3	miR-1	Transient miRNA mimics overexpression	HeLa (ovarian cancer)	NA	SILAC	504	Vinther et al. [74]
4	miR-1	Transient miRNA mimics overexpression	HeLa (ovarian cancer)	Microarray	pSILAC	4962*a)*	Selbach et al. [65]
5	miR-1	Transient miRNA mimics overexpression	Hela (ovarian cancer)	Microarray	SILAC	2312	Baek et al. [64]
6	miR-15a/16-1	Transient miRNA mimics overexpression	MEG-01 (Leukemia)	Microarray	2DE	27	Calin et al. [75]
7	miR-16	Transient miRNA mimics overexpression	HeLa (ovarian cancer)	Microarray	pSILAC	4962*a)*	Selbach et al. [65]
8	miR-17-92	Conditional episomal miRNA vector overexpression	SHEP-TR-miR-17–92 (neuroblastoma)	NA	SILAC	NA	Mestdagh et al. [76]
9	miR-17-92	Stable pre-miR overexpression	BEAS2B (bronchial epithelial cells)	NA	iTRAQ	479	Taguchi et al. [77]
10	miR-17-92 cluster	LNA-anti- miR-19a, miR-20a and miR-92–1 knockdown	SBC-3 (small-cell lung cancer cells)	NA	2DE	112 up-regulated proteins	Kanzaki et al. [78]
11	miR-21	Transient anti-miRNA oligo overexpression	MCF-7 (breast cancer)	NA	iTRAQ	1151	Yang et al. [67]
12	miR-21	Transient anti-miRNA oligo overexpression	MCF-7 (breast cancer)	NA	2D-DIGE	NA	Zhu et al. [79]
13	miR-21	Transient miRNA mimics overexpression	U266 (human myeloma cell line)	NA	SILAC	1498	Xiong et al. [80]
14	miR-29a	Transient miRNA mimics overexpression	DLKP-A cells (Lung carcinoma)	NA	2D-DIGE	75	Muniyappa et al. [81]
15	miR-29a	Transient miRNA overexpression	HEK293T (human embryonic kidney)	NA	SILAC	5074	Bargaje et al. [82]
16	miR-30a	Transient miRNA mimics overexpression	HeLa (ovarian cancer)	Microarray	pSILAC	4962*a)*	Selbach et al. [65]
17	miR-30e-3p	Transient miRNA mimics overexpression	DLD1 (colorectal cancer)	NA	Spectral counting	NA	Schepeler et al. [71]
18	miR-34a	Transient pre-miR mimics overexpression	HepG2 (hepatocellular carcinoma)	NA	2D-DIGE	116*b)*	Cheng et al. [43]

No.	miRNA	Type of perturbation	Cell line	Transcriptomics analysis	Quantitative proteomics	Total protein	Reference
19	miR-34a	Episomal pri-miRNA vector overexpression	SW480 (colon cancer)	Microarray	pSILAC	1206	Kaller et al. [69]
20	miR-34a	Transient miRNA mimics overexpression	IMR32 (neuroblastoma)	Microarray	iCAT	1495	Chen et al. [83]
21	miR-34a	Transient miRNA mimics overexpression	HEK293T (human embryonic kidney)	NA	SILAC	5688	Bargaje et al. [82]
22	miR-122a	Stable lentiviral vector overexpression	HepG2 (Hepatocellular carcinoma)	NA	2DE	10	Diao et al. [84]
23	miR-124	Transient miRNA mimics overexpression	Hela (ovarian cancer)	Microarray	SILAC	2120	Baek et al. [64]
24	miR-143	Transient miRNA mimics overexpression	MiaPaCa-2 (pancreatic cancer)	Microarray	SILAC	1202	Yang et al. [68]
25	miR-155	Transient miRNA mimics overexpression	HeLa (ovarian cancer)	Microarray	pSILAC	3299	Selbach et al. [65]
26	miR-155	Transient miRNA episomal vector overexpression	HEK293T (human embryonic kidney)	NA	SILAC	3148	Lossner et al. [85]
27	miR-155	Transient miRNA mimics overexpression	DHL16 (diffuse large B-cell lymphoma)	NA	pSILAC	2103	Huang et al. [86]
28	miR-181	Transient miRNA mimics overexpression	Hela (ovarian cancer)	Microarray	SILAC	1774	Baek et al. [64]
29	miR-193b	Transient pre-miRNA vector overexpression	MCF-7 (breast cancer)	Microarray	iTRAQ	743	Leivonen et al. [87]
30	miR-210	Transient miRNA mimics overexpression	Human umbilical venous endothelium cells	NA	2DE	28	Fasanaro et al. [88]
31	miR-223	Congenital miRNA knockout	miR-223$^{-/-}$ mouse bone marrow primary culture	Microarray	SILAC	5019	Baek et al. [64]
32	miR-372	Transient miRNA overexpression	CL 1–0 (lung adenocarcinoma cells)	NA	2D-DIGE	19	Lai et al. [89]
33	miR-373	Transient pre-miRNA mimics overexpression	MCF-7 (breast cancer)	NA	SILAC	3666	Yan et al. [90]
34	miR-516a-3p	Stable episomal miRNA vector overexpression	44As3-miR516a-3p (gastric cancer)	NA	iTRAQ	NA	Takei et al. [91]

2-DE: two-dimensional gel electrophoresis; 2-D DIGE: two-dimensional fluorescence difference gel electrophoresis; NA: not available.

a) Total number of proteins from experiments of let-7b, miR-1, miR-16, miR-30a, and miR-155.

b) The number of differentially expressed proteins identified by 2D gels.