Impact of MicroRNA in Normal and Pathological Respiratory Epithelia

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Abstract

Extensive sequencing efforts, combined with ad hoc bioinformatics developments, have now led to the identification of 1222 distinct miRNAs in human (derived from 1368 distinct genomic loci) and of many miRNAs in other multicellular organisms. The present chapter is aimed at describing a general experimental strategy to identify specific miRNA expression profiles and to highlight the functional networks operating between them and their mRNA targets, including several miRNAs deregulated in cystic fibrosis and during differentiation of airway epithelial cells.

Key words: Lung, microRNA, cystic fibrosis, cancer.

1. Introduction

In 1993, a new type of small regulatory RNA was described in the nematode *Caenorhabditis elegans*: the heterochronic gene lin-4, encoding a small RNA with partial antisense complementarity to lin-14 (1, 2), corresponds to the first ever reported miRNA. Extensive sequencing efforts (3, 4), combined with ad hoc bioinformatics developments (5), have now led to the identification of 1222 distinct miRNAs in human and of many miRNAs in other multicellular organisms (accessible through miRBase, version 16, the main miRNA online registry) (6). It is currently postulated that the total number of human miRNAs should not exceed 2000.

Mature miRNAs, approximately 22 nucleotides long (7, 8), are usually derived from a primary transcript called pri-miRNA, usually transcribed by a type II RNA polymerase (transcription by type III RNA polymerase has also been suggested for some miRNAs (9, 10); but see also (11–14)). Pri-miRNAs are cleaved into the nucleus by Microprocessor, a hetero-dimer composed of Drosha, an RNase III endonuclease, and DGCR8 (DiGeorge syndrome critical region gene 8)/Pasha. This first cleavage liberates the pre-miRNA (formed by a hairpin of about 70 nucleotides that includes an overhang of 2–3 nucleotides at the 3′ end). Pre-miRNAs are then exported to the cytoplasm via exportin-5 in a Ran GTP-dependent manner. In the cytoplasm, cleavage of each pre-miRNA near the hairpin by Dicer, a second RNase III endonuclease, generates two short RNA sequences: one sequence corresponds to the mature miRNA, while the second is usually degraded (15–17). The final effector able to interact with the target mRNAs is the RNA-induced silencing complex (RISC). The efficient transfer of nascent miRNAs from Dicer to this complex necessitates that Dicer assembles with the RNA-binding protein TRBP and members of the Argonaute family to form a RISC-loading complex, before loading of the miRNA on Argonautes (18). Each resulting RISC complex can directly interact with its target mRNA(s) (19). The cytoplasmic steps of miRNA biosynthesis largely overlap with those of siRNA biosynthesis. More specifically, human AGO1 and AGO2, but not AGO3 and AGO4, possess strand-dissociating activity of miRNA duplexes. They function as RNA chaperones, capable of performing multiple rounds of strand dissociation, while only AGO2 has target RNA cleavage activity (also called slicer activity) (20).

Animal miRNAs and siRNAs differ by their mode of interaction with their targets: while siRNAs fully match their target mRNA sequences, perfect complementarity is not required between a miRNA and its target(s). Target recognition follows a complex set of rules that is usually dominated by the existence of a perfect match between six and eight nucleotides located in the 5′ region of the miRNA (called the seed) and the target mRNA (21–23). Recent experimental evidences demonstrate convincingly that interactions take place within the 3′-non-coding region in 40% of the cases (especially at proximity of the stop codon and near the poly A tail, 23a) within the CDS in 25% of the cases, and within the 5′-non-coding region in 1% of the cases, the rest of the hybridizations being located within non-coding RNAs and intronic, intergenic, or other sequences (24).

Based on relative complexities of seeds and targets, each miRNA can potentially interact with hundreds of mRNAs. Estimation is that up to 30% of human genes could potentially be regulated by miRNAs (25). This view, implying that miRNAs

can "tune" the expression of most of their putative targets, needs probably to be reappraised, since miRNAs and their predicted targets are not necessarily co-expressed into the same cell, nor within the same subcellular compartment. The ability of the miRNA to interact with many targets, together with the possibility for several miRNAs to share a same target, represents a powerful mechanism to increase tremendously the complexity of biological networks. Challenges in miRNA research are currently to improve the prediction of miRNA targets and to integrate their complex modes of regulation into already existing biological networks.

miRNA regulates protein synthesis at a post-transcriptional level, by affecting mRNA translation or stability. More specifically, miRNA base pairing to a target mRNA can induce post-transcriptional gene repression by deadenylation (26–28), inhibition of translation (29, 30), or mRNA cleavage (31). In some cases, miRNA can also relocalize target mRNA to cytoplasm *foci* called P-bodies for storage or degradation (32). A careful comparison between gene expression and proteomic measurements after overexpression of a specific miRNA has shown that most of the proteins which were deregulated at a protein level were also affected at an mRNA level (33). This observation implies that analysis at a transcript level can often be sufficient to identify relevant miRNA::mRNA complexes.

1.1. miRNAs in Normal and Pathological Respiratory Tissues

Lung development and ageing. Tissue-specific inactivation of Dicer in lung epithelium led to the conclusion that Dicer is essential for proper lung morphogenesis (34). During lung development, a maternally imprinted miRNA cluster located at human chromosome 14q32.21 (mouse chromosome 12F2) is upregulated in neonatal mouse as well as in fetal human lung. This locus includes the miR-154 and miR-335 families and is situated within the imprinted Gtl2-Dio3 domain. Several miRNAs are upregulated in adult compared to neonatal/fetal lung, including miR-29a and miR-29b. Williams et al. observed no significant changes in the expression of 256 miRNAs, over lung ageing up to 18 months of age in female BALB/c mice (35). A parallel by Navarro et al. between embryonic lung and lung tumors reported a downregulation of members of the let-7 family and an overexpression of members of the miR-17-92 cluster and of miR-221 (36). More recently, Marcet et al. (36a) have established miRNA expression profiles specific of in vitro regeneration of airway epithelial cells. The most dramatic variations occurred at the onset of ciliogenesis, with the increased expression of 12 microRNAs (miR-449a, miR-449b, miR-449b*, miR-449c, miR-34a, miR-34b-3p, miR-34b-5p, miR-34c-5p, miR-92b, miR-191, miR-1975, miR-125a), and the decreased expression of 11 microRNAs (miR-17, miR-193b, miR-31, miR-31*, miR-130a, miR-205, miR-21, miR-24-1, miR-24-2, miR-210, miR-29a).

Asthma. In an attempt to identify the role of miRNA during the development of asthma, Williams et al. found no significant differences between normal and mild asthmatic patients, and no effect after 1 month treatment with the corticosteroid budesonide after examining airway biopsies obtained from normal and mild asthmatic patients by quantitative RT-PCR (37). On the other hand, lipopolysaccharides (LPS) alone induced a fast increase in the expression of 46 miRNAs which peaked at 3 h (including miR-21, miR-25, miR-27b, miR-100, miR-140, miR-142-3p, miR-181c, miR-187, miR-194, miR-214, miR-223, and miR-224), while dexamethasone had no effect on miRNA expression.

Lung inflammation. miRNAs, such as miR-10a, miR-106a-363, miR-130a, miR-133, miR-142, miR-146, miR-150, miR-155, miR-181a, miR-17-92, miR-221, miR-222, miR-223, miR-424, or miR-451, can affect the immune responses to infection and the development of diseases of immunological origin. Impact of these miRNAs in the context of the immune system has been well-described elsewhere (38, 39), but recent reports more directly have involved some of these "immunological" miRNAs into respiratory tissues (36, 40–44).

MiR-155 is contained within the only phylogenetically conserved region of BIC RNA (45, 46). It has been linked to cancer (47–50), viral infection (51), and immunity (52–55). MiR-155 has been shown to be induced by pro-inflammatory stimuli such as LPS, Toll-like receptors (TLRs), IL-1, and TNF-α in macrophages and dendritic cells (56–58), in particular through NF-κB and AP-1 transcription factors (56, 59, 60). It has also been detected in fibroblasts from different origins (61) including lung (62) in which miR-155 was also found to be upregulated by IL-1β and TNF-α (63) as well as overexpressed in a mouse model of bleomycin-induced lung fibrosis (63). Multiple targets for miR-155 have been identified in several cell types and linked with the regulation of B- and T-cell differentiation (53, 54, 64–66), TLR signaling in inflammatory cells (58), or cellular adhesion in epithelial malignancies (67). Interestingly, BIC-deficient mice displayed significant remodeling of lung airways with age, associated with increased bronchiolar subepithelial collagen deposition and increased cell mass of sub-bronchiolar myofibroblasts. Recently, Pottier et al. (63) have found that miR-155 targets keratinocyte growth factor (KGF, FGF-7), a paracrine-acting, epithelial mitogen and a central mediator of epithelial–mesenchymal interaction. Overall miR-155 would be involved in the attenuation of the inflammatory signaling pathway (58) and epithelial regeneration (63), making it a potential key player during lung injuries. Bhattacharyya et al. (67a) have shown that miR-155 was also more than 5-fold elevated in CF IB3-1 lung epithelial cells in

culture, compared to control IB3-1/S9 cells. They also detected it in CF lung epithelial cells, and in circulating CF neutrophils. They propose that elevated miR-155 could contribute to pro-inflammatory expression of IL-8 in CF lung epithelial cells by lowering SHIP1 expression and activating the PI3K/Akt signaling pathway.

Viral infection. Respiratory viruses, such as influenza virus, respiratory syncytial virus (RSV), severe acute respiratory syndrome coronavirus (SARS-CoV), rhinovirus, parainfluenza virus, and adenovirus, induce acute infections of the respiratory tract that lead to clinical pictures going from rhinitis, otitis, bronchiolitis or pneumonia. Respiratory epithelial cells represent the primary targets that initially detect the viruses. Their resistance to infection depends on their capacity to detect and restrict virus replication. From that perspective, degradation of the viral genome appears as an ad hoc mechanism for efficient antiviral defense. The contribution of small non-coding RNA to this mechanism has been well-established in plants and invertebrates (68), but rarely in mammals, where the innate antiviral response is rather mediated by a robust type I interferon (IFN-α, β, and λ)-mediated response (69, 70). In that case, virus recognition occurs through the detection of pathogen-associated molecular patterns (PAMPs, usually viral nucleic acids) by pathogen-recognition receptors (PRRs) (71). Interactions within the cytoplasm or at the host cell surface lead to robust cytokine and chemokine responses, via several distinct pathways of activation (72, 73). Interestingly, Otsuka et al. have reported that a mouse mutant with hypomorphic Dicer1 expression (Dicer1(d/d)) was more prone to infection by vesicular stomatitis virus (VSV) (74). These authors detected that host miR-24 and miR-93 were increasing VSV replication in Dicer1-deficient cells after interfering with viral transcripts, without altering VSV genome-derived siRNA pathways and interferon-mediated antiviral responses. miR-122, on the contrary, was required for hepatitis C proliferation in liver (75).

At the moment, there is no report associating any cellular miRNA response to infections by rhinovirus, parainfluenza virus, RSV, or adenovirus in human. On the other hand, Wang et al. have investigated the impact of an infection by the low pathogenic H5N3 influenza virus in chicken. They found a large number of differentially expressed miRNAs between infected and non-infected tissues (73 in lungs and 36 in tracheae) (76). Alteration of the expression of several miRNAs in bronchioalveolar stem cells (BASCs) at the onset of infection by SARS-CoV, which causes acute infectious disease associated with pulmonary fibrosis and lung failure, has been described by Mallick et al., who

suggest that BASCs correspond to the primary site of infection (77). Such variations may participate in the suppression of SARS-CoV replication, thus favoring successful transmission of the virus.

Responses to environmental and external stresses. Expression of several miRNAs appears sensitive to environmental and external stresses. Rat and mouse lungs exposed to environmental cigarette smoke show a downregulation by a factor at least equal to 2 for 30% of the miRNAs tested (78, 79). Schembri et al. have found 28 miRNAs downregulated in human bronchial epithelium from current smokers in comparison to never smokers (80). Four miRNAs were found at the same time in human and in rodent: miR-125b, miR-146, miR-223, and miR-99a. miRNA response to stress has been well-documented in the context of the hypertrophy of adult cardiomyocytes where altered expression of miR-23a, miR-23b, miR-24, miR-195, and miR-214 has been observed. Transgene expression of miR-195 indeed triggers heart failure in mice (81).

miRNAs and lung cancer. A strong link has been established between miRNAs and cancers since the initial report by Calin et al. of frequent deletions of mir-15 and mir-16 in chronic lymphocytic leukemia (82). A large number of studies have demonstrated since then that deregulation of miRNAs is often associated with cancer development and progression (83, 84). Indeed, some miRNAs can be defined as bona fide tumor suppressors or oncogenes. miRNA expression appears in various tumors as a more robust method for classifying cancer subtypes than mRNA expression profiles (85). Numerous publications have documented aberrant expression of miRNAs in cancers (83, 86–89). Specific reviews about miRNAs and lung cancer can be found elsewhere (90–92). Of note, Puisségur et al. (92a) have highlighted the importance of miR-210 and of its transcriptional regulation by the transcription factor hypoxia-inducible factor-1 at late stages of non-small cell lung cancer, and its association with an aberrant mitochondrial phenotype.

Cystic fibrosis. Finally, a paper from Oglesby et al. has just reported variations of miR-126 expression during cystic fibrosis in airway epithelial cells (93) and showed that miR-126 was regulating TOM1, a protein that may have an important role in regulating innate immune responses. Increased expression of miR-155 in CF epithelial cells has been also reported (67a and see above).

The present chapter is aimed at describing a general experimental strategy to identify specific miRNA expression profiles and to highlight the functional networks operating between them and their mRNA targets. This approach is based on several methodological developments previously published by our group (63, 94, 95).

2. Materials

2.1. Identification of miRNome

2.1.1. Total RNA Extraction and Quality Controls

– Trizol® reagent (Invitrogen, the Netherlands),
– Chloroform,
– Ethanol 100%,
– Qiagen RNeasy kit column (Qiagen, France),
– NanoDrop spectrophotometer (Labtech, Palaiseau, France),
– Bioanalyzer System (RNA nano-chip, Agilent Technologies, France).

2.1.2. miRNA High-Throughput Sequencing (HTS)

– SOLiD™ sequencing system (Applied Biosystems, France),
– SOLiD™ Small RNA Expression Kit (Applied Biosystems, France),
– Statistical analysis is based on statistical libraries freely accessible on Bioconductor (http://www.bioconductor.org/).

2.1.3. MicroRNA Microarrays

– Human miRNA Microarray v2 (Agilent Technologies, France),
– miRNA complete labeling and hybridization kit (Agilent Technologies, France),
– Agilent DNA microarray scanner, using Feature Extraction and Analysis software (Agilent Technologies, France).

2.1.4. Quantitative RT-PCR of Mature miRNA

– TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA),
– GeneAmp Fast PCR Master Mix (Applied Biosystems, Foster City, CA),
– Lightcycler 480 (Roche) real-time PCR machine.

2.1.5. In Situ Hybridization of miRNAs

– 4% paraformaldehyde (Electron Microscopy Sciences) solution in sterile PBS,
– acetylation solution (2.33 ml triethanolamine, 500 µl acetic anhydride, volume up to 200 ml in sterile water),
– miRNA digoxigenin-labeled LNA probe (Locked nucleic acid) or scramble miR digoxigenin-labeled LNA probe (Exiqon, Woburn, MA),
– hybridization mixture consisting to 50% deionized formamide, 0.3 M NaCl, 20 mM Tris–HCl pH 8.0, 5 mM

EDTA, 10 mM NaPO$_4$ pH 8.0, 10% dextran sulfate, 1×
Denhardt's solution, and 0.5 mg/ml yeast RNA,

– washing solution: formamide 50%, 0.1% Tween-20, 1× SSC,
for 15 min at RT in 0.2× SSC,

– horseradish peroxidase conjugated with sheep anti-
digoxigenin antibodies (1:100, Roche, Mannheim,
Germany),

– Tyramide Signal Amplification Plus DNP AP System (Perkin
Elmer, Shelton, CT),

– BCIP/NBT substrate (DakoCytomation, Glostrup, Den-
mark),

– Nuclear Fast Red (Sigma-Aldrich),

– Eukitt mounting medium (Electron Microscopy Sciences).

2.2. Combined In Silico and Experimental Approaches to Identify miRNA Targets

2.2.1. Transcriptome Analysis Combined with Ectopic Expression of miRNAs in Cells

2.2.1.1. Ectopic Expression of miRNA

– Lipofectamine RNAi MAX Reagent (Invitrogen) in OPTI-
MEM (Invitrogen, Gibco product)

2.2.1.2. Analysis of RNA Expression Using DNA Microarray

– DNA GeneChip® Human Gene 1.0 ST Array (Affymetrix),

– Whole Transcript (WT) Sense Target Labeling and Control
Reagents (Affymetrix),

– GeneChip Fluidics Station 450,

– GeneChip Scanner 3000 7G (Affymetrix),

– Expression Console software (Affymetrix).

2.2.1.3. Bioinformatics Analysis of miRNA Targets

– Bioconductor statistical suite developed by the R consortium
(http://www.bioconductor.org),

– Mediante (http://www.microarray.fr:8080/merge/index),

– Ingenuity Pathway Analysis (IPA) software (Ingenuity Sys-
tems, Mountain View, CA),

– MicroCible and MicroTopTable (http://www.microarray.fr:
8080/merge/index),

– Sylamer.

2.3. Validation of miRNA Targets

2.3.1. Reporter Plasmid Assay

2.3.1.1. Molecular Constructs

– psiCHECK^{TM}-2 (Promega),

– QuickChange Kit (Stratagene),

– 10× Oligo Annealing Buffer (Invitrogen).

2.3.1.2. Transfection and Luciferase Assays

Pre-miRNA overexpression in cultured cells:

– Pre-miRNA and control miRNA (miR-Neg #1) (Ambion),

– Lipofectamine^{TM} RNAi MAX^{TM} (Invitrogen).

Pre-miRNA and psiCHECK^{TM}-2 plasmid construct co-transfection:

– Lipofectamine 2000^{TM} (Invitrogen),

– Dual-Glo^{TM} Luciferase Assay (Promega),

– Luminometer (Luminoskan Ascent, Thermolab system).

3. Methods

3.1. Identification of miRNome

Abundance of miRNA ranges from a few copies to 50,000 copies per cell. Distinct technologies can detect the abundance of specific miRNAs. Mature miRNAs and their precursors can be analyzed by Northern blot, quantitative real-time PCR [96], microarrays [94, 97–103], flow cytometric assays [85], padlock probes and rolling circle amplification [104], or deep-sequencing technologies [5, 105].

3.1.1. Total RNA Extraction and Quality Controls

Cells are lyzed by the addition of Trizol® reagent (Invitrogen, the Netherlands) directly on the cells. Total RNAs containing small RNA fraction are then purified on a Qiagen RNeasy kit column (Qiagen, France) according to the manufacturer's instructions (*see* **Note 1**). Total RNAs are first evaluated using NanoDrop spectrophotometer (Labtech, Palaiseau, France). Ratios 260/280 and 260/230 are checked to be near a value of 2. Integrity of the RNA is controlled on a Bioanalyzer System (RNA nano-chip, Agilent Technologies, France).

3.1.2. miRNA High-Throughput Sequencing (HTS)

Ad hoc high-throughput sequencing approaches are now commercially available. We use the SOLiD^{TM} Small RNA Expression Kit (Applied Biosystems, France) that provides a simple and robust means to convert small RNAs into a library

of double-stranded DNA molecules. Sequencing is performed according to the manufacturer's instructions. Briefly, total RNAs containing the small RNA fraction are hybridized (65°C for 10 min, then at 16°C for 5 min) and ligated (at 16°C for 2–16 h in a thermal cycler) to Adaptor Mix A to produce template for sequencing the 5′ ends of small RNAs, or to Adaptor Mix B to sequence the 3′ ends. The samples are then reverse transcribed (at 42°C for 30 min) to synthesize cDNA. Small RNA Library Amplification is realized by PCR and size selection of amplified small RNA Library by polyacrylamide gel extraction is carried out as indicated by the manufacturer's instructions (https://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_054973.pdf).
The 105–150 bp material is excised from the gel, eluted, and then re-suspended in nuclease-free water. Multiplexing can be done by using modified adaptors, thus allowing the sequencing of up to 20 different libraries in parallel. In that case, the DNA concentrations of all libraries are normalized by qPCR before operating the final preparation of the analytes.

Statistical analysis is based on statistical libraries freely accessible on Bioconductor (http://www.bioconductor.org/). For each sequenced miRNA, the number of sequences for 5p- and 3p-arm of each miRNA is counted, and the total number of sequences is normalized to 10^6 for each library. Data are normalized following the limma protocol (106). For further analysis, we usually retain those miRNAs whose percent of expression was >1% of the total miRNA expression in at least one condition, with a |$\log_2$ ratio| > 0.5 and an adjusted P-value < 0.05.

3.1.3. MicroRNA Microarrays

Direct and sensitive miRNA profiling from the same total RNA samples can also be performed using miRNA microarrays (Human miRNA Microarray v2, containing 866 human and 89 human viral distinct miRNA sequences, derived from the Sanger miRBase v.12.0, Agilent Technologies, France). Total RNAs are labeled and hybridized using miRNA complete labeling and hybridization kit (Agilent Technologies, France) following the manufacturer's instructions. Slides are then analyzed on Agilent DNA microarray scanner, using Feature Extraction and Analysis software (Agilent Technologies, France).

3.1.4. Quantitative RT-PCR of Mature miRNA

MiRNA expression level can also be assessed by the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) according to the supplier's protocol. Real-time PCR is performed using GeneAmp Fast PCR Master Mix (Applied Biosystems, Foster City, CA) on a Lightcycler 480 (Roche) real-time PCR machine. All reactions are performed in duplicate. Expression levels of mature microRNAs were evaluated using comparative

CT method (2-deltaCT), using transcript levels of RNU44 as endogenous control.

3.1.5. In Situ Hybridization of miRNAs

The exact cellular localization of miRNAs can be determined by in situ hybridization (ISH) (90, 107, 108). Some specific adaptations are, however, necessary, due to the short size of the miRNAs (109). A first method uses LNA oligonucleotide probes (110–114). A second method developed by Thompson et al. used RNA probes to detect mature miRNAs in tissue sections (107, 115). Both approaches may detect primarily mature miRNAs (116).

Ten-micrometer frozen sections of tissues are stored at –80°C until use for in situ hybridization of miRNA. Slides are air-dried for 30 min, and then sections are fixed in 4% paraformaldehyde (Electron Microscopy Sciences) solution in sterile PBS at 4°C for 10 min. After washes in PBS at room temperature (RT), sections are incubated in acetylation solution (2.33 ml triethanolamine, 500 µl acetic anhydride, volume up to 200 ml in sterile water) for 10 min at RT and then washed in PBS. Slides are then exposed overnight at T_m –20°C to either 0.3 ng/µl miRNA digoxigenin-labeled LNA probe (Locked nucleic acid) or 0.3 ng/µl scramble miR digoxigenin-labeled LNA probe (Exiqon, Woburn, MA) in hybridization mixture consisting of 50% deionized formamide, 0.3 M NaCl, 20 mM Tris–HCl pH 8.0, 5 mM EDTA, 10 mM $NaPO_4$ pH 8.0, 10% dextran sulfate, 1× Denhardt's solution, and 0.5 mg/ml yeast RNA. Slides are then sequentially washed for 30 min at T_m –20°C in solution composed of formamide 50%, 0.1% Tween-20, 1× SSC for 15 min at RT in 0.2× SSC and finally for 15 min at RT in PBS. After incubation with horseradish peroxidase conjugated with sheep anti-digoxigenin antibodies (1:100, Roche, Mannheim, Germany) for 1 h at RT, detection of probes is realized using Tyramide Signal Amplification Plus DNP AP System (Perkin Elmer, Shelton, CT) and BCIP/NBT substrate (DakoCytomation, Glostrup, Denmark). Sections are finally counterstained using Nuclear Fast Red (Sigma Aldrich), dehydrated through graded ethanol concentrations, and mounted using Eukitt mounting medium (Electron Microscopy Sciences).

3.2. Combined In Silico and Experimental Approaches to Identify miRNA Targets

Identification of genes targeted by miRNAs in a specific cellular model requires a combination of in silico and experimental approaches. Many computational algorithms have been developed to predict potential genes targeted by miRNAs (21, 22, 117–121). They are generally based on the fact that the "seed" region forms perfect base pairing with the target sites and that this seed pairing is conserved across species (122). It is important to notice that analyses performed with different algorithms usually overlap poorly. This implies that a careful experimental validation of the predicted targets is mandatory.

3.2.1. Transcriptome Analysis Combined with Ectopic Expression of miRNAs in Cells

The easiest approach combines ectopic expression of synthetic miRNA with mRNA expression studies. Enrichments at an RNA level of predicted targets in the set of genes that are downregulated after overexpression of a specific miRNA or in the set of genes that are upregulated after silencing of a specific miRNA have been clearly demonstrated (53, 54, 63, 123), suggesting that many miRNAs can indeed trigger some degradation of their mRNA targets. Transfection of small RNA duplexes at a high copy number does not perturb globally the regulation by endogenous miRNAs (124 , 125). A combination of measurements of the expression profiles at miRNA and mRNA levels is powerful to identify functional miRNA::mRNA relationships and to provide an experimental counterpart to pure computational approaches (126).

3.2.1.1. Ectopic Expression of miRNA

Cells are grown until 30% confluency is reached and then transfected with miRNAs of interest (10 nM) using Lipofectamine RNAi MAX Reagent (Invitrogen) in OPTIMEM (Invitrogen, Gibco product) following the manufacturer's instructions. Transfected cells are lyzed in Trizol$^{®}$ 48 h later for total RNA extraction.

3.2.1.2. Analysis of RNA Expression Using DNA Microarray

Total RNAs are purified, quantified, and quality controlled as above. RNAs are then analyzed on a DNA GeneChip$^{®}$ Human Gene 1.0 ST Array (Affymetrix$^{®}$). Each of the 28,869 genes is represented on the array by approximately 26 probes spread across the full length of the gene (http://www.affymetrix.com). Total RNAs are labeled and hybridized using the Whole Transcript (WT) Sense Target Labeling and Control Reagents, fluidics and scanning instrumentation, and basic analysis software, as indicated by the manufacturer's instructions. Slides are quantified using Expression Console software (Affymetrix). Data analyses are performed using the statistical suite Bioconductor developed by the R consortium (http://www.bioconductor.org) and then data are visualized within Mediante, an information system developed for storing our microarray data (127). For Affymetrix microarrays, the data are processed using the RMA (Robust Multi-Chip Average) algorithm, which performs a background correction, a normalization step, and a probe-level summary. This method has been shown to have higher precision, particularly for low expression values, and higher specificity and sensitivity than many of the other commonly used methods (128). Data sets are further normalized following a linear model and an empirical Bayes method using R software. The Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Mountain View, CA) is utilized to identify networks of interacting genes and other functional groups.

<table>
<tr><td>3.2.1.3. Bioinformatics Analysis of miRNA Targets</td><td>Two in-house bioinformatics tools have been developed to predict miRNA targets (http://www.microarray.fr:8080/merge/index; follow the link to microRNA and bioinformatics tools): (i) MicroCible is a miRNA target predictor that scans transcript sequences for the presence of complementary sequence to the "miRNA seed." This search can be performed for different "seed" match type (129), a minimal free energy binding cut-off, and the location of the potential targeting site (i.e., 3′UTR or the entire transcript); (ii) MicroTopTable is dedicated to the search of putative enrichments in predicted miRNA binding sites among a set of modulated genes, based on several prediction software. MicroTopTable ranks the transcripts into three categories ("upregulated," "downregulated," and "non-modulated"), according to thresholds for expression level and for differential expression. MicroTopTable then calculates the number of predicted targets for each miRNA, according to the prediction software selected, in each of the three categories. Enrichment in miRNA targets in each category is then tested using a hypergeometric law. More systematic analyses can be performed with Sylamer, which measures hypergeometric P-values for all short sequences of fixed length across a ranked gene list (130).</td></tr>
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3.2.2. Other Approaches

Biochemical approaches combining RNA-induced silencing complex (RISC) purification, either combined with cloning (131), microarray analysis (Rip-Chip) (132–136), or high-throughput sequencing approaches (137), have also been proposed. Interestingly, Chi et al. have decoded microRNA::mRNA interaction maps by a technique called "Argonaute HITS-CLIP." These authors used high-throughput sequencing of RNAs isolated by immunoprecipitation of covalent crosslink between the Argonaute protein and RNA complexes (i.e., miRNA::mRNA). This produced two simultaneous data sets – Ago–miRNA and Ago–mRNA binding sites – that were electronically combined to identify interaction sites between miRNA and target mRNA. "Argonaute HITS-CLIP" provides a general platform for exploring the specificity and range of miRNA action in vivo and identifies precise sequences interacting into pertinent miRNA::mRNA interactions. A systematic use of such a technology may help unravel the precise interactions existing in vivo between miRNAs and mRNAs. Azuma-Mukai et al. have used a simpler approach that identifies miRNAs associated with hAgo2 and hAgo3 (138). Proteomic approaches, based on differential labeling of the biological samples, have shown that a single miRNA can repress the production of hundreds of proteins. Surprisingly, this repression appears usually relatively mild (see above) (33, 139). Interestingly, changes in translation efficiencies of targeted mRNAs were highly correlated with changes in the abundance of those RNAs, suggesting a functional link between microRNA-mediated repression of translation and mRNA decay (134).

3.3. Validation of miRNA Targets

When a specific miRNA::mRNA interaction is detected, validations can include direct functional assay, Western blot analysis, and/or reporter plasmid assay. In the latter case, the 3′UTR of the predicted target mRNA is inserted into an expression plasmid to control the level of production of a reporter gene. Experiments are performed in the presence or absence of the miRNA of interest to measure the impact of the miRNA on reporter expression (63, 119, 140).

3.3.1. Reporter Plasmid Assay

3.3.1.1. Molecular Constructs

Molecular constructs are derived from psiCHECK[TM]-2 (Promega) by cloning behind the renilla luciferase ORF sequences from target 3′UTR mRNA in the *Xho*I and *Not*I restriction sites. Mutations are introduced by site-directed mutagenesis of target 3′UTR miRNA putative binding sites using the QuickChange Kit (Stratagene). Complementary oligonucleotides (50 µM final concentrations) are mixed with 10× Oligo Annealing Buffer (Invitrogen), heated to 95°C for 4 min, and allowed to cool at room temperature for 10 min. Diluted (10 nM) dsDNA are subsequently cloned in *Xho*I/*Not*I restriction sites in psiCHECK[TM]-2 (Promega).

3.3.1.2. Transfection and Luciferase Assays

Pre-miRNA overexpression in cultured cells: Pre-miRNA and control miRNA (miR-Neg #1) are purchased from Ambion. Cells are transfected at 50% confluency in 6-well plates using Lipofectamine[TM] RNAi MAX[TM] (Invitrogen) with pre-miRNA at a final concentration of 10 nM.

Pre-miRNA and psiCHECK[TM]-2 plasmid construct co-transfection: Cells are cultured in regular medium until confluency. Then cells are plated into 48-well plates at a density of 26.5×10^3 cells/well and co-transfected using 1 µl of lipofectamine 2000[TM] (Invitrogen) with 0.4 µg of psiCHECK[TM]-2 plasmid construct and pre-miRNA or control miRNA at a final concentration of 10 nM. The medium is replaced 8 h after transfection with fresh medium supplemented with penicillin and streptomycin. Forty-eight hours after transfection, firefly and renilla luciferase activities are assayed using the Dual-Glo[TM] Luciferase Assay (Promega) and measured with a luminometer (Luminoskan Ascent, Thermolab system).

4. Conclusion

Protein-coding genes probably correspond to the tip of an iceberg corresponding to all RNAs generated by a living cell.

With 15,908, 20,158, 22,974, 23,438, and 24,408 protein-coding genes reported by Ensembl (http://www.ensembl.org) in chicken, *Caenorhabditis elegans*, mouse, human, and *Arabidopsis thaliana*, respectively, while genome sizes vary from 0.1×10^9 nucleotides to 3.4×10^9 nucleotides, it is clear that a large fraction of the genome indeed exerts its biological functions independently of its translation into protein(s) ([141], [142]). Though the focus of this review was limited to microRNAs (miRNA), which constitute a tiny subclass of regulatory non-coding RNAs, many more works will be necessary to unravel the many roles played by non-coding RNAs in biological processes. It remains that miRNAs deserve a special interest due to their already established association with many biological processes and to the already ongoing efforts to transfer them into new useful prognosis markers, therapeutic targets, or targeted drugs.

5. Note

1. Total RNAs containing small RNA fraction can be isolated using either miRNeasy Minikit or RNeasy Minikit. Columns (RNeasy Mini Spin Columns) are identical. The point is to add 1.5 volume 100% ethanol to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) upward.

Acknowledgments

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