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Review

A toolbox for miRNA analysis

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ABSTRACT

MicroRNAs (miRNAs) are small regulatory RNAs, which mediate selective repression of gene expression. miRNAs play important roles in many natural and pathological processes. Numerous tools were developed for their detection and functional analysis. There are many excellent articles covering different areas of miRNA biology in detail. At the same time, I think there are many colleagues who face a miRNA-related research problem and would appreciate having an introductory general overview of tools for miRNA analysis, which would help them in considering available options. Accordingly, this review provides an elementary roadmap to navigate among available tools for miRNA analysis. The most common problems and errors observed in miRNA research are also discussed.

1. Introduction

The last decade witnessed explosive growth of the microRNA (miRNA) field. There are countless original and review articles describing all possible aspects of miRNA biology in very great detail. At the same time, I feel it might be difficult for a non-expert to get quickly oriented in the field and find a specific solution to his/her miRNA-related problem. I had two main motivations to write this review, which should be seen as an elementary small scale map of the world of miRNA analysis. First, I wanted to provide an introductory material allowing for understanding the basics of miRNA analysis and starting exploring further details in the referenced literature. Second, I wanted to highlight the problem of stoichiometry between miRNAs and their targets, which needs to be taken into account when studying physiological roles of miRNAs and performing their functional analysis. The review has two parts, which follow a basic introduction into miRNA biogenesis and function. The first part deals with analyzing miRNA presence, abundance, and activity. The second part is focused on functional analysis of miRNAs, where I discuss tools for manipulating miRNA levels and options for identification and validation of miRNA targets.

2. Essentials of miRNA biogenesis and function

miRNAs are genome-encoded short RNAs that regulate gene expression by mediating translational repression and/or degradation of cognate mRNAs. miRNAs play important roles in many processes and are the most common type of small RNAs found in mammalian cells. Biogenesis of canonical and non-canonical miRNAs has been summarized in detail elsewhere [1–4]. Here, I will only briefly summarize essential information concerning canonical miRNA biogenesis in animals (Fig. 1A).

Canonical miRNAs are transcribed by RNA polymerase II as long primary transcripts (pri-miRNAs), which are cleaved by RNase III Drosha, a component of the nuclear "microprocessor" complex, to release short hairpin intermediates (pre-miRNAs). Pre-miRNAs are transported to the cytoplasm where they are further processed by RNase III Dicer (Fig. 1B) into ~22 nucleotide (nt)-long double-stranded RNA molecules. Some animals (e.g. *Caenorhabditis* and vertebrates) have one Dicer producing different types of small RNAs from different substrates while others (exemplified by *Drosophila*) have a specific Dicer isoform dedicated to the miRNA pathway.

One strand of the duplex produced by Dicer is selected and loaded onto an Argonaute protein (Fig. 1C), the miRNA-carrying component of the effector ribonucleoprotein complex, which recognizes and represses target mRNAs. The AGO-containing effector complex has been given different names, here I will refer to it as miRNA-Induced Silencing Complex (miRISC). In mammals, four AGO proteins, AGO1–4, associate with miRNAs and are implicated

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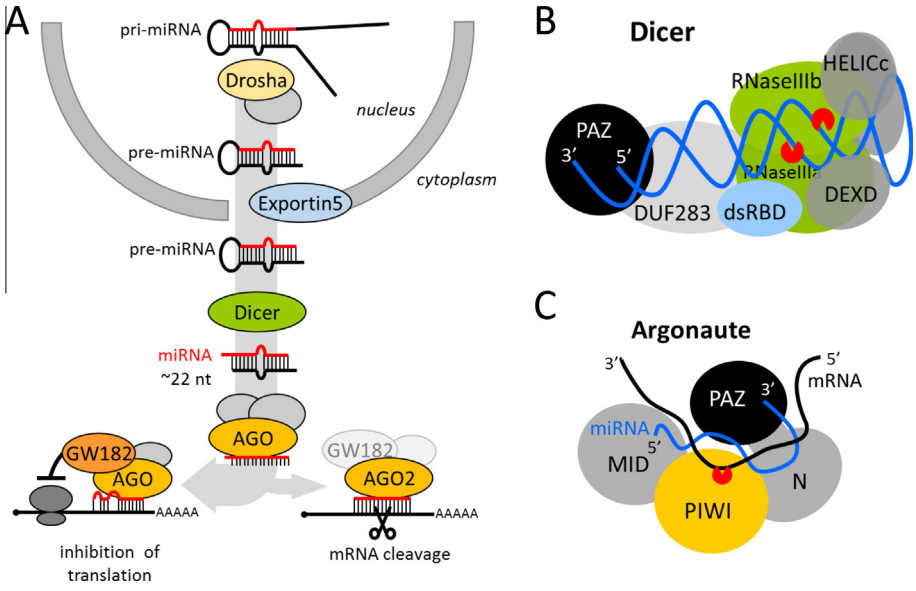

Fig. 1. miRNA pathway in mammals. (A) A schematic overview of miRNA biogenesis and action. (B) Domain organization of Dicer RNase III, the key player in biogenesis of miRNAs and siRNAs (reviewed in [1,90]). Mammalian Dicer is ~215kDa protein containing several conserved domains, including N-terminal DEAD-like and helicase superfamily C domains, the piwi/argonaute/zwille (PAZ) domain, two RNase III domains, and the C-terminal double-stranded RNA binding domain (dsRBD). The PAZ domain binds a 3′ protruding overhang in dsRNAs or miRNA precursors. Two RNase III domains (depicted as red Pac-Men) form a single processing center producing a short dsRNA with a 2 nt 3′ overhang, where each domain cleaves one strand of the duplex. Thus, Dicer acts as a molecular ruler, measuring the substrate length from the PAZ domain to RNase III domains. The scheme of Dicer organization is based on previous structural studies [91,92]. (C) Domain organization of an Argonaute protein based on previous structural studies of AGO2 [5,8]. AGO proteins have four domains (N-terminal (N), PIWI, middle (MID), and PAZ). The small RNA (miRNA or siRNA (blue)) binds with its 3′ end in the PAZ domain and the 5′ binds a binding pocket in the MID domain. The mRNA (black) enters between the N-terminal and PAZ domains and exits between the PAZ and middle domain. A perfect duplex of a small RNA and mRNA fits into a groove in AGO2 such that the active site in the PIWI domain (depicted as a red Pac-Man) cleaves the mRNA opposite the middle of the siRNA guide. Imperfect base pairing or other AGO isoforms mediate translation repression, which requires additional co-factors, including GW182.

in translational repression [5–7]. In addition, AGO2 can mediate specific endonucleolytic cleavage of a target mRNA in the middle of the sequence base paired with a short RNA [5,6,8]. Whether AGO2-loaded short RNA will mediate endonucleolytic cleavage or induce translational repression depends on the extent of base pairing with its mRNA target. The AGO2-mediated endonucleolytic cleavage requires formation of a perfect or nearly perfect miRNA:mRNA duplex, exemplified by mammalian mir-196 and *HoxB8* mRNA [9]. However, complementarity between animal miRNAs and their cognate mRNAs is typically imperfect while the endonucleolytic cleavage has been traditionally attributed to RNA interference (RNAi) pathway. Functional base pairing of miRNAs with their mRNA targets appears to involve little beyond the "seed" region comprising nucleotides 2–8 of the miRNA [10,11].

Imperfect miRNA:mRNA base pairing results in translational repression [12,13] followed by substantial mRNA degradation [14,15], which actually facilitates identification of primary miRNA targets through transcriptome profiling. The molecular mechanism of mRNA degradation induced by imperfect base pairing differs from the above-mentioned AGO2-mediated endonucleolytic cleavage [16] and involves deadenylation and decapping activities [17–20]. Recent data suggest that RNA degradation is actually the dominant component of cognate gene repression [21]. Repressed mRNAs, miRNAs, and AGO proteins localize to cytoplasmic foci known as P-bodies [22,23], which contain mRNA degrading enzymes such as the decapping complex, deadenylases, and the exonuclease XRN1 (reviewed in [24]).

Thousands of miRNAs have been annotated in different species. The central miRNA database miRBase (http://www.mirbase.org) [25] contains 28 645 entries, including 2588 human, 1915 murine, 466 *Drosophila melanogaster* and 434 *Caenorhabditis elegans* mature miRNAs (release 21). miRNAs were implicated in countless cellular and developmental processes and changes in their expression are observed in various diseases. At the same time, the function of most miRNAs and suppression of their cognate genes are poorly understood. One of the bioinformatic estimates suggests that overall miRNAs might directly target over 60% of mammalian genes [26]. Functional analyses in different mammalian cell types, including effects of ectopic expression of miRNAs [15], inhibition of individual miRNAs by antisense molecules [27] or miRNA pathway knock-down [16] or knock-out [28,29] suggest that in one cell type (i) a significant portion of the transcriptome (thousands of genes) is affected (directly and indirectly) by miRNA activity and that (ii) a single miRNA might directly suppress expression of up to several hundred genes.

The set of miRNAs in each cell type forms a combinatorial post-transcriptional regulation system shaping gene expression in an analogy to a trimming stencil. miRNAs have widespread impact on expression and evolution of protein-coding genes (reviewed in [30]). Evolution of miRNA regulations is very fast. For example, there are only a few miRNAs conserved between *Drosophila* and mammals. Animal miRNAs seem to emerge from random formation of Drosha/Dicer substrates. Newly evolving miRNAs likely form a considerable portion of annotated miRNAs, especially in species where miRNAs were intensely studied by next generation sequencing (NGS), which can identify low-abundance miRNAs. The newly emerging miRNAs either acquire significant repressive functions and become retained during evolution or they become lost. Furthermore, target repertoire of individual miRNAs can evolve fast since a single point mutation can weaken an existing regulation or create a new one. This idea is consistent with the data showing that mammalian mRNAs are under selective pressure to maintain and/or avoid specific 7-nucleotide seeding regions

[31]. It is also nicely illustrated on the Texel sheep phenotype where a single mutation creating a novel miRNA target site in myostatin causes the renowned exceptional meatiness of this breed [32]. The number of mRNAs that have functionally important interaction with miRNAs in a studied model system is presumably small and certainly difficult to discern among the all possible interactions. Thus, every search for functionally important interactions between miRNAs and their targets has to face the fact that miRNAs represent a dynamically evolving system with countless random interactions, which are not biologically relevant.

3. miRNA profiling – miRNA detection & quantification

Several methods can be used for detecting individual miRNAs. The oldest method for miRNA detection is Northern blotting [33], which was also adopted as the golden standard for annotating novel miRNAs [34]. There are several online protocols for miRNA Northern blotting using RNA or DNA radiolabeled oligonucleotides [35] or an adaptation using so-called locked nucleic acids (LNA) probes [36], which offer higher hybridization affinity [37]. While Northern blotting is relatively labor-intensive, it is an invaluable tool for obtaining insights into regulation of miRNA biogenesis and degradation. One of the main Northern blot advantages is that it reveals in a single experiment miRNA and pre-miRNA amounts and ratios, miRNA length variability, and covalent modifications.

The more common approach for monitoring individual miRNA levels is nowadays real-time reverse-transcription PCR (qPCR), for which there is a number of protocols and commercial assays (e.g. miRCURY LNA qPCR from Exiqon or TaqMan assays from ABI). qPCR has a high sensitivity and can be performed on a minimal amount of material – for example, a qPCR protocol allowing for a quantitative analysis of 220 miRNAs from a single cell has been described [38]. cDNA preamplification combined with a qPCR array system allows for a rapid analysis of a large number of samples. Sufficient number of replicates is also important for quantitative single-cell qPCR analysis, one has to take into the account stochasticity of single-cell expression and analyze enough cells [39]. Available 48 × 48 and 96 × 96 qPCR array formats (e.g. Fluidigm) allow for analysis of 48 or 96 miRNAs in 48 or 96 samples, respectively. Since analyzing 48 or 96 miRNAs is sufficient for most applications, PCR arrays offer the best option in terms of the total price per analyzed sample. At the same time, qPCR is more prone to artifacts resulting in false positives and quantification errors than a Northern blot, which directly detects miRNAs in a non-amplified RNA sample.

The third common individual miRNA detection approach is in situ hybridization, which is a powerful tool for spatiotemporal resolution of miRNA expression, especially in cases where one has to rely on histology. As miRNAs are short, it has been challenging to optimize detection conditions when adapting traditional in situ RNA hybridization protocols. A major breakthrough was made by introduction oligonucleotides containing LNA nucleotides, which improve hybridization properties since they have higher melting temperature than DNA or RNA oligonucleotides [37]. LNA-containing oligonucleotides are nowadays the standard approach for miRNA in situ detection in tissues or during development [40,41].

In addition to the aforementioned "traditional methods" of miRNA detection, an interesting progress can be observed in electrochemical sensing approaches (reviewed in [42]). These methods offer high sensitivity and great potential for developing instruments for routine assessment of specific miRNA content in biological samples. As such, these approaches will presumably have more use in diagnostic/clinical set up rather than in common molecular biology labs.

3.1. miRNA profiling by microarrays

Microarrays are one of the two most common strategies for profiling a miRNA population in a sample (the second one is NGS, discussed further below). Microarray profiling based on detecting complementarity between nucleic acids in the sample and nucleic acids arranged in an array has been developed for profiling gene expression since mid 90's [43]. Conceivably, microarrays were rapidly adapted for miRNA profiling [44–47] and a number of microarray platforms has been developed, some of which are available commercially. Detailed discussion of different platforms and experimental designs is beyond the scope of this review and can be found elsewhere [48,49]. Here, I would only briefly highlight main advantages and disadvantages of miRNA microarrays for gene-by-gene or genome-wide profiling and underscore the importance of the right experimental design including appropriate controls.

Detection on microarrays is restricted for probes, which are arrayed on them. Thus, microarrays are generally suitable for comparing expression of annotated miRNAs but cannot reveal novel miRNAs. A general problem of miRNA hybridization is that optimal hybridization temperature varies for different miRNAs, which makes it difficult to find hybridization conditions optimal for all microarray probes. A possible solution to this problem was provided by LNA, which offer higher melting temperature than DNA or RNA duplexes [37]. Thus, oligonucleotide probes on an array can be designed to contain various amounts of LNA nucleotides to adjust optimal hybridization temperatures for all miRNAs. However, when using microarrays, one should always keep in mind the risk of cross-hybridization among closely related miRNAs and that microarrays are less informative than NGS.

miRNA profiling by microarrays typically requires 100 ng–1 μg of total RNA, which is similar to NGS. In contrast to NGS, microarray profiling traditionally offered a faster and cheaper solution with a lower resolution and a lower dynamic range [50]. However, with constantly diminishing costs of sequencing per nucleotide combined with multiplexing options, NGS might easily become financially superior option. Therefore, I recommend calculating all presently available options before one commits to one high-throughput system. For the choice, it is also important, what one expects from the profiling and how much information is willing to process and analyze. NGS is superior to microarrays and qPCR because it reveals the entire miRNA population, including rare and unannotated miRNAs, heterogeneity of miRNA species produced from a single pre-miRNA, and allows to precisely distinguish, which miRNA family members are expressed.

This might be important especially in diagnostics and for screening a large number of samples for biomarkers.

3.2. miRNA profiling by next generation sequencing (NGS)

NGS revolutionized miRNA profiling. This is unsurprising because short miRNAs with defined (i.e. annotated) sequences are an optimal material for NGS. There are several different NGS platforms available (for a comparison, see the reference [51]). Platform selection is the matter of availability and cost; these are constantly changing variables as new instruments and sequencing services are available while the cost per base is constantly declining. Because of the constantly changing landscape of NGS one needs to survey presently available options when considering NGS analysis. The cost of commercial NGS services typically breaks down into library preparation, sequencing, and data analysis and often needs to be directly inquired/negotiated.

In my lab, we have had a good experience with both, SOLiD (by Sequomics in Hungary) and Illumina (EMBL, Germany) sequencing platforms over the past few years. However, as bioinformatic

analysis of SOLiD data was significantly more complex, we prefer Illumina sequencing now. For detailed analysis of small RNAs, we used multiplexing of 50-nt single-end (50SE) sequencing on HiSeq 2000, which yielded depths in tens of millions reads at the cost of ~400 Euros/sample for 6–8 RNA samples. This depth was sufficient to detect rare small RNAs (including endo-siRNA) [52,53]. A lower NGS depth (up to ten million reads) would be sufficient for routine miRNA profiling (especially if the studied cell population is homogeneous). Furthermore, development of NGS platforms and multiplexing options offers highly reduced costs per sample at excellent sequencing depths.

Before miRNA profiling by NGS, I highly recommend to consult normalization options with the NGS provider and include spiking controls whenever possible because having an external normalization standard is invaluable. Next, it is important to be prepared for data management and analysis. NGS providers typically offer data analysis (usually for extra charge), which should satisfy common needs for simple differential expression analysis. A recommended solution, however, is to find local bioinformatics support or to learn how to analyze the data. The last option is more plausible than it sounds thanks to web-based platforms for miRNA expression analysis, such as oasis [54], MAGI [55] or older and more complex Galaxy [56,57]. Importantly, NGS is a great tool, however, it is also prone to artifacts, which might distort miRNA representation in the original sample [58]. Therefore, one should not rely solely on NGS data when inferring a specific miRNA abundance in a sample but, whenever suitable, complement the analysis with Northern blot or qPCR analyses.

4. Monitoring biological activity of miRNAs with reporters

A common way of monitoring miRNA activity employs reporters carrying miRNA binding sites in the 3′UTR. One option is to use a natural 3′UTR of a known miRNA target. For example, lin-41 3′UTR targeted by let-7 miRNA has been combined with coding sequences of EGFP [59,60], *lacZ* [61], or luciferase [62]. A neat system for monitoring let-7 activity in *C. elegans* was developed by co-expressing non-targeted mCherry and let-7-targeted GFP in the distinctly shaped pharynx [63]. In the absence of let-7, both red and green fluorescence is observed. When let-7 is expressed, the GFP reporter is repressed and only red fluorescence is observed. This sensor system can be used to study regulation of let-7 activity and allows for high-throughput screening using fluorescence-activated worm sorting.

The most common reporter systems in cultured mammalian cells are those using dual luciferase reporters. Typically, one luciferase reporter (e.g. firefly) serves as a normalization control, while the other one (e.g. *Renilla*) is targeted by a selected miRNA through miRNA binding sites in the 3′UTR (Fig. 2A and B). Reporters can also carry artificial miRNA binding sites, either with perfect or partial complementarity to a miRNA [12,16,23]. The number and the type of miRNA binding sites allow to modulate reporter sensitivity/degree of downregulation by endogenous miRNAs. The reporter system ideally includes a negative control made of a mutated targeted reporter where point mutations are introduced into miRNA binding sites to disrupt miRNA-mediated repression. The mutant control is better than using a completely unrelated 3′UTR (e.g. commercial luciferase reporter with SV40 3′UTR) because the difference between targeted and mutated reporter 3′UTRs is small and the effect of miRNA-mediated repression can be attributed to specific 3′UTR nucleotides. Using a negative control carrying an unrelated 3′UTR in the control reporter increases the risk that the negative control reporter will generate artifacts because its 3′UTR would influence translation and/or mRNA half-life distinctly from the targeted 3′UTR but in a miRNA-independent fashion.

Finally, adding MS2 binding sites to the luciferase reporter allows for visualization of localization of targeted RNA to P-bodies by co-expressing MS2-YFP [62] (Fig. 2C). In this case, 12 MS2 binding sites were inserted into a miRNA-sensitive luciferase reporter and the visualization is achieved by co-expression of MS2-YFP fusion protein carrying a nuclear localization signal, so the unbound MS-YFP is sequestered in the nucleus [62,64]. This type of reporter might be very helpful for target validation because it reveals two distinct miRNA effects at the same time – translational repression (monitored by luciferase assay) and miRNA-dependent localization into P-bodies (revealed by microscopy).

5. Approaches to manipulate miRNA levels

5.1. miRNA overexpression

To increase miRNA levels in cells, common RNAi tools (reviewed for example in [65,66]) can be easily adapted. In mammals, RNAi and miRNA pathway essentially converge upon cleavage by Dicer. Therefore, short interfering RNA (siRNA) and short hairpin RNA (shRNA) expression can be directly employed for miRNA upregulation. A transient increase can be achieved by transfecting siRNA carrying the miRNA sequence (so-called miRNA mimic) or using shRNA-expressing plasmid where one has a choice between polymerase III-driven shRNAs, which are Drosha-independent, or polymerase II-driven shRNAs, which are processed like endogenous miRNAs [67]. Such miRNA expression systems allow for stable or inducible expression in cultured cells or entire organisms. Importantly, miRNA overexpression data must be carefully interpreted since miRNA overexpression can easily generate false positives due to (i) non-physiological stoichiometry of the overexpressed miRNA resulting in artificial repression and (ii) miRISC saturation by the overexpressed miRNA, which results in relieved repression of natural targets of other miRNAs.

5.2. Global suppression of the miRNA pathway

Several strategies can be used for modulating the miRNA pathway. It can be inhibited by knocking-down or knocking-out individual miRNA pathway components, such as Dicer or Drosha (e.g. [16,29,68]), or by plant virus-encoded repressors [69]. However, these strategies have certain limits. For example, the knock-down/knock-out strategy is constrained by the targeted protein-product half-life, thus the inhibition cannot be rapidly induced. This might be a problem, for example, when addressing the role of miRNAs in rapidly changing model systems. The knock-out approach used to be restricted to embryonic stem cells (ESCs) and cells derived from ESCs or from mutant animals (e.g. mouse embryonic fibroblasts). However, the recent progress in guided nucleases [70,71] makes it feasible to produce knock-outs also in commonly used cell lines. In our experiments with miRNA reporters in HeLa, HEK293 and 3T3 cell lines, knock-down of *Ago2* or Dicer significantly relieved the miRNA-mediated repression (2–3-fold) while expression of plant virus repressors P19 and P21 had minimal effects.

A possible future alternative to the above-mentioned approaches could be small compound modulators. Small compound modulators of Dicer or miRISC identified through chemical biology would be a great tool for studying RNAi and miRNA pathways in vivo and in vitro. In fact, several putative small compound inhibitors and stimulators of the miRNA pathway were reported (reviewed in [72]) but their specificity for the miRNA pathway was not demonstrated and none of them has been developed into an experimentally useful tool, so far.

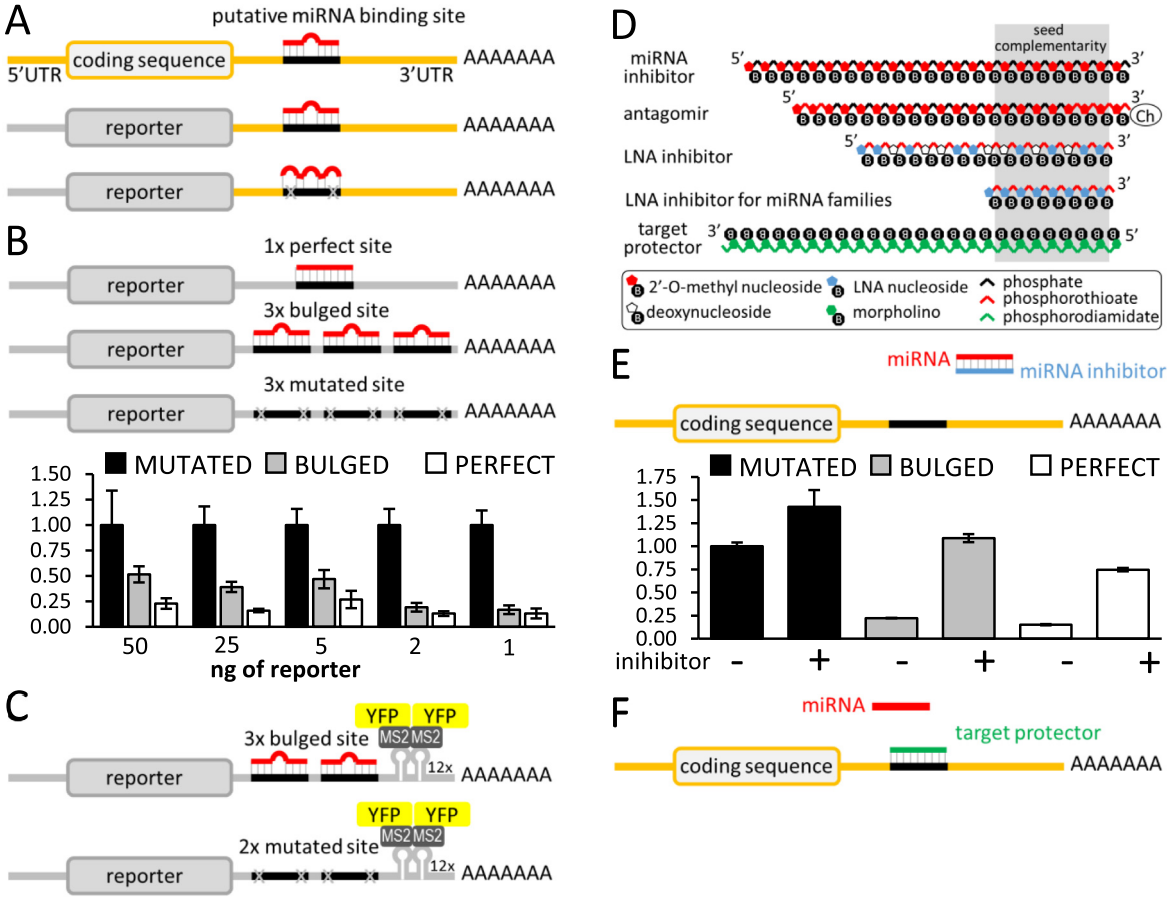

Fig. 2. Tools for functional analysis of miRNAs. (A) miRNA-sensitive reporters based on fusing 3′UTR of a targeted mRNA with a reporter. The targeted endogenous mRNA is labeled with orange color, the targeting miRNA is shown in red. The miRNA binding site is depicted as a black region in the 3′UTR. Below the endogenous cognate mRNA are schemes of two reporter mRNAs, where the upper one is repressed by the miRNA and the lower one is resistant to miRNA repression. A gray X represents a mutation disrupting base pairing between the miRNA and the reporter. (B) Artificial reporters carrying bulged and perfect complementarity types of miRNA binding sites. miRNA binding sites are depicted as black regions in the 3′UTR, miRNAs are shown in red. The graph below is an example of reporter utilization. In this particular example, reporter plasmids were transfected into 3T3 cells per well in a 24-well plate and luciferase activities were assayed 48 h post-transfection. SV40 promoter-driven reporters contained either one perfect or four bulged or four mutated miR-30 sites [64]. The *y* axis show relative expression formalized to the non-targeted firefly luciferase and set to one for the mutated reporter. Error bar = S.E.M. (C) miRNA-repressed reporters, whose cytoplasmic localization can be visualized in cells through MS2 binding sites (depicted as three stem loops downstream of miRNA binding sites), which are recognized by a fluorescent protein (here YFP) fused with MS2 binding domain [62]. (D) Examples of oligonucleotide inhibitors of miRNA function described in the text. Different chemistries are indicated in the lower panel. Ch indicates cholesterol moiety at the 3′ end of an antagomir. (E) Selective miRNA inhibitor. The scheme depicts the principle of action of miRNA inhibitors – an oligonucleotide binds a miRNA and prevents repression of a cognate mRNA. The graph depicts effects of a Let-7 family short LNA inhibitor (Exiqon) on expression of luciferase reporters. HeLa cells were transfected with Let-7 reporters [16] (1 ng/well/24-wellplate) and 1 μM of LNA inhibitor. Cells were assayed 24 h post-transfection and data were normalized as in Fig. 2B. (F) Target protector. The scheme depicts the principle of action of target protectors – a morpholino oligonucleotide (in green) binds a miRNA binding site (black) and thus blocks miRNA-mediated repression of the cognate mRNA.

5.3. Selective inhibition of miRNA function

A traditional approach for selective miRNA inhibition would be a knock-out. Several years ago, including knock-out strategy among the experimental tools for selective miRNA inhibition would be a formal acknowledgment of the approach rather than a useful advice. However, guided nucleases mentioned above [70,71] allow for relatively simple generation of knock-out models, including common cultured cell lines. Therefore, genetic manipulation of a miRNA is certainly worth of considering nowadays.

Other strategies to selectively block miRNAs include sequestering miRNAs with so-called miRNA sponges or employing various complementary oligonucleotides (Fig. 2D), which bind miRNAs and render them non-functional or even destabilize them.

5.3.1. Oligonucleotide inhibitors – antagomirs, target protectors and others

miRNAs can be selectively and irreversibly inhibited by stoichiometric amounts of 2′-O-methyl oligoribonucleotides (Fig. 2D) in vitro and in vivo [73,74]. Initial experiments in cultured cells used 24-nt 2′-O-methyl oligoribonucleotides that contained a 3′C7 aminolinker (to enable postsynthetic conjugation of non-nucleotidic residues such as biotin) [73] or 31-nt 2′-O-methyl oligoribonucleotides [74]. The term antagomir, which is often used as a common name for oligonucleotide miRNA inhibitors, was introduced for 21-nt cholesterol-conjugated 2′-O-methyl oligoribonucleotides carrying phosphorothioate linkages between several nucleotides at 5′ and 3′ ends (Fig. 2D). Antagomirs are suitable for in vivo applications in animal models as well [27]. Similar

effects can be achieved by miRCURY LNA miRNA inhibitors, which use different chemistry and which can be obtained as mixmers of LNA and DNA oligonucleotides with phosphodiester or phosphorothioate bonds. As LNAs offer highly stable base pairing, short oligonucleotides (8-mers) can be used to inhibit entire miRNA families through targeting the common seed regions (Fig. 2D and E).

Target protectors [75] represent a different class of oligonucleotide inhibitors as they selectively disrupt inhibition of a single cognate mRNA (Fig. 2D and F). Target protectors represent an alternative use of morpholino oligonucleotides, which have been developed to suppress mRNA translation by binding to the translation start site. As morpholinos are stable and do not destabilize mRNAs, they can be effectively used to mask miRNA binding sites in the 3′UTR without disturbing normal gene function [75]. Target protectors are great tools for target validation and delineating miRNA-dependent effects as their effects are directly aimed at specific miRNA targets.

5.3.2. miRNA sponges

miRNA sponges (reviewed in [76]) are a cost-effective alternative to oligonucleotide inhibitors, which also allows for establishing long term repression or tissue-specific repression in vivo. The principle of a sponge is simple – it is a transcript carrying multiple miRNA binding sites, which is highly expressed and sequesters specific cellular miRNAs [76,77]. Sponges allow for inducing selected miRNA suppression in vivo [78] and can achieve suppression of several selected miRNAs in a specific tissue – exemplified by repression of three miRNAs in the retina of a transgenic mouse model [79].

6. Identification and validation of miRNA targets

Target identification & validation is a common problem in the miRNA field. Numerous experimental strategies can be used to solve it [80,81]. Below, I will provide suggestions to avoid being entangled in the complexity of the problem and discuss which strategies are less likely to produce artifacts.

6.1. Target identification

Several biochemical approaches were developed for direct isolation and identification of target mRNAs associated with miRISC (reviewed in detail in [80,81]). A powerful methods is crosslinking followed by AGO immunoprecipitation and NGS known as high throughput sequencing of crosslinking immunoprecipitation (HITS-CLIP) [82,83]. HITS-CLIP allows for narrowing down a miRNA bound region and, when combined with selective miRNA inhibition (see below), it offers a great tool to identify natural miRNA targets regardless of their conservation. A further improvement of the approach came with crosslinking using photoactivatable nucleosides such as 4-thiouridine, a CLIP modification known as photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) [84]. PAR-CLIP offers more efficient crosslinking, hence up to three orders of magnitude better RNA recovery than HITS-CLIP [84]. Importantly, PAR-CLIP also allows for precise localization of miRNA binding site as cross-linked 4-thiouridine marks the cross-linked site with frequent thymidine to cytidine transition, which is revealed by NGS [84].

While CLIP approaches provide outstanding tools for miRNA target identification, they are not always accessible to a common user mainly because they require significant expertise in proteomics and bioinformatics, good antibodies, and sufficient funding. Therefore, common users usually opt for fallible bioinformatic target prediction and become entangled in the ever-growing list of prediction tools [85]. Discussing complexity of miRNA target prediction is beyond the scope of this article, so I will only briefly touch upon this topic from the perspective of a common user. Prediction algorithms incorporate in one way or another the presence of the "seed" region in the 3′UTR and inter-species conservation [10,11]. The miRBase lists targets predicted for each miRNA by six different sources and experimentally validated ones [25]. Target prediction distinguishes canonical (conserved), and non-canonical targets. The first category is easier to identify computationally as the prediction can rely on the higher conservation of the miRNA binding site (particularly the seed complementarity). The second category comprises relatively recently acquired miRNA targets that are not commonly suppressed in different species. I suggest to start bioinformatics target prediction by surveying data deposited in the miRBase and then combine it with analysis of putative targets using TargetScan PCT [26] and MIRZA-G-C, which performed well in a recent study [86].

6.2. Target validation

When miRNA transfection or expression is used for target validation, one has to be extremely cautious about data interpretation. Consider the following (common) scenario: miRNA profiling of a biological model system reveals a highly differentially expressed miRNA. Target prediction leads to an interesting gene. Its 3′UTR is used in a luciferase reporter, which is co-transfected with the miRNA and it is shown that the presence of the miRNA leads to repression of the reporter, which is in turn results in the interested gene to be proclaimed a validated miRNA target and the differentially expressed miRNA is implicated in the studied process. What could be wrong?

First, current profiling techniques are extremely sensitive and can quantify truly minuscule expression levels, which may not be physiologically relevant. Thus, when considering normal miRNA-mediated target interaction, one has to consider how well the physiological stoichiometry supports the proposed model. For example, rice miRNAs were reported to reach femtomolar levels in plasma of subjects consuming rice [87]. Regardless whether or not miRNAs from food emerge intact in the bloodstream, such amounts essentially rule out physiologically relevant target repression by normal miRNA function. 10 fM corresponds to approximately 6×10^9 mol/L while a volume of a somatic cell is within picoliters. Thus, there would be (much) less than one miRNA molecule per cell at 10 fM concentration. For comparison, concentrations of siRNAs for inducing efficient and specific RNAi are typically within nanomolar range, some six orders of magnitude higher (e.g. [88]).

Therefore, a good research practice requires insights into the stoichiometry of miRNA and target abundancies. As a rule of a thumb, most abundant miRNAs exert strongest effects on the transcriptome, so a highly abundant cell-specific miRNA is the first best candidate for a physiologically important role while the probability of a strong physiological effect of a miRNA, which constitutes <1% of all cellular miRNAs is small. The argument that rare miRNA can still efficiently repress rare targets is generally invalid because a rare miRNA would bind to all accessible binding sites including those on highly abundant mRNAs, which will effectively outcompete binding sites in rare targets. Therefore, for differential miRNA profiling, I highly recommend not to rely on filtering based just on a relative change. Instead, after obtaining fold-change and *P*-value data for differentially expressed miRNAs, inspect the corresponding MA plot or scatter plot indicating miRNA abundance and focus first on significant changes of the 20–30 most abundant miRNAs. Importantly, at the same time, one has to take into the account also sample heterogeneity where highly abundant, functionally significant miRNAs might have an apparent low

abundance in the studied miRNA population because cells expressing such miRNAs would constitute only a small fraction of the sample.

Second, target validation by using a reporter targeted by over-expressed miRNA is prone to two sources of artifacts yielding false positive results: (i) Hundreds of genes, which are primary targets of an overexpressed miRNA [15,28], may affect expression of many other genes (secondary targets) and one of the targets can affect the reporter by a mechanism independent of direct miRNA interaction with the reporter. Accordingly, one needs to use appropriate controls. It is not enough to monitor a reporter carrying a tested 3′UTR in the presence and absence of a miRNA. Adding and unrelated small RNA does not provide a complete remedy because if the first miRNA is causing an artifact, which affects the reporter, a second control miRNA most likely will not have such an effect. This is an analogy to RNAi off-targeting in RNAi experiments, where each siRNA has its own and specific off-target set of repressed mRNAs [88]. Therefore, a better control for target validation is using a second reporter, in which the predicted miRNA binding site(s) are mutated by point mutations. Such reporter should have the same expression level as the one with the wild-type 3′UTR in the miRNA absence but it should not be repressed in the presence of the miRNA. (ii) Delivering both, the miRNA and the reporter can produce a positive, but non-physiological outcome. In my opinion, a much more convincing result is obtained when one uses a cell expressing physiological levels of the miRNA, titrates levels of the reporter to reveal the range of effective repression by the endogenous miRNA and then uses a miRNA-specific inhibitor and, if being truly meticulous, a target protector.

7. Conclusions and perspectives

miRNAs are ubiquitous regulators implicated in almost every studied biological process in animals. At the same time, not every promising correlation reveals a direct miRNA-mediated repression, not every miRNA has a function, and not every important reprogramming process must involve miRNAs [64,89]. Prudence should accompany the search for biologically significant miRNA-mediated regulations. As a rule of a thumb, such regulation needs to be supported by co-expression and inverse correlation between miRNA and its target levels, experimental validation of miRNA targeting, and evidence that physiological miRNA-mediated effects are responsible for observed biological effect.

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