MicroRNA

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MicroRNAs (miRNAs) are small endogenous RNAs that regulate gene-expression posttranscriptionally. MiRNA research in allergy is expanding because miRNAs are crucial regulators of gene expression and promising candidates for biomarker development. MiRNA mimics and miRNA inhibitors currently in preclinical development have shown promise as novel therapeutic agents. Multiple technological platforms have been developed for miRNA isolation, miRNA quantitation, miRNA profiling, miRNA target detection, and modulating miRNA levels *in vitro* and *in vivo*. Here we will review the major technological platforms with consideration given for the advantages and disadvantages of each platform. (J Allergy Clin Immunol 2018;141:1202-7.)

Key words: *Allergy, antagomiRs, antimiRs, CRISPR, CRISPR/Cas9, gene silencing,* in situ *hybridization, microarray, microRNA, microRNA methods, miRNA, miRNA expression, miRNA inhibitors, miRNA isolation, miRNA mimics, miRNA profiling, miRNA quantitation, miRNA targets, qPCR, stem-loop*

MicroRNAs (miRNAs) are short RNA molecules 19 to 25 nucleotides in size that regulate posttranscriptional silencing of target genes. A single miRNA can target hundreds of mRNAs and influence the expression of many genes often involved in a functional interacting pathway. MiRNAs has been shown to be involved in the pathogenesis of many allergic diseases including asthma, eosinophilic esophagitis, allergic rhinitis, and eczema.[1-5] We refer our readers to references 6 to 9 for further readings on details of miRNA biology.[6-9] This article will review methods used in miRNA isolation, expression level detection, and target identification, as well as potential ways to target miRNAs experimentally and therapeutically.

Abbreviations used
Cas9: CRISPR associated protein 9
CRISPR: Clustered regularly interspaced short palindromic repeats
miRNA: MicroRNA
qPCR: Quantitative PCR
UTR: Untranslated region

MiRNA ISOLATION

MiRNA can be isolated from cells, tissues, and body fluids such as serum, plasma, tears, or urine.[10] The early work published in the field used the traditional phenol-chloroform extraction followed by RNA precipitation (Fig 1, *A*). A widely used reagent is TRIzol (Thermo Fisher Scientific, Waltham, Mass). However, there is often the presence of a high level of contaminants using this method. In addition, it has been found that miRNAs with a low guanine-cytosine content are selectively lost during phenol-chloroform extraction from a low quantity of cells, and this is due to the inefficiency of precipitating small nucleic acids compared with long nucleic acids. Using a column-based RNA adsorption method during miRNA isolation avoids these issues.[11] The initial column-based method involves loading the aqueous phase from the phenol-chloroform extraction on to an RNA adsorption column followed by wash and elution of miRNA (Fig 1, *B*). The mirVana (Thermo Fisher) and miRNEasy (Qiagen, Hilden, Germany) kits are 2 widely used kits for this method. Newer commercially available kits such as the Direct-zol kits (Zymo Research, Irvine, Calif) skip the phase separation step. The phenol reagent containing miRNA can be directly loaded onto an RNA adsorption column followed by wash and elution of miRNA (Fig 1, *B*). Care should be taken to avoid overloading the columns, as overloading significantly reduces RNA yield and quality. Most commercial kits designed for miRNA isolation provide a protocol for isolation of total RNA containing miRNA and an alternative protocol for separation of the small RNA (<200 nucleotides) enriched fraction and the large RNA (>200 nucleotide) fraction. The wash buffer RW1 included in the Qiagen RNEasy total RNA isolation kit washes away all small nucleic acids during the buffer wash step, and therefore this kit is not suitable for miRNA isolation. However, if samples were already lysed in the lysis buffer RLT included in the Qiagen RNEasy kit, modified protocols are available to recapture the miRNA.[12] Isolation of miRNA from body fluids presents additional challenges. The yield of miRNA in body fluids is much lower than that of cells or tissues. A large amount of starting body fluid sample is often needed that exceeds the sample input volume limit of some commercially available kits.[13] With both the miRNEasy serum/plasma kit (Qiagen) and the miRCURY RNA isolation kit (Exiqon), the maximum biofluid sample input is 200 μL. The

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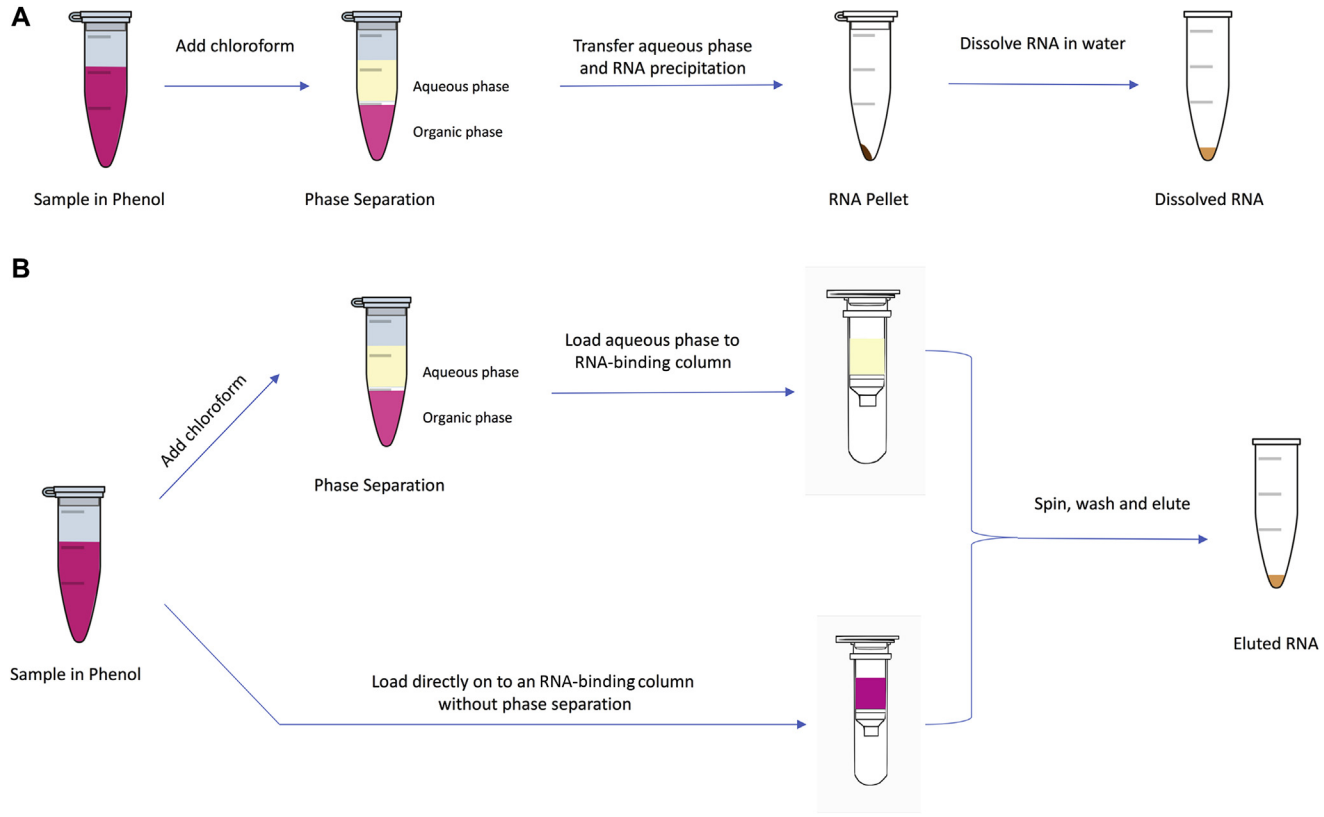

FIG 1. MiRNA extraction methods. **A,** Phenol chloroform-based phase separation followed by pelleting of RNA and redissolving in nuclease-free water. **B,** The aqueous phase from the phenol chloroform can be loaded onto an RNA-adsorbing column. After wash steps, RNA is eluted in nuclease-free water. Alternatively, newer miRNA extraction kits allow the user to skip the phase-separation step. The samples homogenized in phenol can be loaded directed onto an RNA-adsorbing column, followed by wash steps and elution of RNA. A proprietary lysis buffer may be provided by the manufacturer instead of the phenol-based reagent.

mirVana PARIS kit (Thermo Fisher) has been designed to isolate RNA from up to 625 μL of the liquid sample. For larger sample input volume, the QIAmp circulating nucleic acid kit (Qiagen) can be used and enables miRNA isolation from up to 3 mL of starting sample.[13] Care should be taken to specifically use the miRNA protocol and use the lysis buffer without the carrier RNA.

MiRNA EXPRESSION DETECTION

MiRNA expression can be detected in both tissue samples and cell-free biological fluids such as serum or plasma. Current methodologies used for detecting miRNAs include quantitative PCR (qPCR), *in situ* hybridization, microarrays and RNA sequencing. Because the length of miRNA is typically only 21 to 23 base pairs, it is technically challenging to design PCR primers as the conventional PCR primer is about 20 base pairs long. The solution is to extend the length of miRNA during the reverse transcription step by either utilizing a miRNA-specific stem loop primer for transcription (Fig 2, *A*) or universal reverse transcription by adding a 3′poly-A tail to the miRNA and then using a poly-T primer with a universal sequence appended at the 3′ end for reverse transcription (Fig 2, *B*). Subsequently qPCR is performed with forward primer and probe that are specific to each miRNA and the reverse primer complementary to the stem-loop or the universal sequence of the poly-T primer.[14,15] A universal adapter can also be added to the 5′ end to allow an optional universal preamplification prior to qPCR for detection of very low abundance targets with primer/probe (Fig 2, *C*). Compared with the universal reverse transcription, the stem-loop primer-based method has a higher specificity but the reverse transcription step is limited to 1 miRNA at a time.[16] Multiplex stem-loop primer pools are available to overcome this limitation.[17] qPCR of a specific template will typically give 10- to 100-fold higher amplification signal than template with a single nucleotide difference, although distinguishing miRNAs that differ by only 1 to 2 nucleotides with PCR remains a challenge.[18] The microarray-based methods include multiplex qPCR-based arrays and hybridization-based arrays. The qPCR microarrays use pre-plated PCR primer/probes distributed across 96- or 384-well plates. For low amount of input material, a microfluidic card is available that requires as little as 1 ng of total RNA, and microfluidic systems are available that enable single-cell miRNA profiling.[16] A list of available PCR-based arrays can be found in Table I. The hybridization-based arrays have the advantage of allowing a large number of parallel measurements per sample at a relatively low cost. Due to limited specificity, findings from hybridization-based arrays are typically validated with a second method such as qPCR or *in situ* hybridization.[16] *In situ* hybridization has the advantage of determining the tissue origin of the miRNA of interest and the relative expression level across

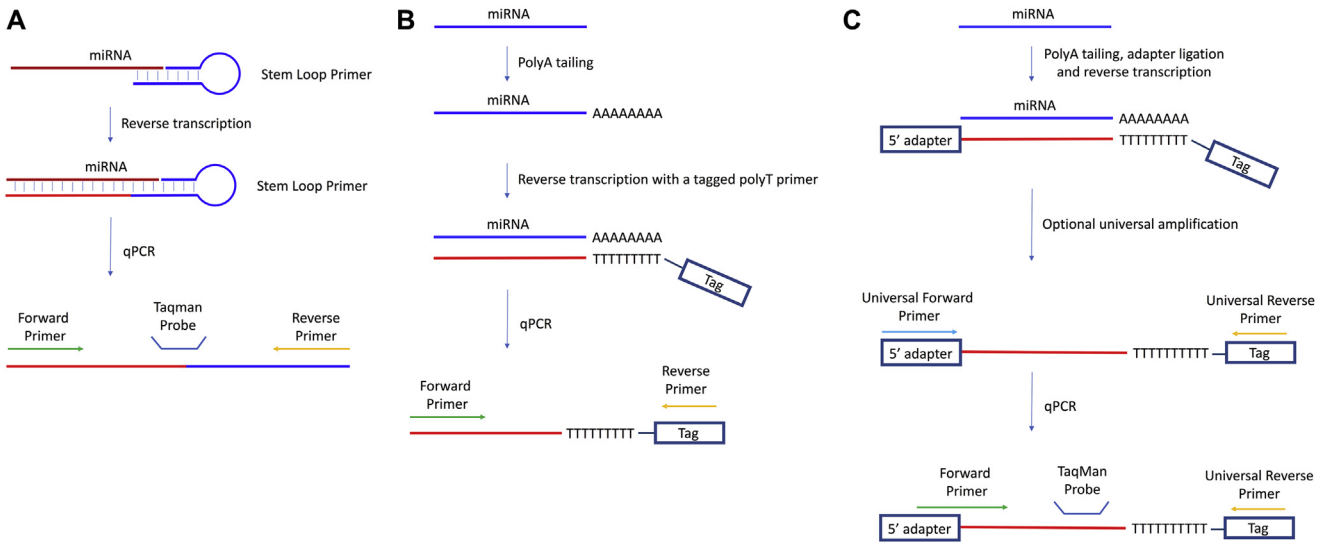

FIG 2. Quantitative RT-PCR for miRNA detection. **A,** Reverse transcription with miRNA-specific stem-loop primer, followed by qPCR using a miRNA-specific probe, a miRNA-specific forward primer *(green)* and reverse primer complementary to the stem-loop sequence *(orange)*. **B,** Universal reverse transcription by adding poly-A tails to 3′ end of miRNA, followed by reverse transcription with a poly-T primer with a universal sequence (Tag) appended at the 3′ end and then qPCR with miRNA-specific forward primer *(green)* and universal reverse primer *(orange)*. **C,** Universal reverse transcription by adding a 5′ adapter sequence and poly-A tails to 3′ end of miRNA, followed by reverse transcription with a poly-T primer with a universal sequence (Tag) appended at the 3′ end, followed by optional universal amplification step with 5′ primer binding to the adapter sequence *(blue)* and 3′ primer binding to the tagged sequence *(orange)*, followed by qPCR with a miRNA-specific probe, a miRNA-specific forward primer *(green)*, and a universal reverse primer *(orange)*.

TABLE I. Major qPCR array platforms used for miRNA profiling

Platform	Vendor	Recommended input amount	Reaction volume
MiScript miRNA PCR arrays	Qiagen	125-250 ng of total RNA	25 μL for 96-well plate, 10 μL for 384-well plate
MiProfile miRNA qPCR arrays	Genecopoeia (Rockville, Md)	500-1000 ng of small RNA	20 μL
TaqMan array human microRNA cards	Thermo Fisher Scientific	1-350 ng of total RNA with preamplification, 350-1000 ng of total RNA without preamplification	1 μL
TaqMan open array microRNA panels	Thermo Fisher Scientific	50-200 ng of total RNA	33 nL
MiRNA ready-to-use PCR panels	Exiqon (Woburn, Mass)	20-40 ng of total RNA	10 μL
MiRNome microRNA profilers	System Biosciences (Palo Alto, Calif)	100-800 ng of total RNA	5 μL
BioMark HD system	Fluidigm (San Francisco, Calif)	20 ng of total RNA, single cell as input when used with the C1 single-cell prep system	5 μL

different tissue compartments, although the dynamic range is limited. Locked nucleic acid probes are frequently used to increase binding affinity and mismatch discrimination.[19,20] Using high throughput sequencing, a small RNA sequencing library can be constructed and sequenced to enable quantitative identification of all small RNA species in a particular sample and to enable discovery of novel miRNAs and other small noncoding RNAs.[16] A starting material of 10 to 50 ng of small RNA is required for the library construction.

TARGET DETECTION

MicroRNAs mediate posttranscriptional gene silencing of target genes by targeting the 3′ untranslated region of mRNA, with the seed region (shown in Fig 3, *A*) in nucleotides 2 to 7 in the 5′ end of miRNA being the crucial sequence. From a transient double-stranded miRNA duplex, the guide strand (mature miRNA strand) is incorporated into the RNA-induced silencing complex to mediate gene silencing and the passenger strand is degraded.[9] The *in silico*-based target prediction methods use a variety of factors including complementarity to seed region of miRNA, conservation through evolution, energetically favorable hybridization of miRNA/mRNA, etc. Several algorithms are available through web interface including Targetscan.org[21-24] and microRNA.org,[25-28] which generate a large number of predicted targets, with many of them presumed to be false targets.[29] The *in silico*-predicted targets can be verified by cloning 3′ untranslated region (UTR) of target genes into the 3′ UTR region of a reporter vector, followed by cotransfection with

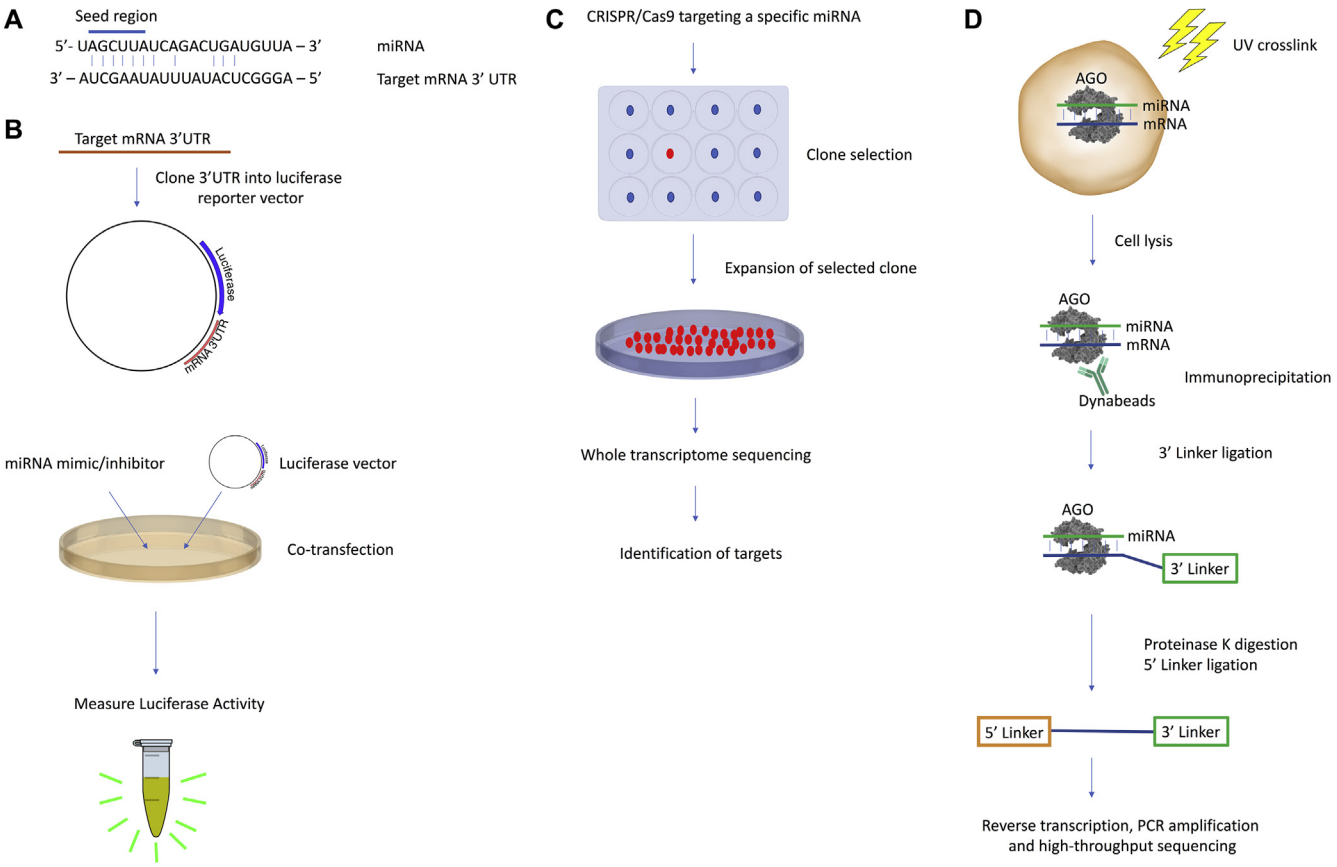

FIG 3. MiRNA target detection. **A,** An illustration of a miRNA/mRNA base pair, highlighting the critical seed region required for miRNA targeting. **B,** Target verification by cloning the 3′ UTR of the target mRNA into a luciferase reporter vector, followed by cotransfection of miRNA mimics/inhibitors and reading the luciferase activity. **C,** Specific miRNAs are targeted by transfecting CRISPR/Cas9 targeting sequence, followed by puromycin selection and single clone isolation. The clone with loss of miRNA expression *(red)* is expanded and whole transcriptome sequencing is performed to identify direct and indirect targets. **D,** Protein/DNA in cells are UV cross-linked, followed by cell lysis and immunoprecipitation of Argonaute protein in the RNA-induced silencing complex. A 3′ linker is ligated, the RNA-induced silencing complex is digested by proteinase K, and a 5′ linker is ligated. This is followed by reverse transcription, PCR amplification and high-throughput sequencing.

targeting miRNA and demonstrating knock-down of reporter activity (Fig 3, *B*). Specificity is demonstrated by mutating the region of target 3′ UTR that binds the seed region of miRNA and demonstrating that the targeting effect no longer exists after the seed region is mutated. Another method is to transiently transfect an miRNA mimic or an miRNA antagonist into the cell of interest, followed by whole transcriptome sequencing to identify both direct and indirect targets.[30,31] MiRNA mimics are double-stranded RNA molecules that imitate the endogenous miRNA duplexes. The transfection of miRNA mimics should be used with caution. It has been reported that transfection of miRNA mimics at low concentrations fails to suppress target gene expression and transfection of miRNA mimics at high concentrations leads to nonspecific changes in gene expression.[32] This may be due to the failure of the guide strand from the transfected miRNA mimics to be incorporated into the RNA-induced silencing complex and the accumulation of high-molecular weight RNA species.[32,33] Significant incorporation of the passenger strand instead of the guide strand is another source for discrepancy.[34] Using plasmid transfection or lentiviral transduction appears to avoid these problems because the biogenesis of miRNA from these methods likely use the same cellular processing pathways as endogenous miRNAs do.[32] The transfection of miRNA inhibitors is less problematic because the miRNA inhibitors do not need to undergo cellular processing. Chemical modifications are incorporated into the miRNA inhibitor to increase its stability and protect it from degradation by endogenous nucleases.[35] MiRNA inhibitor cross-reactivity for closely related miRNA family members has been reported where all family members with closely related sequences were inhibited by a particular miRNA inhibitor.[36] The recent development of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) technology has enabled *in vitro* targeting of the genomic loci that encode the miRNA of interest. Whole transcriptome sequencing can then be performed to identify both the direct and indirect targets of the miRNA of interest (Fig 3, *C*).[37] Advantages of this method include identifying targets in the cell of interest, identifying both direct and indirect targets, and stability of the targeting effect. Some mature miRNAs are

generated from multiple separate loci. These miRNAs represent a challenge for genetic deletion using CRISPR/Cas9 technology because multiple loci have to be targeted simultaneously. The binding of miRNA to mRNA requires a protein complex called RNA-induced silencing complex with the core of the complex containing a miRNA guiding an Argonaute protein to target mRNA. Another method to identify global miRNA-mRNA interaction is to cross-link the RNA-protein complex followed by immunoprecipitation of the RNA-induced silencing complex. The protein is then digested by proteinase K and the remaining RNA is sequenced to identify miRNA-target interaction (Fig 3, *D*).[38]

MODULATING miRNA LEVELS *IN VITRO* AND *IN VIVO*

MiRNA levels can be modulated *in vitro* by using transient transfections of miRNA mimics or miRNA antagonists. The transfection efficiency and the transient nature of the transfection limits the type of functional study that can be performed. Stable clones can be generated *in vitro* by using lentiviral vectors that encode miRNA mimics or miRNA antagonists.[39,40] The promoters driving the expression of miRNAs need to be carefully selected to make sure that the cellular miRNA processing machinery is not overwhelmed by the strong promoter-driven expression of the transduced miRNA. The CRISPR/Cas9 technology enables stable targeting of miRNA without the use of a lentiviral facility. However, clone selection is laborious, and targeting miRNAs generated from multiple loci remains a challenge. Experimentally, gene-targeted mice in which many individual miRNAs are either constitutively or conditionally knocked out have been developed.[41] This enables the study of the function of miRNA in either the animal disease model of interest or the tissue of interest, provided that the miRNA and its binding sites within its target mRNAs are conserved between mice and humans. Another way to modulate miRNA levels *in vivo* is to use cholesterol conjugated antimiRNAs that can be administered via an intravenous infusion using a weight-based regimen, and the antimiRNAs are efficiently up-taken by all tissues except the brain. The knock-down is stable for up to 21 days *in vivo*.[42] Other methods to deliver miRNAs *in vivo* include liposome-mediated delivery or using polymer-based nanoparticle delivery vehicles.[43] Intranasal topical delivery of the miRNA mimics/inhibitors to the lung that requires a much lower dose than systemic delivery has been described, and this can potentially be used in the study or treatment of allergic disease.[44]

SUMMARY

MiRNAs have been shown to regulate multiple allergic diseases and represent an exciting area of research. Several miRNAs have been shown to target key pathogenic pathways involved in allergic inflammation and have the potential to develop into novel therapeutic targets. The methods reviewed will aid researchers who are beginning to explore the field of miRNA in allergic inflammation and help to avoid potential pitfalls involved in miRNA research.

REFERENCES

1. Rebane A. Chapter 17: microRNA and Allergy. MicroRNA: Medical Evidence From Molecular Biology to Clinical Practice. Advances in Experimental Medicine and Biology, volume 888. Switzerland: Springer International Publishing. 2015;pp. 331-52.
2. Dissanayake E, Inoue Y. MicroRNAs in allergic disease. Curr Allergy Asthma Rep 2016;16:67.
3. Lu TX, Rothenberg ME. Diagnostic, functional, and therapeutic roles of microRNA in allergic diseases. J Allergy Clin Immunol 2013;132:3-13; quiz 4.
4. Liu Z, Zhang XH, Callejas-Diaz B, Mullol J. MicroRNA in united airway diseases. Int J Mol Sci 2016;17:pii:E716.
5. Zhang XH, Zhang YN, Liu Z. MicroRNA in chronic rhinosinusitis and allergic rhinitis. Curr Allergy Asthma Rep 2014;14:415.
6. Ha M, Kim VN. Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol 2014;15:509-24.
7. Jonas S, Izaurralde E. Towards a molecular understanding of microRNA-mediated gene silencing. Nat Rev Genet 2015;16:421-33.
8. Gulyaeva LF, Kushlinskiy NE. Regulatory mechanisms of microRNA expression. J Transl Med 2016;14:143.
9. Vishnoi A, Rani S. MiRNA biogenesis and regulation of diseases: an overview. Methods Mol Biol 2017;1509:1-10.
10. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. Clin Chem 2010;56:1733-41.
11. Kim YK, Yeo J, Kim B, Ha M, Kim VN. Short structured RNAs with low GC content are selectively lost during extraction from a small number of cells. Mol Cell 2012;46:893-5.
12. Eldh M, Lotvall J, Malmhall C, Ekstrom K. Importance of RNA isolation methods for analysis of exosomal RNA: evaluation of different methods. Mol Immunol 2012;50:278-86.
13. Moldovan L, Batte KE, Trgovcich J, Wisler J, Marsh CB, Piper M. Methodological challenges in utilizing miRNAs as circulating biomarkers. J Cell Mol Med 2014;18:371-90.
14. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005;33:e179.
15. Shi R, Chiang VL. Facile means for quantifying microRNA expression by real-time PCR. Biotechniques 2005;39:519-25.
16. Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. Nat Rev Genet 2012;13:358-69.
17. Le Carre J, Lamon S, Leger B. Validation of a multiplex reverse transcription and pre-amplification method using TaqMan(®) MicroRNA assays. Front Genet 2014;5:413.
18. Balcells I, Cirera S, Busk PK. Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers. BMC Biotechnol 2011;11:70.
19. Nielsen BS. MicroRNA in situ hybridization. Methods Mol Biol 2012;822:67-84.
20. Kloosterman WP, Wienholds E, de Bruijn E, Kauppinen S, Plasterk RH. In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. Nat Methods 2006;3:27-9.
21. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120:15-20.
22. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009;19:92-105.
23. Shin C, Nam JW, Farh KK, Chiang HR, Shkumatava A, Bartel DP. Expanding the microRNA targeting code: functional sites with centered pairing. Mol Cell 2010;38:789-802.
24. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. Elife 2015;4; https://doi.org/10.7554/eLife.05005.
25. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in Drosophila. Genome Biol 2003;5:R1.
26. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. PLoS Biol 2004;2:e363.
27. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. Nucleic Acids Res 2008;36:D149-53.
28. Betel D, Koppal A, Agius P, Sander C, Leslie C. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. Genome Biol 2010;11:R90.
29. Witkos TM, Koscianska E, Krzyzosiak WJ. Practical aspects of microRNA target prediction. Curr Mol Med 2011;11:93-109.
30. Hodzic J, Sie D, Vermeulen A, van Beusechem VW. Functional screening identifies human miRNAs that modulate adenovirus propagation in prostate cancer cells. Hum Gene Ther 2017;28:766-80.
31. Di Martino MT, Amodio N, Tassone P, Tagliaferri P. Functional analysis of microRNA in multiple myeloma. Methods Mol Biol 2016;1375:181-94.
32. Jin HY, Gonzalez-Martin A, Miletic AV, Lai M, Knight S, Sabouri-Ghomi M, et al. Transfection of microRNA mimics should be used with caution. Front Genet 2015;6:340.
33. Thomson DW, Bracken CP, Szubert JM, Goodall GJ. On measuring miRNAs after transient transfection of mimics or antisense inhibitors. PLoS One 2013;8:e55214.

34. Sokilde R, Newie I, Persson H, Borg A, Rovira C. Passenger strand loading in overexpression experiments using microRNA mimics. RNA Biol 2015;12:787-91.
35. Lennox KA, Behlke MA. Chemical modification and design of anti-miRNA oligonucleotides. Gene Ther 2011;18:1111-20.
36. Robertson B, Dalby AB, Karpilow J, Khvorova A, Leake D, Vermeulen A. Specificity and functionality of microRNA inhibitors. Silence 2010;1:10.
37. Ho TT, Zhou N, Huang J, Koirala P, Xu M, Fung R, et al. Targeting non-coding RNAs with the CRISPR/Cas9 system in human cell lines. Nucleic Acids Res 2015;43:e17.
38. Hausser J, Zavolan M. Identification and consequences of miRNA-target interactions—beyond repression of gene expression. Nat Rev Genet 2014;15: 599-612.
39. Zollner H, Hahn SA, Maghnouj A. Lentiviral overexpression of miRNAs. Methods Mol Biol 2014;1095:177-90.
40. Scherr M, Venturini L, Battmer K, Schaller-Schoenitz M, Schaefer D, Dallmann I, et al. Lentivirus-mediated antagomir expression for specific inhibition of miRNA function. Nucleic Acids Res 2007;35:e149.
41. Park CY, Jeker LT, Carver-Moore K, Oh A, Liu HJ, Cameron R, et al. A resource for the conditional ablation of microRNAs in the mouse. Cell Rep 2012;1:385-91.
42. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature 2005;438:685-9.
43. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 2017;16: 203-22.
44. Takyar S, Vasavada H, Zhang JG, Ahangari F, Niu N, Liu Q, et al. VEGF controls lung Th2 inflammation via the miR-1-Mpl (myeloproliferative leukemia virus oncogene)-P-selectin axis. J Exp Med 2013;210:1993-2010.