CHAPTER 21

MicroRNAs—Basic Biology and Therapeutic Potential

A. Katrina Loomis* and **Graham J. Brock****

Contents

ABBREVIATIONS

3′UTR	3′untranslated region
Ago	Argonaute
BMBC	brain metastatic breast cancer
CLIA	Clinical Laboratory Improvement Amendments
HCV	hepatitis C virus
HITS-CLIP	high-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation
LNA	locked nucleic acid
mRNA	messenger RNA

* Human Genetics and Epigenetics, PharmaTx Precision Medicine, Pfizer, Inc., Groton, Connecticut, USA
** Molecular Profiling, Department of Drug Discovery, Biogen Idec, Inc., Cambridge, Massachusetts, USA

Annual Reports in Medicinal Chemistry, Volume 46
ISSN: 0065-7743, DOI: 10.1016/B978-0-12-386009-5.00006-0

NGS	next-generation sequencing
nt	nucleotide
PARE	parallel analysis of RNA ends
PK	pharmacokinetic
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNA Pol II	RNA polymerase II
TARBP	transactivation-responsive RNA binding protein 2
T_m	melting temperature

1. INTRODUCTION

MicroRNAs are single-stranded RNA molecules, approximately 22 nucleotides (nt) long that play a significant role in the post-transcriptional regulation of gene expression. This chapter outlines what is currently known about microRNAs, recent advances in our understanding, their potential therapeutic uses, and future directions for investigation.

1.1. Background

MicroRNAs were originally discovered in *Caenorhadbitis elegans* [1], and due to their high sequence conservation, their subsequent discovery in many other organisms including mammals was facilitated. MicroRNA genes are frequently located in intronic regions of protein-coding genes but may also be found in intergenic regions of the genome and can occur singly or in clusters. When microRNAs are located in introns, their expression is thought to be co-regulated with that of their host gene. MicroRNA genes are transcribed by RNA polymerase II (RNA Pol II) to form primary microRNAs (pri-miRNAs) which are then capped and polyadenylated [2]. These primary transcripts are subsequently processed into ~70 nt precursor microRNAs (pre-miRNAs) by Drosha, an RNase III endonuclease [3] and exported from the nucleus by Exportin-5 [4] (see Figure 1). Significantly, the ~70 nt pre-microRNA folds into a distinct hairpin conformation, and many microRNA sequences are highly conserved across multiple species. Consequently, to identify other putative microRNAs, many computational methods utilize algorithms to scan the genome for sequences capable of forming these characteristic hairpin structures and to identify sequences that are highly similar if not identical to microRNA sequences from other species. As of 2011, the microRNA registry contained predictions of over 15,000 microRNAs, over a thousand of which are in the human genome [5]. Experimental methods are

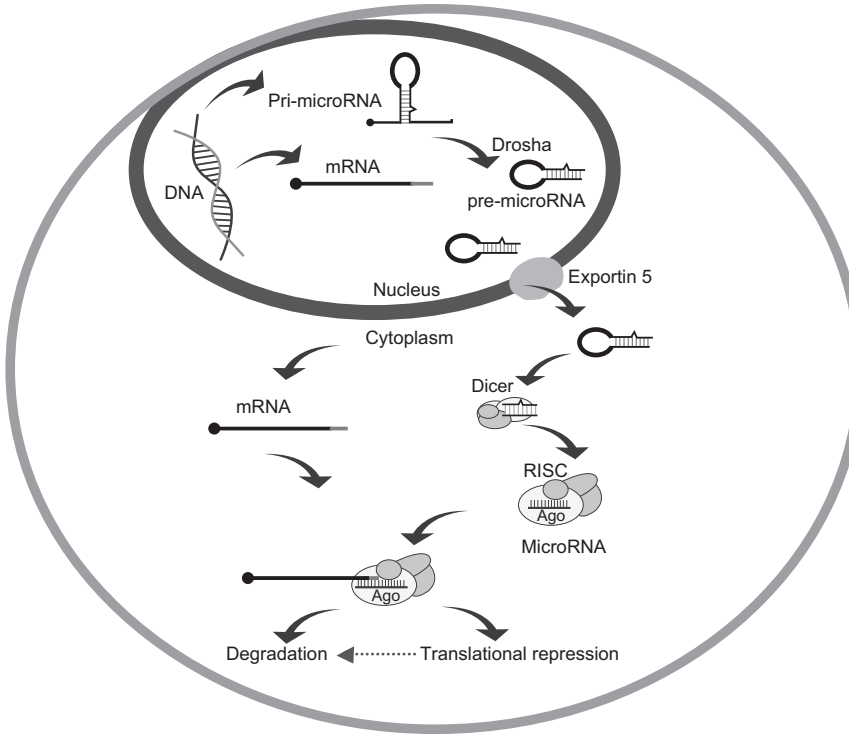


Figure 1 Schematic outline of the main steps in microRNA biogenesis. MicroRNAs are initially synthesized as pri-microRNA transcripts by RNA Pol II in the nucleus. Processing by Drosha results in ~70 nt hairpin pre-microRNA molecules which are exported to the cytoplasm by Exportin 5. Dicer processing leads to the formation of mature microRNAs which are then loaded into the RISC. The passenger strand microRNA is cleaved and degraded, while the guide strand microRNA is retained and used by RISC to identify its target mRNA. Regulation of gene expression is through either translational repression or an mRNA cleavage/degradation mechanism.

needed to confirm the existence of these predicted microRNAs. As with some protein-coding genes, some microRNAs belong to large microRNA families with members differing in sequence by 1 or 2 nt (Table 1).

Once in the cytoplasm, the double-stranded pre-microRNAs are processed by Dicer, another member of the RNase III endonuclease family, into mature ~22 nt microRNAs [3] (Figure 1). This mature double-stranded microRNA species is made up of the guide strand (designated as miR) and its complementary passenger strand (designated as miR*). The regulation of mRNAs by a mature microRNA requires incorporation into the RNA-induced silencing complex (RISC), which comprises multiple proteins including Argonaute, Dicer, and TAR RNA binding proteins (TRBP) (reviewed in Ref. [6]). Following incorporation, the passenger

Table 1 The hsa-let-7 family of microRNAs showing high sequence similarity

MicroRNA	Sequence 5′ to 3′
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU
hsa-let-7b	UGAGGUAGUAGGUUGU**G**U**G**GUU
hsa-let-7c	UGAGGUAGUAGGUUGUAU**G**GUU
hsa-let-7d	**A**GAGGUAGUAGGUUG**C**AUAGUU
hsa-let-7e	UGAGGUAG**G**AGGUUGUAUAGUU
hsa-let-7f	UGAGGUAGUAG**A**UUGUAUAGUU
hsa-let-7g	UGAGGUAGUAG**U**UUGUA**C**AGUU
hsa-let-7i	UGAGGUAGUAG**U**UUGU**GCU**GUU

The seed region (nts 2–7) of this microRNA family is underlined and nucleotides that differ from hsa-let-7a are shown in bold.

microRNA strand (miR*) is degraded and released from the RISC. The relative thermodynamic stability of each end of the double-stranded microRNA species plays a large role in determining which strand becomes the guide strand and is retained, and which strand is degraded [7,8]. The recognition of mRNA targets by the RISC occurs through the guide strand microRNA and is thought to be based on the perfect (or almost perfect) sequence complementarity to the ''seed region,'' defined as nucleotides 2–7 at the 5′end of the microRNA. Previously, suppression of gene expression was thought to result either from cleavage of the targeted mRNA followed by its degradation or from translational repression of the target [9–11]. For the cleavage/degradation mechanism to occur, extensive complementarity between the microRNA and its target is required, whereas translational repression is facilitated primarily through complementarity with the seed region. As the majority of microRNAs do not have extensive matches with their predicted mRNA targets, this suggests translational repression as the main mode of action of microRNAs. However, it has recently been reported that degradation of the mRNA target frequently follows translational repression [12].

The limited complementarity between a miRNA and its target mRNAs (with complementarity focusing on the seed region) has hindered the identification of mRNA targets of microRNA action. Consequently, the function of large numbers of microRNAs remains unknown. Nonetheless, current understanding of their basic mechanism of action has established them as an important class of regulatory molecules, adding a new level of eukaryotic gene regulation at the posttranscriptional level [13,14]. Furthermore, microRNA expression patterns may be altered during the progression of many diseases. Therefore, their potential utility as biomarkers in human diseases has been an area of intense investigation, and a database of reported microRNA disease associations is available [15].

To date, studies of altered microRNA expression patterns have mainly focused on oncology, but recent reports have indicated that microRNA expression can be disrupted in other human diseases as well. Questions surrounding the feasibility of using such molecules as biomarkers relate to the technical challenges inherent in discriminating between such highly similar sequences. Despite these challenges, microRNA signatures have proven to be more robust prognostic markers in oncology than mRNA signatures [16,17].

1.2. Technical considerations when studying microRNA expression

The initial step in a microRNA analysis is usually to undertake genome-wide expression profiling to determine expression levels in various cell types or disease states. This will identify changes in the expression of subsets or even individual microRNAs that can then be evaluated as biomarkers. However, as noted, the relatively short length and highly similar sequence characteristics of microRNAs make profiling technically demanding. Nevertheless, several commercially available platforms with varying degress of sensitivity, specificity, and coverage are available. Recently, the advent of next-generation sequencing (NGS) techniques has offered the potential for validating predicted microRNA sequences [18] as well as quantitating microRNA levels in various cell types [19]. In addition, such sequencing methodologies allow *de novo* identification of microRNAs not previously predicted. However, technical issues remain, such as the potential for bias during construction of the sequencing library [20]. Prior to the advent of NGS, genome-wide analysis methods involved microRNA arrays with hybridization to hundreds or even thousands of probes generated using conventional deoxyribonucleotide or locked nucleic acid (LNA) chemistries [21–24]. Alternatively, although less comprehensive in their coverage, methods that use multiple qPCR primer sets, such as the TaqMan Human MicroRNA Array or a similar system from Roche/Exiqon that uses LNA primers, have been widely used. While the numbers of interrogated microRNAs are currently smaller than most hybridization arrays, these qRT-PCR arrays have increased sensitivity, require considerably less starting material, and/or do not require a preamplification or purification step. Regardless of the method chosen, these techniques all have to address the highly varied melting temperatures (T_m, 45–74 °C) and short sequences (~22 nt) differing by only 1–2 nt found in currently known mature microRNAs. The issue of sequence similarity requires that some array platforms truncate the probes to obtain equivalent T_m between the microRNAs represented on an array. As an alternative, LNA-based arrays add modified nucleotides that increase the thermostability of the RNA/DNA duplexes.

Depending on the number of nucleotides modified, the T_m can be altered by 2–10 °C allowing for a more uniform hybridization temperature across the array. The alternative TaqMan and other qRT-PCR-based methods also have to account for the mature microRNA being only slightly longer than the primer sequence used for amplification. The TaqMan system uses a probe to increase specificity, whereas the Roche/Exiqon method uses shorter LNA-based primer sequences while allowing products to be cloned and verified if needed. Finally, in addition to the issue of similarity, there is the added complication of cross-hybridizing with precursor microRNA species. In the case of arrays, this can be minimized through additional steps to enrich for mature microRNAs while excluding larger pri- and pre-microRNAs.

Recent studies have examined array platforms and compared the results with qRT-PCR and/or NGS and they revealed good intra-platform reproducibility for all technologies [25,26]. However, when differential expression of a subset of microRNAs included in all platforms was examined there was little or no concordance [25]. That there are several potential explanations for these discrepancies (bias due to hybridization or amplification, for example) is perhaps illustrative of the relatively novel nature of the technology and the inherent technical difficulties.

1.3. Mode of action, mRNA target recognition, and outcome

The first microRNA families examined in detail were reported to interact with the 3′UTRs of the mRNAs they regulated [27,28]. In a manner analogous to the development of algorithms for pri-microRNA hairpin identification, algorithms have been developed to identify potential mRNA targets of microRNA interaction [27,29]. These algorithms had to account for the fact that the majority of animal microRNAs (unlike plant microRNAs) lack perfect base-pairing to their mRNA targets. In most cases, the regulation of an mRNA is thought to require an interaction between the 6–8 nt of the microRNA ''seed region'' and the target mRNA sequence, referred to as the ''seed match.'' However, other factors such as the secondary structure of the target mRNA's 3′UTR or synergistic action involving other microRNAs may also be important. Consequently, bioinformatic analysis and prediction of microRNA binding sites may produce high numbers of false-positive predictions. Although various prediction algorithms are predicated upon similar principles, it is not uncommon to find poor agreement in the results obtained from different prediction algorithms (reviewed in Ref. [30]). Such prediction programs may ultimately be improved as a consequence of experimental investigation and a better understanding of microRNA–mRNA interactions. This will enable

more accurate identification of multiple microRNA target interactions, critical to the advancement of the field.

Historically, validation of predicted microRNA–mRNA interaction has involved cell-based experimental techniques, including cotransfection of the microRNA and a reporter construct, which may not accurately reflect biological mechanisms. The validation of these individual putative microRNA–mRNA interactions is time consuming and usually involves luciferase reporter constructs containing the 3′UTR of interest for each target [31]. In many cases, a microRNA can have several hundred predicted mRNA targets. These reporter constructs, which aim to provide evidence that a particular microRNA can downregulate protein synthesis through interaction with the target mRNA, are not definitive. Indeed in some cases, artificially high levels of the transfected microRNA and the use of a fragment of 3′UTR potentially lacking secondary structure may call into question the validity of the results.

The majority of studies involving microRNA function have focused on the 3′UTRs of mRNAs. However, there is emerging evidence that microRNAs may also target coding regions and introns of an mRNA [32,33]. Another dogma for predicting microRNA binding sites, the importance of cross-species conservation, has also been challenged. Recently, several mouse microRNA binding sites have not been found to be conserved in other organisms [32].

Additional evidence suggesting the importance of non-3′UTR microRNA binding sites comes from a study identifying microRNA–mRNA interactions *in vivo*, which found that 25% of the mRNA binding sequences identified in the mouse brain were in coding regions of the mRNA [34]. This study used an alternative method to the widely used reporter construct assays for identification of microRNA–mRNA interactions. The technique is called high-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation (HITS-CLIP) [34]. In this method, Argonaute (an important component of the RISC) is covalently cross-linked to mRNA and immunoprecipitation of these complexes is followed by high-throughput sequencing of the cross-linked RNA species. Bioinformatic analysis is then used to identify microRNAs as well as potential seed matches in mRNAs. While this method does not identify one-to-one relationships for microRNA–mRNA interactions, it is a significant improvement over existing prediction programs. It greatly reduces the number of false positives and restricts potential mRNA target sites to the 45–60 bases of the Argonaute interaction [34].

In cases where microRNAs mediate endonucleolytic cleavage of their target mRNAs, another experimental approach for identifying microRNA–mRNA interactions has been developed. Called Degradome-Seq or parallel analysis of RNA ends (PARE) [35], this technique utilizes high-throughput sequencing and bioinformatics tools to create and

analyze libraries that contain 3′ cleavage products of mRNAs. These cleavage products are a result of Argonaute activity and are unique in that they have a ligation-competent mRNA end with a 5′-phosphate which is not found in other mRNA species. This difference is exploited to create specific libraries of cleavage products. The sequences of these cleavage products are then mapped back to the genome and used to identify matches to known or potential microRNAs [35]. Other methods that identify microRNA–mRNA interactions have also been described using variations of this experimental approach [36–39]. As an alternative strategy to ''Argonaute immunoprecipitation,'' labeled synthetic microRNAs have also been used in pull-down assays to elucidate the mRNA targets of each microRNA [40–42]. Finally, in the tandem affinity purification of microRNA target mRNAs (TAP-Tar) method [43], a two-step procedure is used to isolate microRNA–mRNA complexes from cells expressing a flag-tag version of Argonaute that have been transfected with biotinylated microRNA. The microRNA–mRNA complexes are pulled down with anti-FLAG antibodies and then purified on streptavidin beads. The authors claim that this method reduces the background often seen with single-step pull-down methods.

Recently, an integrated database was developed to facilitate the annotation and analysis of microRNA–mRNA targets that are being identified by some of these techniques. Called starBase (sRNA target Base), this database will document and integrate HITS-CLIP and Degradome-Seq data from several organisms, including humans [44]. At the time of writing, the methods listed above are relatively novel, and rigorous technological evaluation is still required to determine any drawbacks and to identify the most appropriate use for each technique.

2. MICRORNAS IN HUMAN DISEASE

Given the critical role microRNAs are thought to play in the regulation of cell development, division, and survival, it is not surprising that their dysregulation has been associated with human diseases. Genome-wide microRNA and targeted expression profiling experiments have both identified microRNAs with potential involvement in disease. To date, most reports have focused on cancer, for example, leukemias [45], hepatocellular carcinomas [46], melanoma [47], and ovarian cancers [48] (also reviewed in Ref. [49]). The microRNA profiles generated in these studies have been reported to be able to identify a tumor's origin, classify its subtype, and predict survival or response to specific therapies [49,50]. The majority of these studies simply show associations between microRNA levels and diseases, but there are examples where the function of a microRNA has been elucidated and is reported to be involved in the

development or progression of a disease. One such example is miR-95. Its upregulation has been associated with colorectal cancer, and it reportedly functions as an oncogene by increasing cell proliferation via targeting, and thus downregulating, expression of Sorting Nexin 1 [51].

In addition to oncology, recent reports have focused on other areas including diabetes [52], cardiovascular [53,54], and autoimmune diseases. Reports have, for example, suggested a role for miR-375 in type 2 diabetes [55–57], and its expression profile may ultimately serve as a biomarker of this disease [57]. Many studies have reported identifying microRNA profiles associated with various types of cardiac disease [58–61]. Cardiac-expressed microRNAs are sensitive to changes in clinical cardiac status, and studies indicate that these changes may be measurable in peripheral blood, making them potentially valuable biomarkers of heart disease (reviewed in Ref. [53]).

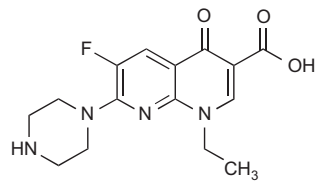
Emerging evidence also indicates a role for microRNAs in a diverse range of autoimmune disorders as well as in the maintenance of normal immune function [62]. Recent reviews have covered developments in lupus [63] and rheumatoid arthritis [64]. The level of miR-181a, which is reportedly important in the regulation of B cell and T cell function [62], is downregulated in pediatric systemic lupus erythematosus patients in comparison to control subjects [65]. Finally, the significance and therapeutic potential of disrupting microRNAs reported to regulate Toll-like receptors (TLRs) was also recently reviewed [66].

One advantage of investigating microRNA profiles as biomarkers or for diagnostic applications is their reported stability in both fresh and archived serum and plasma [67]. The use of microRNA profiles in the field of diagnostics is also arguably preferable to the use of mRNAs due to this stability, especially in samples that are difficult to process. For example, a study has reported that microRNA profiles were superior to mRNA profiles when comparing formalin-fixed, paraffin-embedded (FFPE) samples to fresh malignant melanoma samples [68]. In another analysis, classification of poorly differentiated tumors by the comparatively less complex microRNA profile was reported to be more accurate than classification using mRNA profiles (generated using standard mRNA microarray techniques) when applied to the same samples [69]. Another report claimed that tumor-derived microRNAs in serum or plasma are very stable and may be used as biomarkers in blood-based detection tests for cancer [70]. Indeed, the potential for using circulating microRNAs in cancer detection has resulted in a commercial CLIA (Clinical Laboratory Improvement Amendments) laboratory test offered by Exiqon/Oncotech. This test is reported to be capable of classifying stage II colon cancer patients who may be at significantly higher risk for recurrence and for whom adjuvant chemotherapy may be warranted. This feature of

circulating microRNAs and their potential applications is an area of current intense investigation and is further reviewed in Ref. [67].

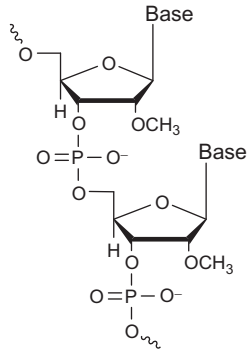
3. MICRORNAS AS POTENTIAL THERAPEUTICS

Because of the strong association of microRNAs with cancer progression and prognosis as well as the regulation of cell proliferation, they have been investigated as potential therapeutics in cancer treatment (reviewed in Ref. [71]). However, from a therapeutic intervention point of view, there is a need to first understand the mechanism by which these microRNAs could be contributing to the disease etiology. For example, the upregulation of microRNAs could result in inhibition of a tumor suppressor gene or a microRNA itself that functions as a tumor suppressor could be downregulated. This gain or loss of microRNA expression could be attributed to gene amplification, transcriptional deregulation, or disruption of epigenetic mechanisms. Any intervention strategy would then have to be tailored to the effect of the microRNA, that is, inhibition of tumor promoters or enhancement of tumor suppressors. Several studies have attempted to restore the expression of downregulated microRNAs. For example, stable expression of miR-1258 in brain metastatic breast cancer (BMBC) cells inhibited the *in vitro* activity of heparanase, a prometastatic enzyme that is overexpressed in BMBC cells and is associated with a highly aggressive cancer phenotype [72]. In another example, expression of miR-16 was reported to be significantly reduced in most prostate tumors compared to normal prostate tissue, and this reduced expression was associated with rapid tumor growth [73]. Systemic delivery of miR-16 using atelocollagen (a highly purified low immunogenicity form of collagen) as a delivery vehicle resulted in restoration of miR-16 expression in a mouse model of bone-metastatic human prostate cancer [73]. Finally, since most cancers reportedly show a decrease in global microRNA expression, a recent study used the fluoroquinolone antibacterial compound enoxacin, **1**, to enhance microRNA biogenesis. The restoration of global microRNA expression was shown to inhibit the growth of several cancer cell types and is supposedly a consequence of enoxacin binding to the TARBP (TAR RNA binding protein 2) [74].

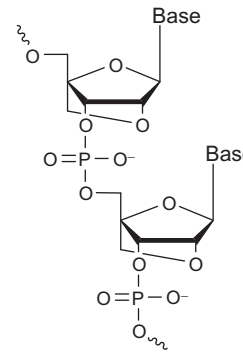


1

Approaches for disrupting the function of aberrantly upregulated microRNAs involve the use of antisense oligonucleotides that are complementary in sequence to the mature microRNAs they are intended to target and inhibit [75,76]. Called antagomirs, these single-stranded oligonucleotides typically incorporate modified nucleosides such as 2′-methoxyribonucleosides or LNAs, **2** and **3,** respectively (below). These chemical modifications increase the thermal stability of the antagomir–microRNA duplex. They also help protect the antagomir from nuclease degradation. Since a single microRNA can potentially have multiple target mRNAs, an antagomir against a single microRNA could potentially increase the expression of multiple genes and increase the activities of multiple associated pathways. Thus, in addition to the issue of antagomir delivery *in vivo*, the potential for producing undesired effects will need to be addressed if antagomirs are to be a safe and effective therapeutic.



2



3

Antagomirs that are complementary to the entire 22 nt microRNA can potentially partially hybridize to multiple microRNAs. Incorporation of LNAs can help address this specificity issue. Since LNA oligonucleotides have reduced conformational flexibility and hybridize to target microRNAs with increased thermal stability [40], LNA-containing antagomirs as short as 15 nt in length are capable of inhibiting microRNA function. Recently, such a 15-nt LNA-containing antagomir targeted against miR-122 was shown to successfully suppress viremia in chimpanzees chronically infected with hepatitis C virus (HCV), demonstrating the feasibility of using this type of microRNA inhibitor as a therapeutic [77]. Since miR-122 is a host microRNA which appears to be essential for HCV infection, it may be an ideal therapeutic target in that viral escape through adaptive mutations is unlikely. The miR-122 LNA antagomir exhibited good pharmacokinetic (PK) and safety profiles in primates in this study [77]. In Phase I clinical studies, the antagomir was well tolerated and

demonstrated dose-dependent pharmacology. It is currently being evaluated in HCV infected patients in Phase II studies.

Another approach reported to inhibit microRNA function in cell lines and transgenic organisms uses microRNA ''sponges.'' These are transcripts expressed from strong promoters that contain multiple tandem binding sites specific for the seed region of the microRNA of interest [78,79]. Sponges can be designed with binding sites that are complementary to the seed region. Alternatively, these binding sites may contain mismatches in the middle of the seed match. Sponge designs with mismatches are thought to be more effective because they reportedly form a more stable interaction with the microRNA [79]. Testing microRNA sponges with 6–18 microRNA binding sites indicated that increasing the number of binding sites above 6 produces a marginal increase in activity [78]. While the approach has potential, it should be noted that because the inhibition is based on the seed match, this method may inhibit activity of whole families of microRNAs which share a common seed region. This could significantly increase any off target effects by disrupting other pathways and/or mRNAs.

4. CONCLUSION

MicroRNAs have a significant regulatory role in post-transcriptional gene expression. While some microRNAs are only required during development, other microRNAs appear to be important for normal cell homeostasis and as such, their dysregulation may contribute to human disease [80–83]. A deeper understanding of their ability to act as regulatory switches which control individual mRNAs and associated pathways is beginning to emerge. However, it should also be noted that this is a relatively novel and rapidly developing field and one for which the rules are still being defined.

The identification of aberrant expression of an individual or small group of microRNAs suggests the potential of microRNAs to serve as biomarkers. MicroRNAs are relatively stable, their expression may not fluctuate as much as that of mRNA molecules, and as they are fewer in number, they present a possibly less complex profile. However, current laboratory techniques for accurately quantitating and discriminating between microRNAs, particularly closely related family members, may need to be improved.

While the importance of microRNAs as a class of regulatory molecules is undisputed, the function of most individual microRNAs remains to be determined. Current techniques for determining microRNA function can be slow and labor intensive. In addition, methods for identifying their putative mRNA targets are fraught with many difficulties and challenges.

Fortunately, these areas are currently the subjects of intense scientific focus, and new techniques such as HITS-CLIP and PARE have been developed. Continued research in these areas will undoubtedly lead to a better understanding of the role of microRNA dysregulation in human disease and facilitate the identification of new gene targets and therapeutic modalities for their treatment.

REFERENCES

[1] R. C. Lee, R. L. Feinbaum and V. Ambros, *Cell*, 1993, **75**, 843.
[2] X. Cai, C. H. Hagedorn and B. R. Cullen, *RNA*, 2004, **10**, 1957.
[3] Y. Lee, C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim and V. N. Kim, *Nature*, 2003, **425**, 415.
[4] R. Yi, Y. Qin, I. G. Macara and B. R. Cullen, *Genes Dev.*, 2003, **17**, 3011.
[5] S. Griffiths-Jones, *Nucleic Acids Res.*, 2004, **32**, 109.
[6] V. N. Kim, J. Han and M. C. Siomi, *Nat. Rev. Mol. Cell Biol.*, 2009, **10**, 126.
[7] A. Khvorova, A. Reynolds and S. D. Jayasena, *Cell*, 2003, **115**, 209.
[8] D. S. Schwarz, G. Hutvagner, T. Du, Z. Xu, N. Aronin and P. D. Zamore, *Cell*, 2003, **115**, 199.
[9] J. G. Doench and P. A. Sharp, *Genes Dev.*, 2004, **18**, 504.
[10] P. H. Olsen and V. Ambros, *Dev. Biol.*, 1999, **216**, 671.
[11] S. M. Hammond, E. Bernstein, D. Beach and G. J. Hannon, *Nature*, 2000, **404**, 293.
[12] H. Guo, N. T. Ingolia, J. S. Weissman and D. P. Bartel, *Nature*, 2010, **466**, 835.
[13] V. Ambros, *Cell*, 2001, **107**, 823.
[14] M. Lagos-Quintana, R. Rauhut, W. Lendeckel and T. Tuschl, *Science*, 2001, **294**, 853.
[15] Q. Jiang, Y. Wang, Y. Hao, L. Juan, M. Teng, X. Zhang, M. Li, G. Wang and Y. Liu, *Nucleic Acids Res.*, 2009, **37**, 98.
[16] M. Jung, A. Schaefer, I. Steiner, C. Kempkensteffen, C. Stephan, A. Erbersdobler and K. Jung, *Clin. Chem.*, 2010, **56**, 998.
[17] Y. Xi, G. Nakajima, E. Gavin, C. G. Morris, K. Kudo, K. Hayashi and J. Ju, *RNA*, 2007, **13**, 1668.
[18] R. D. Morin, M. D. O'Connor, M. Griffith, F. Kuchenbauer, A. Delaney, A. L. Prabhu, Y. Zhao, H. McDonald, T. Zeng, M. Hirst, C. J. Eaves and M. A. Marra, *Genome Res.*, 2008, **18**, 610.
[19] P. Landgraf, M. Rusu, R. Sheridan, A. Sewer, N. Iovino, A. Aravin, S. Pfeffer, A. Rice, A. O. Kamphorst, M. Landthaler, C. Lin, N. D. Socci, L. Hermida, V. Fulci, S. Chiaretti, R. Foa, J. Schliwka, U. Fuchs, A. Novosel, R. U. Muller, B. Schermer, U. Bissels, J. Inman, Q. Phan, M. Chien, D. B. Weir, R. Choksi, G. De Vita, D. Frezzetti, H. I. Trompeter, V. Hornung, G. Teng, G. Hartmann, M. Palkovits, R. Di Lauro, P. Wernet, G. Macino, C. E. Rogler, J. W. Nagle, J. Ju, F. N. Papavasiliou, T. Benzing, P. Lichter, W. Tam, M. J. Brownstein, A. Bosio, A. Borkhardt, J. J. Russo, C. Sander, M. Zavolan and T. Tuschl, *Cell*, 2007, **129**, 1401.
[20] G. Tian, X. Yin, H. Luo, X. Xu, L. Bolund and X. Zhang, *BMC Biotechnol.*, 2010, **10**, 64.
[21] C. G. Liu, G. A. Calin, S. Volinia and C. M. Croce, *Nat. Protoc.*, 2008, **3**, 563.
[22] M. Castoldi, S. Schmidt, V. Benes, M. W. Hentze and M. U. Muckenthaler, *Nat. Protoc.*, 2008, **3**, 321.
[23] J. Q. Yin, R. C. Zhao and K. V. Morris, *Trends Biotechnol.*, 2008, **26**, 70.
[24] P. T. Nelson, D. A. Baldwin, L. M. Scearce, J. C. Oberholtzer, J. W. Tobias and Z. Mourelatos, *Nat. Methods*, 2004, **1**, 155.

[25] A. Git, H. Dvinge, M. Salmon-Divon, M. Osborne, C. Kutter, J. Hadfield, P. Bertone and C. Caldas, *RNA*, 2010, **16**, 991.
[26] Y. Chen, J. A. Gelfond, L. M. McManus and P. K. Shireman, *BMC Genomics*, 2009, **10**, 407.
[27] B. P. Lewis, I. H. Shih, M. W. Jones-Rhoades, D. P. Bartel and C. B. Burge, *Cell*, 2003, **115**, 787.
[28] M. C. Vella, K. Reinert and F. J. Slack, *Chem. Biol.*, 2004, **11**, 1619.
[29] M. Kiriakidou, P. T. Nelson, A. Kouranov, P. Fitziev, C. Bouyioukos, Z. Mourelatos and A. Hatzigeorgiou, *Genes Dev.*, 2004, **18**, 1165.
[30] P. Alexiou, M. Maragkakis, G. L. Papadopoulos, M. Reczko and A. G. Hatzigeorgiou, *Bioinformatics*, 2009, **25**, 3049.
[31] J. L. Clancy, M. Nousch, D. T. Humphreys, B. J. Westman, T. H. Beilharz and T. Preiss, *Methods Enzymol.*, 2007, **431**, 83.
[32] Y. Tay, J. Zhang, A. M. Thomson, B. Lim and I. Rigoutsos, *Nature*, 2008, **455**, 1124.
[33] J. R. Lytle, T. A. Yario and J. A. Steitz, *Proc. Natl. Acad. Sci. USA*, 2007, **104**, 9667.
[34] S. W. Chi, J. B. Zang, A. Mele and R. B. Darnell, *Nature*, 2009, **460**, 479.
[35] M. A. German, M. Pillay, D. H. Jeong, A. Hetawal, S. Luo, P. Janardhanan, V. Kannan, L. A. Rymarquis, K. Nobuta, R. German, E. De Paoli, C. Lu, G. Schroth, B. C. Meyers and P. J. Green, *Nat. Biotechnol.*, 2008, **26**, 941.
[36] R. Jain, T. Devine, A. D. George, S. V. Chittur, T. E. Baroni, L. O. Penalva and S. A. Tenenbaum, *Methods Mol. Biol.*, 2011, **703**, 247.
[37] G. Malterer, L. Dolken and J. Haas, *RNA Biol.*, 2011 8.
[38] L. P. Tan, E. Seinen, G. Duns, D. de Jong, O. C. Sibon, S. Poppema, B. J. Kroesen, K. Kok and A. van den Berg, *Nucleic Acids Res.*, 2009, **37**, 137.
[39] F. V. Karginov, C. Conaco, Z. Xuan, B. H. Schmidt, J. S. Parker, G. Mandel and G. J. Hannon, *Proc. Natl. Acad. Sci. USA*, 2007, **104**, 19291.
[40] U. A. Orom and A. H. Lund, *Methods*, 2007, **43**, 162.
[41] W. Zheng, H. W. Zou, Y. G. Tan and W. S. Cai, *Mol. Biotechnol.*, 2011, **47**, 200.
[42] R. J. Hsu, H. J. Yang and H. J. Tsai, *Nucleic Acids Res.*, 2009, **37**, 77.
[43] N. Nonne, M. Ameyar-Zazoua, M. Souidi and A. Harel-Bellan, *Nucleic Acids Res.*, 2010, **38**, 20.
[44] J. H. Yang, J. H. Li, P. Shao, H. Zhou, Y. Q. Chen and L. H. Qu, *Nucleic Acids Res.*, 2011, **39**, 202.
[45] G. A. Calin, C. G. Liu, C. Sevignani, M. Ferracin, N. Felli, C. D. Dumitru, M. Shimizu, A. Cimmino, S. Zupo, M. Dono, M. L. Dell'Aquila, H. Alder, L. Rassenti, T. J. Kipps, F. Bullrich, M. Negrini and C. M. Croce, *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 11755.
[46] Y. Murakami, T. Yasuda, K. Saigo, T. Urashima, H. Toyoda, T. Okanoue and K. Shimotohno, *Oncogene*, 2006, **25**, 2537.
[47] D. W. Mueller and A. K. Bosserhoff, *Br. J. Cancer*, 2009, **101**, 551.
[48] R. Eitan, M. Kushnir, G. Lithwick-Yanai, M. B. David, M. Hoshen, M. Glezerman, M. Hod, G. Sabah, S. Rosenwald and H. Levavi, *Gynecol. Oncol.*, 2009, **114**, 253.
[49] E. Chan, D. E. Prado and J. B. Weidhaas, *Trends Mol. Med.*, 2011, **17**, 235.
[50] C. A. Andorfer, B. M. Necela, E. A. Thompson and E. A. Perez, *Trends Mol. Med.*, 2011, **17**, 313.
[51] Z. Huang, S. Huang, Q. Wang, L. Liang, S. Ni, L. Wang, W. Sheng, X. He and X. Du, *Cancer Res.*, 2011, **71**, 2582.
[52] D. Ferland-McCollough, S. E. Ozanne, K. Siddle, A. E. Willis and M. Bushell, *Biochem. Soc. Trans.*, 2010, **38**, 1565.
[53] G. W. Dorn 2nd, *Curr. Cardiol. Rep.*, 2010, **12**, 209.
[54] J. Ai, R. Zhang, Y. Li, J. Pu, Y. Lu, J. Jiao, K. Li, B. Yu, Z. Li, R. Wang, L. Wang, Q. Li, N. Wang, H. Shan and B. Yang, *Biochem. Biophys. Res. Commun.*, 2010, **391**, 73.
[55] M. N. Poy, J. Hausser, M. Trajkovski, M. Braun, S. Collins, P. Rorsman, M. Zavolan and M. Stoffel, *Proc. Natl. Acad. Sci. USA*, 2009, **106**, 5813.

[56] Y. Li, X. Xu, Y. Liang, S. Liu, H. Xiao, F. Li, H. Cheng and Z. Fu, *Int. J. Clin. Exp. Pathol.*, 2010, **3**, 254.
[57] H. Zhao, J. Guan, H. M. Lee, Y. Sui, L. He, J. J. Siu, P. P. Tse, P. C. Tong, F. M. Lai and J. C. Chan, *Pancreas*, 2010, **39**, 843.
[58] S. Ikeda, S. W. Kong, J. Lu, E. Bisping, H. Zhang, P. D. Allen, T. R. Golub, B. Pieske and W. T. Pu, *Physiol. Genomics*, 2007, **31**, 367.
[59] C. Sucharov, M. R. Bristow and J. D. Port, *J. Mol. Cell. Cardiol.*, 2008, **45**, 185.
[60] S. V. Naga Prasad, Z. H. Duan, M. K. Gupta, V. S. Surampudi, S. Volinia, G. A. Calin, C. G. Liu, A. Kotwal, C. S. Moravec, R. C. Starling, D. M. Perez, S. Sen, Q. Wu, E. F. Plow, C. M. Croce and S. Karnik, *J. Biol. Chem.*, 2009, **284**, 27487.
[61] S. J. Matkovich, D. J. Van Booven, K. A. Youker, G. Torre-Amione, A. Diwan, W. H. Eschenbacher, L. E. Dorn, M. A. Watson, K. B. Margulies and G. W. Dorn 2nd, *Circulation*, 2009, **119**, 1263.
[62] E. Sonkoly, M. Stahle and A. Pivarcsi, *Semin. Cancer Biol.*, 2008, **18**, 131.
[63] R. J. Rigby and C. G. Vinuesa, *Curr. Opin. Rheumatol.*, 2008, **20**, 526.
[64] V. Furer, J. D. Greenberg, M. Attur, S. B. Abramson and M. H. Pillinger, *Clin. Immunol.*, 2010, **136**, 1.
[65] Y. A. Lashine, A. M. Seoudi, S. Salah and A. I. Abdelaziz, *Clin. Exp. Rheumatol.*, 2011, **10**, 41.
[66] E. J. Hennessy, A. E. Parker and L. A. O'Neill, *Nat. Rev. Drug Discov.*, 2010, **9**, 293.
[67] G. Reid, M. B. Kirschner and N. van Zandwijk, *Crit. Rev. Oncol. Hematol.*, Epub Dec, 2010, in press.
[68] A. Liu, M. T. Tetzlaff, P. Vanbelle, D. Elder, M. Feldman, J. W. Tobias, A. R. Sepulveda and X. Xu, *Int. J. Clin. Exp. Pathol.*, 2009, **2**, 519.
[69] J. Lu, G. Getz, E. A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B. L. Ebert, R. H. Mak, A. A. Ferrando, J. R. Downing, T. Jacks, H. R. Horvitz and T. R. Golub, *Nature*, 2005, **435**, 834.
[70] P. S. Mitchell, R. K. Parkin, E. M. Kroh, B. R. Fritz, S. K. Wyman, E. L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K. C. O'Briant, A. Allen, D. W. Lin, N. Urban, C. W. Drescher, B. S. Knudsen, D. L. Stirewalt, R. Gentleman, R. L. Vessella, P. S. Nelson, D. B. Martin and M. Tewari, *Proc. Natl. Acad. Sci. USA*, 2008, **105**, 10513.
[71] M. Angelica Cortez, C. Ivan, P. Zhou, X. Wu, M. Ivan and G. A. Calin, *Adv. Cancer Res.*, 2010, **108**, 113.
[72] L. Zhang, P. S. Sullivan, J. C. Goodman, P. H. Gunaratne and D. Marchetti, *Cancer Res.*, 2011, **71**, 645.
[73] F. Takeshita, L. Patrawala, M. Osaki, R. U. Takahashi, Y. Yamamoto, N. Kosaka, M. Kawamata, K. Kelnar, A. G. Bader, D. Brown and T. Ochiya, *Mol. Ther.*, 2010, **18**, 181.
[74] S. Melo, A. Villanueva, C. Moutinho, V. Davalos, R. Spizzo, C. Ivan, S. Rossi, F. Setien, O. Casanovas, L. Simo-Riudalbas, J. Carmona, J. Carrere, A. Vidal, A. Aytes, S. Puertas, S. Ropero, R. Kalluri, C. M. Croce, G. A. Calin and M. Esteller, *Proc. Natl. Acad. Sci. USA*, 2011, **108**, 4394.
[75] J. Krutzfeldt, N. Rajewsky, R. Braich, K. G. Rajeev, T. Tuschl, M. Manoharan and M. Stoffel, *Nature*, 2005, **438**, 685.
[76] M. Yang and J. Mattes, *Pharmacol. Ther.*, 2008, **117**, 94.
[77] R. E. Lanford, E. S. Hildebrandt-Eriksen, A. Petri, R. Persson, M. Lindow, M. E. Munk, S. Kauppinen and H. Orum, *Science*, 2010, **327**, 198.
[78] M. S. Ebert, J. R. Neilson and P. A. Sharp, *Nat. Methods*, 2007, **4**, 721.
[79] M. S. Ebert and P. A. Sharp, *RNA*, 2010, **16**, 2043.
[80] J. T. Mendell, *Cell Cycle*, 2005, **4**, 1179.
[81] J. S. Mattick and I. V. Makunin, *Hum. Mol. Genet.*, 2005, **14**(Spec. No. 1), 121.
[82] A. M. Krichevsky, K. S. King, C. P. Donahue, K. Khrapko and K. S. Kosik, *RNA*, 2003, **9**, 1274.
[83] I. Alvarez-Garcia and E. A. Miska, *Development*, 2005, **132**, 4653.