Review Article

A Potential of microRNAs for High-Content Screening

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In the last years miRNAs have increasingly been recognised as potent posttranscriptional regulators of gene expression. Possibly, miRNAs exert their action on virtually any biological process by simultaneous regulation of numerous genes. The importance of miRNA-based regulation in health and disease has inspired research to investigate diverse aspects of miRNA origin, biogenesis, and function. Despite the recent rapid accumulation of experimental data, and the emergence of functional models, the complexity of miRNA-based regulation is still far from being well understood. In particular, we lack comprehensive knowledge as to which cellular processes are regulated by which miRNAs, and, furthermore, how temporal and spatial interactions of miRNAs to their targets occur. Results from large-scale functional analyses have immense potential to address these questions. In this review, we discuss the latest progress in application of high-content and high-throughput functional analysis for the systematic elucidation of the biological roles of miRNAs.

1. Introduction

miRNAs (microRNAs) are 17-nt to 24-nt long noncoding RNAs that regulate gene expression in metazoans. miRNAs act by partially or completely complementary binding to their target mRNAs, resulting in translational repression and/or mRNA degradation [1, 2]. miRNAs are predicted to affect the expression of nearly 60% of protein-coding mammalian genes [3, 4] and, thereby, to control many, if not all, biological processes. Fundamental changes at the cellular and organismal level, including development [5], aging [6], the stress response [7], cell proliferation [8, 9], and apoptosis [10, 11], were shown to be regulated by miRNAs. Furthermore, miRNAs have been implicated in various diseases, such as diabetes [12–14], cancer [15, 16], hepatitis C [17], neurodevelopmental (reviewed in [18]), and mental [19] disorders. Rapidly growing knowledge of miRNAs as potent regulators in health and disease makes miRNAs attractive as targets for therapeutic intervention [20, 21] as well as for diagnostic markers [22, 23].

Numerous previous publications have addressed miRNA biogenesis and action (for detailed reviews see [24, 25]). Briefly, miRNAs are transcribed as long primary transcripts (pri-miRNAs), most of which are polyadenylated and capped. Pri-miRNAs are initially cleaved in nucleus by a multiprotein complex, called Microprocessor, yielding ~70-nt long stem-loop structured precursor miRNAs (pre-miRNAs). The key components of the Microprocessor complex are the RNase III enzyme Drosha and the double-stranded RNA-binding protein DGCR8/Pasha [26]. The excised pre-miRNA hairpin is then exported to the cytoplasm by Exportin-5 complexed with Ran-GTPase [27]. In the cytoplasm, the pre-miRNA is further processed to a 20–22-nt long miRNA/miRNA* duplex by a second RNase III enzyme, Dicer, which is in a complex with the TRBP and PACT proteins [28, 29]. Subsequently, the miRNA duplex is unwound by multiple helicases, which may be miRNA-specific and may regulate miRNA activity [30, 31]. The mRNA-targeting miRNA strand (guide strand) is loaded into the miRNA-induced silencing complex (miRISC). Until recently, it was assumed that the complementary miRNA* strand (passenger strand) is degraded, but there is now evidence that a substantial cohort of miRNA* species is functionally active [32]. The core components of miRISC are proteins of the Argonaute (AGO) [33] and GW182 protein families [34]. Individual miRNAs might need specific maturation steps [35–37].

Once incorporated into miRISC, the miRNA brings the complex to its target mRNAs by interacting with complementary binding sites, which can be present in multiple

copies [38–40]. Each miRNA can usually affect more than one transcript and, as a consequence, many proteins simultaneously [41, 42]. On the other hand, multiple miRNAs can repress expression of a single target mRNA [43–46]. miRNAs are postulated to preferentially bind to the 3′ untranslated regions (3′UTRs) of transcripts [47]. However, recent experimental evidences prove the existence of a new class of miRNA targets containing miRNA binding sites in both their 5′UTR and 3′UTR [48] or within the coding region of mRNA [49].

The complexity of miRNA-mediated modulation of gene expression is only beginning to be appreciated, and much research needs to be done in order to understand miRNA global and adaptive regulatory functions. In this review, we summarize available methodologies for modulating expression levels of endogenous miRNAs, as well as on the application of these strategies for high-content and high-throughput functional studies.

2. RNA Silencing

The discovery that small ncRNAs (noncoding RNAs) play pivotal roles in fundamental biological processes has considerably widened our knowledge of mechanisms of gene regulation in the last years [50, 51]. siRNAs (small interfering RNAs) and miRNAs are the two best characterized classes of ncRNAs. Both are derived from dsRNA (double-stranded RNA) precursors and exert their inhibitory function on gene expression by Watson-Crick base-pairing to complementary sequences in target RNA molecules: an effect commonly referred to as gene silencing [2]. Moreover, both siRNAs and miRNAs share some components of the cellular effector machinery involved in gene silencing [52]. Usually, miRNAs modify expression of endogenous genes whereas siRNAs have evolved to defend genome integrity against foreign invaders, like viruses or transposons [53]. In mammals siRNAs are 21-22 nt long fragments often, but not always, derived from foreign dsRNA by Dicer [52, 54]. siRNAs are incorporated into the siRNA-induced silencing complex (siRISC) [55] and bind to perfectly matched sequences in target molecules. Typically, this induces degradation of the bound RNA, a function called RNAi (RNA interference) [53], and this property was widely implicated in functional studies over the last decade (see what follows).

Binding of miRNA to perfectly matched sequences in mRNA can also result in degradation of the mRNA [56], but usually, miRNAs bind to sites in mRNAs with only partial sequence complementarity. This results primarily in translational repression rather than degradation [57] but can also cause secondary nucleolytic degradation of the mRNA [24]. Conversely, binding of siRNAs to partly unmatched sequences in mRNAs can result in translational blockade-potentially interfering with the results from siRNA-based screening experiments [56].

3. siRNA/shRNA-Based Screens

In the last years RNAi has developed into a powerful tool for systematic studies of fundamental physiological and pathological processes. A number of large-scale screens have been completed, analysing diverse cellular processes. Recent impressive examples of genome-wide RNAi-based screens in human cells are provided by the work of Collinet et al. [58], who performed a high-content survey of genes involved in endocytosis, and by the study of Neumann et al., who used time-lapse microscopy in living cells to identify genes that play a role in cell division [59].

siRNA-based screens have also been performed in several model organisms. *C. elegans* and Drosophila are especially receptive to this type of genetic screening [60]. In *C. elegans*, for example, gene silencing can be accomplished by feeding bacteria that express long dsRNA (about 200–2000-bp-long) or by providing such dsRNA in the medium [61, 62]. RNAi in cultured cells of Drosophila can similarly be induced by adding *in vitro* transcribed dsRNA to the culture medium [63]. In mammalian cells, in contrast, short RNA duplexes of 21–29 bp have to be administered, because long dsRNA evokes a response of the innate immune system, which ultimately leads to apoptosis [64]. The short siRNAs can be chemically synthesized or derived from transcribed PCR products by digestion with recombinant Dicer or bacterial RNase III (esiRNAs-endoribonuclease-prepared short interfering RNAs) [64, 65]. siRNA-mediated knock-down can attain close to 100% reduction in the target mRNA. However, the effectiveness of an individual siRNA is hard to predict and, therefore, several siRNAs targeting different regions of the target mRNA have to be tested. Some authors suggest checking 4–6 siRNAs per gene to obtain reliable results in a screening experiment [66].

If stable knock-down is needed or if cells are difficult to transfect, short hairpin RNAs (shRNAs) can be used. The sequences encoding the shRNA can be cloned into plasmids or virus-derived vectors (lenti-, retro-, or adenoviral origin) [67–69]. In the case of "second generation" shRNAs, so called shRNA-miRs, the RNAi-triggering small RNA sequence is cloned into the backbone of a pri-miRNA [70]. This design principle, together with improved selection of the small RNA targeting sequence, improves both the production levels of small RNAs and the silencing efficiency. shRNA-miRs can be cloned into constructs carrying different promoters and used for tissue-specific or inducible expression [70].

4. Modulation of miRNA Function as an Approach for Functional Screening

Clearly, their biological importance *per se* makes analyses of miRNA expression and identification of their target mRNAs a focus of the current research. Yet specific features of miRNAs give them strong potential as tools in functional genomics. Firstly, miRNAs are able to change the translation of hundreds of mRNAs simultaneously and, by doing so, add another layer of regulation to gene expression [71]. In fact, miRNAs can influence the whole biological programs, including development, apoptosis, proliferation, and differentiation, not only through direct interactions with target mRNAs but also indirectly by altering expression of, for example, components of the translation or RNAi machinery [42].

In general, miRNAs effect only subtle modulation of target gene expression. Their pronounced effect on cellular behaviour might, therefore, be a consequence of their ability to influence multiple genes involved in a single pathway. Indeed, miRNA-based screens can provide lists enriched in functionally related targets: for example, a recent RNAi screen identified three target genes that phenocopy miR-19 and cooperate in the regulation of phosphatidylinositol-3 kinase-mediated survival signalling [72]. miRNA-based screens and, especially, a combination of miRNA- and siRNA-based screens, though laborious, might have a clear advantage over siRNA-based screens alone, since with siRNAs, downregulation of only single individual mRNAs is expected. On the other hand, enrichment of target genes with specific functions is not always found using current bioinformatic prediction tools [73]; therefore, the potential to organise hits of miRNA-based screens directly into functional networks is as yet unproven. The fact that a single mRNA can be inhibited by different miRNAs [74] also means that cellular phenotypes result from synergistic effects, complicating interpretation. Having an infrastructure for large-scale experimentation, complex and previously unanticipated regulatory patterns might be identified.

In contrast to the vast number of mRNAs that have to be knocked-down in comprehensive functional genomic approaches using siRNAs/shRNAs, the number of miRNAs that have to be analysed in a genome-wide screen is relatively low. To date, 1424 miRNAs have been identified in the human genome (miRBase database, release 17; http://www.mirbase.org/). Various *in silico* methods, developed to predict targets of miRNAs, estimate that between 10% and 60% of all mRNAs might be influenced by miRNAs [3, 4, 75, 76]. Hence, the analysis of the comparatively small number of miRNAs could, in principle, cover the function of substantial fraction of human genes. To modulate the expression of the same number of genes by siRNA/shRNA would require about 44,000 to 66,000 different siRNA/shRNAs, taking into account the need for multiple reagents targeting the same mRNA (see above).

5. Tools to Modulate miRNA Function

Here, we will briefly describe approaches that are currently in use to modulate miRNA function and discuss their potential and limitations (Table 1). Basically, it is possible either to interfere with miRNA expression (loss-of-function assays) or to induce ectopic (over-) expression of miRNA (gain-of-function).

One possibility for analysis of miRNA would be a complete knock-out. This very precise intervention will result in a complete loss of function. The resulting phenotypes are, therefore, often stronger than those seen after knock-down. For some time, generation of knock-out phenotypes was feasible in mammals only by exploiting *homologous recombination* technology in mice [77]. More recently, germline competent embryonic stem cells have also been established in rat, so that homologous recombination can now be performed in this species as well [78]. The development of genome editing approaches, using engineered *zinc finger nucleases*, opened up the possibility to extend such analyses to other organisms and tissue culture cells [79]. Still, a gene knock-out is laborious and, if the work is given to a contractor, expensive. Many miRNAs are located in introns of protein coding genes; so knock-out of a miRNA can result in simultaneous inactivation of the "host" protein coding gene with potentially detrimental effects that are unrelated to miRNA action. Finally, as discussed below, it is often desirable to downregulate the function of several miRNAs together, which is not feasible through knock-out. Interference with multiple miRNAs that are not synthesized from a single polycistronic gene is much easier to achieve using a knock-down approach.

The knock-down of miRNAs can be performed by application of *antisense oligonucleotides (ASOs)*. Usually, ASOs exert their effects independently of the cellular silencing machinery: their potential in biological assays was described as early as 1978 [80]. Since then, lot of experience has been gained in the design of stable, specific, and potent antisense reagents for research and therapeutic use. Since unmodified oligonucleotides are quickly degraded by nucleases when administered to cells, chemical modification is necessary to enhance stability and potency. (For a review on chemical modification used in antisense approaches, see [81].) Inactivation of complementary RNA can be achieved, for example, by delivery of DNA oligonucleotides. RNA/DNA hybrids are then recognized and cleaved by RNase H [82]. Alternatively, oligonucleotides can be coupled to ribozymes or DNAzymes that inactivate bound RNA by cleavage [81]. For the inactivation of miRNAs, RNA-based oligonucleotides that do not carry enzymatic activity have gained popularity. It is not clear whether these oligonucleotides work by inducing degradation of the targeted miRNA [83] or by forming stable ASO/miRNA heteroduplexes and, thereby, blocking miRNA function [84, 85].

ASOs and ribozymes that interfere with miRNA function are commonly referred to as antagomiRs and antagomiRzymes, respectively, and can be obtained from commercial sources. In addition, plasmid- and virus-derived vectors exist that both allow for simultaneous and permanent expression of antogomiRs targeting different miRNAs and permit measurement of expression levels by coexpression of a fluorescent reporter protein [86].

In principle, miRNA function can be inhibited at different stages [87]. For example, ASOs have been used to interfere with the maturation of pri-miRNA [88]. Targeted degradation of pri-miRNAs in the nucleus with RNase H-based ASOs has also been tried [87]. Strategies to target pri-miRNAs could have the advantage of combined inhibition of several miRNAs transcribed from the same polycistronic gene locus. Experiments with siRNAs targeting the loop region of pre-miRNA have been reported [89], but the approach has not acquired much popularity: the loop region might be difficult to access, leading to inefficient knock-down of miRNA [87].

With regard to knock-down of protein-coding mRNAs, siRNA are nowadays in much wider use than ASOs, probably because siRNAs provide a more potent and efficient mode

TABLE 1: Ways to analyse function of miRNA.

Aim	Method	Features	
		Pros	Cons
Knock-out of miRNA gene	Homologous recombination/Gene editing with zinc finger nucleases	(i) Precise intervention (ii) Complete loss-of-function	(i) Laborious and time consuming (ii) Simultaneous knock-out of protein encoded by the same transcriptional unit
Knock-down of miRNA	Antisense oligonucleotide Ribozymes/DNAzymes	(i) Can attack mature miRNA and miRNA precursors (ii) Stable expression from vector-constructs (iii) Commercially available (iv) Transfection/transduction of multiple oligonucleotides is possible	(i) OTEs and unspecific secondary effects may occur (ii) Efficiency of knock-down is difficult to predict (iii) Multiple knock-downs might be needed to produce a phenotype
	miRNA sponges	(i) Easy design (ii) Simultaneous knock-down of multiple miRNAs (iii) Expression can be verified by fluorescent reporter	(i) Efficiency of knock-down is difficult to predict (ii) Incomplete knock-down of individual miRNAs
Over-expression of miRNA	miRNA mimics	(i) Efficient silencing of target mRNA (ii) Oligonucleotides mimicking mature miRNAs or miRNA precursors could be used (iii) Transfection/transduction of multiple mimics is possible (iv) Stable transfection from vector-constructs	(i) Oversaturation of RNAi machinery can lead to secondary effects
	Conditional release of miRNA from riboswitch constructs	(i) Controlled expression of mature miRNA (ii) Possibly less toxic side effects	(i) Laborious design (ii) Lack of availability (iii) Not yet adaptable to high-throughput analysis
Release of target mRNA from repression by miRNA	Target protectors	(i) Release of specific mRNA from regulation by miRNA possible	(i) Target mRNA has to be known

of intervention. In some model organisms, like zebrafish, however, a certain class of ASOs, called "morpholinos", is still indispensable for knock-down of gene expression and so far could not be replaced by siRNA-based approaches [90].

More recently, vector constructs have been devised that contain multiple binding sites for miRNAs, in order to compete with endogenous mRNAs for miRNA binding. Such *miRNA sponges* appeared to be a versatile tool for miRNA research [91]. Loya and colleagues analysed phenotypes when miR-7, miR-8, and miR-9a in Drosophila were down-regulated by miRNA sponges. The authors found that miRNA sponges evoked the same phenotype as produced by homologous recombination, but in a milder form. This is expected: miRNA sponges do not completely sequester target miRNAs, because of the competitive binding of miRNAs to mRNAs. Still, this approach enables not only simultaneous down-regulation of several miRNAs but also inducible expression of miRNA sponges in specific tissues and at different time points, allowing spatiotemporal analysis of miRNA function. For instance, it was possible to assign the known role of miR-8 in neuromuscular junction formation by expression of miRNA sponge that targeted miR-8 in muscle cells [92].

As an alternative to, or to complement, loss-of-function studies, *miRNA-mimics* can be used to achieve *de novo* or enhanced expression of miRNA (gain-of-function). miRNA-mimics can be administered as synthetic small dsRNA with sequences identical to those of endogenous miRNAs [93, 94]. They resemble siRNA molecules and are readily incorporated into the cellular RNA silencing machinery. If stable ectopic expression of miRNA is desired, constructs are at hand that allow expression of single or multiple miRNAs, and measurements of miRNA expression level by cotranscription of fluorescent reporter protein are possible (e.g., [95]). As for other vector-based constructs, the choice of suitable promotors can render tissue- and/or time-specific expression of miRNA-mimics possible. Importantly, molecules mimicking different maturation stages of endogenous miRNAs can be overexpressed and analysed.

Spatial and temporal regulation of miRNA expression can also be attained by allosteric ribozymes, *riboswitches* [96]. These are modular constructs containing an aptamer domain (an RNA sequence that specifically binds a chemical compound) embedded within a ribozyme and fused to a pri-miRNA analogue. This construct is functionally inactive in the absence of the appropriate chemical trigger. Upon application of the trigger substance (e.g., theophylline in the case of the cited work) a conformational change is induced in the ribozyme leading to its activation. The ribozyme will then cleave the modular RNA in *cis,* with pri-miRNA released from the construct and processed [96]. The large number of known aptamers and their endogenous and exogenous chemical triggers could develop this approach of *conditional RNA interference* into a versatile alternative for inducible vector constructs, especially if toxic side effects in knock-down or knock-out approaches are of concern [97].

Finally, specific mRNAs can be released from miRNA-mediated inhibition by application of *target protectors*. In experiments performed by Choi et al. in zebrafish [98],

morpholino-based ASOs were used, which prevented miR-430 binding by blocking seed-matched sites and neighbouring nucleotides in two target mRNAs. In this way, the authors succeeded in specifically interfering with miR-430-mediated translational repression. To conduct this type of experiment, it is necessary to know the targets of the miRNA of interest, but target protectors can be valuable in target validation and in characterization of miRNA: mRNA interactions.

6. miRNA-Based Functional Screens

The established infrastructure for siRNA/shRNA screens (i.e., robotics for large-scale sample preparation, automated data acquisition and analysis, data storage capacities) can easily be applied for high-throughput studies of miRNA function. As for siRNA-based screens, lipid-based transfection is most commonly used to achieve a transient overexpression [99] or inhibition [100] of miRNAs in cell culture. A number of screens were completed under conditions of miRNA stable overexpression [40, 45, 101, 102], achieved by transduction with retroviral vectors encoding specific miRNA genes [40]. Alternatively, constitutive overexpression or down-regulation of non-coding RNAs can be obtained by adenoviral vector-based systems [69, 103]. The advantages of the reverse transfection method, coupled with an automatic liquid handling system, have been also utilized in miRNA-based screens by several groups with a high success rate [104, 105].

The first miRNA was described in the early 1990s [50], but the initial accumulation of data concerning regulatory roles was rather slow [106–108]. This in turn delayed the availability of reagents to modulate the activity of endogenous miRNAs, and so the first functional miRNA-based screen [100] was completed a couple of years later than siRNA-based screens [109]. As a consequence, though, miRNA-based screens could benefit a lot from prior experience accumulated with siRNA-based screens.

7. Biological Processes Analysed in miRNA-Based Screens

A number of miRNA-based screens have been completed during the last 5 years (Table 2). Two major groups of biological processes have been investigated so far, namely, (i) cell viability, proliferation, and apoptosis and (ii) gene transcription and/or activity regulation.

Numerous miRNAs were shown to inhibit (let-7, miR-34a, miR-143, miR-145, miR-221, miR-222) [117–121] or to stimulate (miR-21, miR-133) [122, 123] cell proliferation. Moreover, recent studies demonstrated that a well-studied cluster of miR-17-92 can act both as oncogenes [124–127] and as tumour suppressors [9, 128], and these roles are probably cell type- and/or environment-dependent. Context-dependent activity was also reported for miR-24: down-regulation of miR-24 inhibited proliferation of A549 cells but increased growth of HeLa cells [100].

A considerable increase in the numbers of miRNAs that regulate cell proliferation and apoptosis was obtained

TABLE 2: miRNA-based functional screens.

Model system	Number of miRNAs screened	Type of regulation	Phenotype measured	Assay	Year	Reference
HeLa and A549	95	Loss-of-function by miRNA inhibitors	Cell proliferation and apoptosis	Cell counting and caspase-3/7 activity assay	2005	[100]
Primary hTERT-immortalized BJ-EHT fibroblasts	~450	Gain-of-function by stable miRNA expression	Sustained proliferation	miR-Array	2006	[40]
HeLa	~450	Gain-of-function by stable miRNA expression	p27^{Kip1} regulation	GFP reporter assay and miR-Array	2007	[45]
MDA-MB-453	187	Gain-of-function by miRNA precursors	TRAIL-induced caspase-3 activation	Caspase-3/7 activity assay	2007	[104]
Drosophila clone 8 cells	77/78 miRNA loci	Gain-of-function by plasmid-based miRNA expression	Wg signaling pathway regulation	Luciferase reporter assay	2007	[110]
Neuroblastoma cell lines	8	Gain- and loss-of-function by miRNA mimics and inhibitors	Proliferation	Change in electrical impedance	2008	[111]
MCF7	~450	Gain-of-function by stable miRNA expression	Cell migration	Trans-well cell migration assay	2008	[101]
HeLa	91	Gain-of-function by miRNA mimics	p53 gene activity	Luciferase reporter assay	2009	[38]
HeLa	~450	Gain-of-function by stable miRNA expression	*Per* gene family regulation	GFP reporter assay and miR-Array	2009	[102]
Primary ovarian granulosa cells	80	Gain-of-function by miRNA precursors	Progesterone, testosterone and estradiol release	Enzyme immunoassay (EIA)	2009	[112]
HEK 293	266	Gain-of-function by miRNA mimics	p21Cip/Waf1 regulation	Luciferase reporter assay	2010	[46]
HCT-16	810	Gain-of-function by miRNA mimics	Cell viability in the presence of Bcl-2 family inhibitor ABT-263	CellTiter-Glo Luminescent Cell Viability Assay	2010	[99]
HEK 293T	107	Gain-of-function by miRNA mimics	p53 gene regulation	Luciferase reporter assay	2010	[39]
HCT116 p53$^{+/+}$, H460 and MCF7	5	Gain-of-function by miRNA precursors	p53 gene regulation	Western blot	2010	[10]
Huh-7	327	Gain-of-function by miRNA precursors	Lipid droplet formation and growth	Immunocytochemistry and fluorescence microscopy	2010	[105]
Primary ovarian granulosa cells	80	Gain-of-function by miRNA precursors	Proliferation and apoptosis	Immunocytochemistry and fluorescence microscopy	2010	[113]
DLD-1	319	Gain-of-function by miRNA precursors	Cell viability	CellTiter-Glo Luminescent Cell Viability Assay	2010	[11]
HMEC	328	Gain-of-function by miRNA precursors	Proliferation	Fluorescence microscopy	2010	[114]

TABLE 2: Continued.

Model system	Number of miRNAs screened	Type of regulation	Phenotype measured	Assay	Year	Reference
MIA PaCa-2	445	Gain-of-function by stable miRNA expression	Proliferation	Custom-made microarray	2010	[115]
HeLa and HeLa P4	8	Gain- and loss-of-function by miRNA precursors and inhibitors	Cell proliferation and trafficking	Fluorescence microscopy	2011	[116]

[40, 99, 105] since Cheng et al. reported the first large-scale screen to identify mammalian miRNAs involved in these processes [100]. In 2007, Ovcharenko et al. performed a screen with 187 synthetic oligonucleotides to capture the modulators of TRAIL-induced apoptotic pathway [104]. They found that 34 of tested miRNAs modulate the activity of caspase-3. Recently, a gain-of-function miRNA-based screen was completed in human colorectal cancer DLD-1 cells [11]. By measuring cell viability, authors not only confirmed function of already known oncogenic miR-372 and miR-373 [40] but also discovered novel miRNAs involved in cell proliferation and apoptosis. miR-491 was among the strongest antiproliferative miRNAs, and further experimental analysis revealed that it induces apoptosis via direct down-regulation of antiapoptotic Bcl-xL [129]. miRNAs regulating expression of another member of Bcl-2 protein family, Mcl1, were identified by screening a library of 810 human miRNAs for the ability to confer resistance of cancer cells to ABT-263, an inhibitor of Bcl-2 family members [99]. Viability measurements of human colorectal cancer HCT-16 cells transiently transfected with miRNA mimics revealed 19 miRNAs that sensitized cells to ABT-263. 15 of these miRNAs showed the same phenotype in melanoma CHL1 cells. Furthermore, 10 out of 12 strong sensitizer miRNAs that were analysed for targeting 3′URT of Mcl1 were confirmed as direct regulators of the gene. These examples of the screens demonstrate that miRNAs that modulate sensitivity to chemotherapeutic agents can be identified and potentially, in future, used in cancer therapy [21, 130]. The feasibility of such screens has vastly improved by the evolution of methods to quantify cell proliferation from straightforward cell counting [100] to recording of electrical impedance over 96 hours [111].

miRNAs acting on transcription and/or gene activity regulation are usually identified in so-called "target-based screening". In the most cases, luciferase or green fluorescent protein (GFP) is fused to the gene of interest and the strength of the detected signal is used to gauge the expression or activity of the test gene [38, 39, 45, 46, 102]. Using a functional genetic approach with stable expression of individual miRNAs [40], miR-221 and miR-222 were demonstrated to specifically regulate expression of tumour suppressor p27^{Kip1} [45], miR-192/194 cluster-modulate expression of Per gene family [102].

The repertoire of cellular processes analysed by miRNA functional screens is expanding rapidly (Table 2). Doing miRNA-based screens in appropriate cellular contexts, for instance, for miRNAs regulating steroidogenesis in ovarian cells [112], and for miRNAs regulating lipid droplet formation in hepatocytes [105] helps to ensure acquisition of physiologically relevant information.

8. Gain-of-Function versus Loss-of-Function in miRNA-Based Screens

Although reagents for gain-of-function and loss-of-function miRNA experiments are available to similar extents, nearly all reported screens to assess the function of miRNAs utilized the gain-of-function approach (Table 2). This trend probably arose because of the ease with which ectopic expression of miRNAs can be confirmed. For instance, exogenous reporter gene assays [116, 131], qRT-PCR, and Northern blotting and ribonuclease protection assay [40] are easily applicable methods to measure over-expression of miRNAs. In contrast, the only assays that have been extensively used so far to show the inhibition of endogenous miRNA activity have involved reporters [38, 100, 128, 132]. The popularity of gain-of-function screens might additionally be explained by potentially easier evaluation. Over-expression of miRNAs might induce accentuated phenotypes, which might be not related to the levels of endogenous miRNAs, whereas the evaluation of data obtained under the conditions of miRNA down-regulation is possible only then one knows expression level of endogenous miRNAs in the test system. Acquisition of these data sets, therefore, needs thorough additional experimentation.

9. Fluorescence Screening Microscopy for miRNA-Based Screens

There are virtually no reasons why the read-out strategies in miRNA-based screens should be different from the ones established in siRNA-based screens. Nevertheless, we consider fluorescence microscopy screens to be highly advantageous. Features that make such microscopy ideal to analyse regulatory potential of miRNAs include the following: (i) rapid collection of large amount of data, (ii) feasibility of phenotype multiplexing, (iii) the possibility to acquire quantitative data on a cell-by-cell basis and/or population-based basis, and (iv) detection of subtle phenotypes [133, 134]. One of the first studies applying this

technology for functional miRNA studies was by Sirotkin and colleagues, who reported an immunocytochemistry- and fluorescence-microscopy-based screen to identify miR-NAs regulating proliferation and apoptosis [113]. Primary human ovarian cells were transfected with synthetic miRNA precursors, and PCNA and cyclin B1 proteins were used as markers of proliferation. The expression levels of Bax and caspase-3, in combination with the TUNEL assay, were used to determine the extent of apoptosis. The power of technology is demonstrated by another microscopy-based screen: using automated image analysis and nucleus classification software, a novel antiproliferative activity of miR-320a was discovered [116]. The screen to identify miRNAs controlling lipid droplet formation in hepatocytes [105] illustrated the sensitivity of fluorescence microscopy-based approach, which was comparable to the laborious biochemical assay—11 out of 327 transiently overexpressed human miRNAs were selected via an automated work flow as the most potent regulators of intracellular lipid content [105].

10. Validation of Hits in miRNA-Based Screens

In current screens, the list of primary hits involved in regulation of a biological pathway can be obtained reasonably fast, if necessary infrastructure is on place. The primary hit lists in siRNA/shRNA-based screens are usually validated by (i) repeating the assay with different types of reagents, and (ii) secondary assays, and (iii) rescue experiments, in which a phenotype caused by knock-down of a certain gene product is rescued by the over-expression of a construct that cannot be attacked by siRNA (either due to mutation or because of origin from another species). Currently, there is no clear agreement on criteria for successful validation of primary hit miRNAs, except from reproducing the phenotype with different reagents. Additionally, the effects caused by miRNA over-expression and down-regulation could be compared. Since this can be laborious, lists of miRNAs that cause a particular phenotype in screens may usefully be published without further analysis [100, 112, 113], allowing follow-up by others.

In order to perform a follow-up research on hit miRNAs, prediction of their target genes needs to be done first. Many computational algorithms have been developed over the last decade to predict miRNA targets [135], and a combination of multiple algorithms is frequently used to narrow down the candidates. Interesting strategy was used in the studies of TRAIL-induced apoptosis when hits of miRNA-based screen were compared with those of siRNA-based screen in order to identify plausible interactions between miRNAs and their target mRNAs [104].

Many of the screens that have been completed so far have been extended to experimental identification and validation of the targets of the hit miRNAs (Table 3). The most widely used method to test a direct regulation of gene expression by miRNAs is based on reporter assays. The 3′UTR, the 5′UTR, or the whole gene is cloned immediately downstream of a reporter gene encoding luciferase or fluorescent protein. The construct is then transiently cotransfected with miRNA mimics or antimiRs into host cells and luciferase activity or fluorescence is measured after 24–48 hours of incubation [10, 11, 38, 99, 110]. Additional evidence that the target gene is directly regulated by an miRNA can be provided by mutating [38, 110] or deleting [10, 101] predicted miRNA binding sites in the 3′UTR of a reporter vector. As controls, the entire 3′UTR can be inserted in the reverse orientation [136] or truncated [110]. Frequently, qRT-PCR has been used to show degradation of target mRNAs in miRNA overexpressing cells [10, 45, 46, 101].

Since miRNAs can execute their regulatory action by repressing translation and/or promoting mRNA decay of target genes [137], measurement of the product protein level is an additional means to validate miRNA action [11, 45, 46]. Biochemical techniques for the isolation of target mRNAs were also shown to be rewarding [138–140]. Eventually, the action of miRNA can be tested by down-regulation of known or putative target genes and monitoring the resulting phenotypes [72, 105]. Observation of the same phenotype after both miRNA over-expression and down-regulation of its putative target genes strengthens evidence that the target identification was valid. Such tests might include down-regulation of a single target as well as multiple ones. Complex evaluations are possible if combining several validation approaches. For example, changes in target protein level without any changes in a reporter assay would suggest that the miRNA does not directly target an affected gene but rather regulates its modulator or the whole pathway [99].

11. Challenges of siRNA-, shRNAs-, and miRNA-Based Screens

RNAi-based screens have turned to be extremely useful for studies in various fields of biology and medicine [59, 141–143]. A robust assay, careful design of the experiment, reliable controls, and exhaustive testing of the reagents are crucial for successfully completing a screen, and several excellent reviews, describing the planning of RNAi screen experiments, have been published (e.g., [60, 144–146]). We will now focus on some practical aspects of siRNA/shRNA-based screens that could be relevant while performing miRNA-based screens.

The analysis of siRNA-based screens is often complicated by off-target effects (OTEs): phenotypes caused by unspecific down-regulation of mRNAs. One source of OTEs is partial homology of siRNAs with unintended mRNAs. A different type of OTE arises from induction of cytokine production by siRNA/shRNA with certain sequence motifs [147], which ultimately results in apoptosis. Therefore, in the last years considerable effort has been invested in improving the performance of siRNAs/shRNAs. For example, induction of an interferon response can be mitigated by excluding GU-rich sequence motifs in siRNA molecules [148]. Specificity can be considerably enhanced by altering the chemical backbone of siRNAs: examples are LNA (locked nucleic acid) nucleotide analogues or modifying ribosyl groups by addition of a 2′-O-methyl group at specific positions [149, 150].

TABLE 3: Validation assays in miRNA-based screens.

Effector miRNA	Target genes	Validation assay					Year	Reference
		Expression[a]	Reporter[b]	qRT-PCR	IS[c]	siRNA[d]		
miR-372 and -373	LATS2	+	+	+	+		2006	[40]
miR-221 and -222	p27[Kip1]		+		+		2007	[45]
miR-315	Axin and Notum		+				2007	[110]
miR-34[a]	Bcl2 and MYCN				+		2008	[111]
miR-373 and -520c	CD44	+	+				2008	[101]
miR-29[a,b,c]	p85α and CDC42		+		+		2009	[38]
miR-192 and -194	Per1, 2 and 3		+				2009	[102]
28 miRNAs	p21Cip/Waf1		+		+		2010	[46]
10 miRNAs	MCL1		+				2010	[99]
miR-1285	p53		+	+	+		2010	[39]
miR-504	p53		+		+		2010	[10]
11 miRNAs	Multiple genes					+	2010	[105]
miR-491	Bcl-xL		+	+	+		2010	[11]
28 miRNAs	p21Cip/Waf1		+		+		2010	[114]

[a] mRNA expression profiling.
[b] Luciferase and/or GFP reporter assays.
[c] Immunostaining.
[d] Recapitulation of miRNA-mediated phenotype by selected siRNAs.

Secondary or even toxic effects caused by transfection reagents alone and/or in combination with silencing RNA cannot be completely ruled out. Fortunately, current formulations contain 5 to 10 times less reagents than were required several years ago. Another approach is to pool several siRNAs against the same target, while using reduced amounts of each. The idea behind this approach is that the target mRNA will be attacked by all, or the majority of, siRNAs in the pool, and the specific down-regulations will act cumulatively, whereas OTEs of individual siRNAs will be too weak to affect results. The same concept of improved signal-to-noise ratio in observed phenotypes forms also the basis for the application of esiRNAs [65].

Despite these strategies, though, OTEs and unspecific secondary effects cannot be avoided completely. It is, therefore, essential to control for both. One option is to use several different siRNAs to target the same mRNA at independent sites. The probability that an observed phenotype corresponds to an on-target silencing effect rises with the number of siRNAs causing the same phenotype [151]. Moreover, it is of advantage to measure several cellular parameters (e.g., cell shape and size, number of cells) in a screen. Multiparametric analysis allows generation phenotypic profiles of individual siRNAs. A high degree of overlap in these profiles justifies strong confidence in the specificity of an observed phenotype [58, 151].

One of the most serious concerns in functional screens is possible oversaturation of the cellular silencing machinery by exogenous siRNA/shRNA and miRNA mimics. This can result in derepression of mRNAs that are regulated by endogenous miRNAs [152, 153]. Various strategies have been developed to deal with these problems (for a detailed compilation of strategies see [154]). Briefly, overloading the cellular RNA silencing machinery can be avoided by applying the lowest possible amounts of siRNA/shRNA to the cells. For instance, transcription from RNA Pol II-driven promotors, and use of inducible constructs, can be useful to control levels of vector-encoded shRNAs [70, 155]. Using vectors derived from adeno-associated virus can also be a way to induce a moderate level of stable shRNA expression in cells [21, 156]. Coexpression of AGO-2 has also been described as an effective approach to overcome the problem of saturating the RNAi machinery by ectopic siRNA, shRNA, and miRNA [157]. Enhanced amounts of AGO-2 were shown to drive RNAi interference toward preferential knock-down of perfectly matched target mRNAs in diverse mammalian cell lines. Because, in addition, less siRNA/shRNA was needed for specific knock-down, and unspecific targeting of mRNAs was significantly reduced in the presence of ectopic AGO-2, the authors suggest that this strategy might result in reduced rates of false negatives and false positives in RNAi-based screening approaches. On the other hand, the suitability of the approach should be tested rigorously, as AGO-2 was recently shown to play a role in biogenesis of particular type of miRNAs [36, 37].

Finally, miRNA-based functional screens might suffer from neutral repression, when reduced transcript levels do not lead to measurable changes in cellular behaviour. Sometimes, residual protein is still sufficient to fulfil the required function; alternatively, feedback mechanisms exist that compensate functionally for the knock-down [158].

Usually, screens encompass thousands of genes or even the whole genomes; so it is hardly possible to look for functions that might be conferred by redundant gene function. Often, cellular functions are not controlled by just one protein, but by two or more isotypes or evolutionary-related forms of proteins to ensure higher reliability of the process, and metabolic pathways may be redundant.

The need to use several siRNAs per protein, and multiple possible combinations of distinct siRNAs, limits studies that aim to identify redundant gene products to relatively small, directed screens. Conversely, highly homologous gene products, like, for example, splice variants, might have distinct cellular functions, but siRNAs might target all forms together (e.g., [159]). Similar concerns pertain to miRNA-based screens, as redundant activities of miRNAs in regulation of many biological processes is well documented [160]: Miska et al. reported that most miRNAs in *C. elegans* are individually dispensable [161], and Voorhoeve et al. found that miR-372 and miR-373 cooperate with oncogenic RAS in the development of testicular germ cell tumours [40]. A way to deal with miRNA redundancy could be the use of sensitized genetic backgrounds as described by Brenner et al. [162]. By deleting one of the two AGO genes in *C. elegans* the authors partially disabled the RNA silencing machinery. In addition, worms with defects in chromatin modification or transcriptional regulation were generated. Using these organisms allowed definition of biological roles for several individual miRNAs [162]. Additionally, synthetic phenotypes might be used to analyse functions of miRNAs [160].

The complexity of miRNA action presents a challenging task for high-throughput functional analysis. However, given our experience from RNAi screens over the last decade, with siRNAs and shRNAs in different model organisms, careful experimental design and exhaustive target validation makes this powerful technology indispensable for understanding the biological roles of miRNAs.

Acknowledgments

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