Getting to the Heart of microRNA Control of Ribosome Function

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The past decade has seen microRNAs (miRNAs) emerge as a major new class of gene regulators present in most eukaryotic organisms. These ~22 nucleotide non-coding RNAs fine-tune the availability of mRNAs as templates for protein synthesis by the ribosome. The human genome encodes at least 940 distinct miRNAs (miRbase version 15; ref. 1), collectively thought to target the majority of cellular mRNAs. Many miRNAs are evolutionarily conserved and/or exhibit distinct spatio-temporal expression patterns. miRNAs were therefore found to be involved in most biological processes where this has been investigated, and they have been implicated in a multitude of disease pathologies (reviewed in ref. 2). The scope of miRNAs in both health and disease is clearly evident in cardiac biology where a large body of work has shown the dependence of cardiac development and function on miRNA action. There is little margin for error or compromised function of the heart. This need for continuous optimal function requires precise control of the function and structure of cardiomyocytes and extremely coordinated development of this organ. This is provided by exquisitely controlled and robust regulation of gene transcription and protein translation in the heart, and it is now recognised that miRNAs are integral to this process (reviewed in ref. 3).

Unsurprisingly, miRNAs are commonly deregulated in cardiac disease (reviewed in ref. 4). The core role of miRNAs in heart function is highlighted by the discovery that enforced expression or down-regulation of specific miRNAs can cause heart disease (see **Table 1**). For example, miRNA-195 overexpression is sufficient to induce hypertrophy in mice, which progress to dilated cardiomyopathy and heart failure in early life. Conversely, miRNA-208a null mice failed to develop hypertrophy after cardiac pressure overload or sustained calcineurin signalling. These and similar studies in other models of heart disease have sparked interest in manipulating miRNAs for future cardiac therapy, either by directly targeting the miRNAs underlying progression to disease or by disrupting miRNA targeting of genes determined to be the basis of cardiac dysfunction (reviewed in ref. 5). While a large portion of research effort is directed towards identifying miRNAs involved in disease, we still have a way to go in defining the mechanisms miRNAs employ to change the cellular complement of mRNAs translated by the ribosome. When we manipulate miRNAs for therapy, can we be sure we are going to get the outcome we want, and do we understand how changes in the cellular environment might alter the action of a miRNA?

Table 1. Cardiac phenotypes resulting from *in vivo* manipulation of miRNAs. Adapted from (4).

miRNA	Modulation	Phenotype
miR-1	Knockout	Electrophysiological abnormality, embryonic lethality, thickened ventricular wall
miR-1	Mimic	Increased arrhythmia
miR-1	Antisense	Fatal arrhythmia following myocardial infarction
miR-21	Antagomir	Inhibition of interstitial fibrosis and attenuation of cardiac dysfunction after thoracic aorta constriction
	2′0Me	Decreased neointimal formation, cell proliferation, and apoptosis
miR-29	Antagomir	Enhancement of the fibrotic response
miR-126	Knockout	Lethal embryogenesis due to vascular leakage; defective cardiac neovascularisation following myocardial infarction in surviving adults
miR-133	Antagomir	Cardiac hypertrophy
miR-133a1/133a2	Knockout	Embryonic-postnatal lethality, ventricular septal defect, atrial and right ventricular enlargement, dilated heart, increased fibrosis
133a2	Transgenic	Embryonic-postnatal lethality, impaired cardiomyocyte proliferation
133	Mimic	Long QT, arrhythmia
195	Mimic	Cardiac hypertrophy
208	Knockout	No basal phenotype, failure to undergo stress-induced cardiac remodeling after thoracic aorta constriction

Therapeutic use of RNA interference (RNAi, see below) has also become a strong focus in the development of RNA-based therapies, with the goal of removing specific mRNAs from ribosome association. While several phase I and II clinical trials are already under way, there are still some concerns and challenges to overcome, including delivery of miRNA or small interfering (si)RNA into cells, inadvertent triggering of an immune response and the potential for off-target effects (reviewed in ref. 6). In this article, we wish to highlight additional issues in rational miRNA/siRNA-based design, namely the predictability and robustness of miRNA action (whether an intended or off-target effect) on different targets in changing cellular environments.

So what do we know about the action of mammalian miRNAs? miRNAs act to guide large repressive protein complexes, called either the RNA-induced Silencing Complex (RISC) or the miRNA ribonucleoprotein complex (miRNP), to target mRNAs, typically leading to attenuation of ribosome association. The degree of miRNA/mRNA base pairing in large part determines the mode of post-transcriptional control. Near-perfect complementarity between miRNA and target region favours endonucleolytic cleavage of the mRNA as in the RNAi mechanism induced by siRNA. Mismatches, particularly in the central target region, preclude cleavage and instead lead to repression of protein synthesis from the mRNA and cleavage-independent destabilisation. Animal miRNAs typically only partially match to sequences in the 3′ untranslated regions (UTR) of their targets, often exhibiting contiguous Watson-Crick base pairing only in the miRNA 5′ seed region (positions 2-8) (7). When a therapeutic miRNA is designed to induce RNAi on a perfectly complementary target, it can also bind imperfectly to other mRNAs and repress them, leading to off-target effects.

The details of miRNP assembly and the full complement of miRNP components are still being discovered; however, we do know that the core component of the miRNP is an Argonaute (Ago) protein, of which there are four isoforms in humans. Studies of human Ago1-4 proteins suggest that they may all have similar roles in miRNA-mediated repression, as they associate with similar sets of proteins and miRNAs in immunoprecipitation studies (8). Artificially tethering any of the human Agos to the 3′UTR of mRNAs also leads to similar levels of repression, suggesting that while all human Agos function similarly, the individual Agos may come to the fore in different tissues and times in development (8). Conversely, in *Drosophila,* where there are two Ago proteins loaded with different subclasses of miRNAs, clear differences were reported in the modes of miRNA-mediated repression enacted by each Ago (although the picture is complicated by Ago2 predominantly enacting RNAi). Ago1 represses translation by shortening of the poly(A) tail of its mRNA targets and blocking a step after cap recognition, while Ago2 blocks the interaction of the eukaryotic translation initiation factor (eIF) eIF4E with eIF4G and inhibits mRNA cap function. This suggests that differences in the actions of the different mammalian Agos may emerge with further study. Alternatively, miRNA pathways between different species may be divergent, consistent with the concept that differences in non-coding RNA significantly contribute to the evolution of complexity (9).

Recently, great focus has fallen on another component of the miRNP, GW182 (of which there are three isoforms in humans), as an integral effector of miRNA-mediated repression (reviewed ref. 10), as tethering any of the three human GW182 paralogs to the 3′UTR of an mRNA leads to translational repression and mRNA destabilisation independent of miRNAs and Agos. Other less well-characterised members of the miRNP include the fragile X mental retardation protein, FMRP, the RNA helicase RCK/p54, Importin 8 and the TRIM-NHL family of proteins (11). It remains to be seen how robust the association of these proteins is with the miRNP and what their respective functions in miRNA-mediated translational control and mRNA destabilisation are.

There have been many *in vivo* and *in vitro* studies into the mechanisms of miRNA suppression of protein synthesis, predominantly using reporters. Regulation of mRNA translation is integral to the control of gene expression (reviewed in refs. 12 & 13). Translation takes place on the ribosome and is divided into three phases: initiation, elongation (when polypeptide synthesis takes place), and termination. Actively translating mRNAs are associated with multiple ribosomes, referred to as a polysome. Initiation typically begins with binding of the small (40S) ribosomal subunit near the mRNA 5′ end, followed by scanning along the mRNA 5′ UTR. Start codon recognition triggers joining of the large (60S) subunit to form the elongation-competent 80S ribosome. A multitude of eIFs facilitate translation initiation. Of note here, 40S subunit recruitment is promoted by the 5′ cap structure and the 3′ poly(A) tail of the mRNA, and their cognate binding proteins, eIF4E and poly(A)-binding protein (PABP). Both interact with eIF4G, thus bridging between the mRNA ends to form a 'closed loop' (**Fig. 1**). eIF4G in turn co-ordinates recruitment of the 40S subunit and additional eIFs. The components that form the mRNA closed loop are common targets of control mechanisms including miRNA-mediated translational repression.

There is significant evidence that the miRNP targets components of the closed loop and essential regulators of the initiation phase of translation to repress protein synthesis (11). First, in several studies, miRNA-targeted mRNAs lost association with ribosomes (measured as a shift to lighter fractions in density gradient ultracentrifugations), indicative of repression of ribosomal recruitment to the mRNA during initiation. Second, several studies have shown that mRNAs engineered such that translation is independent of the canonical 5′ cap structure (encoding 5′ cap variants or virally derived internal ribosome entry site [IRES] elements) are refractory to miRNA-mediated translational repression (14-16), and these observations have also been supported by numerous *in vitro* studies. These reports further suggest that the function of eIF4E is targeted by the miRNP; however, efforts to elucidate the specifics of this mechanism have been inconclusive. Initial reports that Ago directly bound the 5′ cap have been refuted and the interaction of *Drosophila* Ago2 with eIF4E was found

not to be evolutionarily conserved in mammals (11). Third, several studies have also shown that full miRNA-mediated translational repression requires a poly(A) tail on the mRNA (14,15,17,18). It is well established that miRNA action stimulates deadenylation of target mRNA, and this has usually been attributed to deadenylation representing an early stage of mRNA decay. However, we have shown that inhibition of deadenylation significantly reduces the ability of a miRNA to inhibit translational repression (15). Thus, the poly(A) tail of an mRNA, and its associated PABP, are likely targets of the miRNP in repression of translation as well as stimulation of mRNA decay. In fact, recent studies have shown that the miRNP component GW182 interacts directly with the PABP, which has implications for both translational control and recruitment of deadenylases by the miRNP (10). In summary, many studies support a mechanism of miRNA-mediated repression where the initiation phase of translation is targeted, probably through inhibition of 5′cap/eIF4E and poly(A) tail function and concomitant inhibition of the formation of the closed loop (**Fig. 1**).

However, there is still controversy as to whether miRNAs can also target post-initiation phases of translation. Using polysome sedimentation, several persuasive studies have shown mRNAs still in association with a similar number of ribosomes after being repressed by miRNAs (see ref. 19). While a full description of these studies is outside the scope of this article, one of the most intriguing instances of miRNA-dependent initiation versus post-initiation translational control occurred when an otherwise identical reporter was seen to switch between mechanisms depending on the promoter that drove expression. The basis of this mechanistic difference is not known. There are also reports that in different cellular circumstances, miRNAs can activate translation. So are there multiple mechanisms of miRNA action? One possibility is that miRNAs can affect repression using multiple mechanisms in a tissue- or development-specific context. Another likely scenario is that the experimental conditions (including cell type, translational extract components and design of reporter constructs) have led to one form of translational repression being favoured over another in each study. For example, the choice of transfection reagents has been shown to influence the degree of repression observed and different *in vitro* systems have shown differences in the timing of translational repression versus deadenylation (11,15).

Although findings were not always in complete agreement, the reporter studies outlined above have provided great insight into the way miRNAs can act on their targets. Now that we have begun to understand how miRNAs can act, the next question is how do endogenous miRNAs act on their endogenous targets? Answering this question is no trivial feat, as endogenous miRNAs may often have only subtle effects on the majority of their targets, creating technical difficulties in reproducibly measuring the consequences of miRNA action. To overcome these technical challenges, several groups have looked at global changes to endogenous mRNA translation and stability resulting from altering miRNA levels in cell culture. While this gives greater power to observe wider trends in the transcriptome, it is at the expense of sensitivity and measures all expressed endogenous mRNA variants (e.g., splice and UTR variants) as one composite mRNA. Several major studies have reported on endogenous global effects of miRNA action using either polysome profiling (20), dual isotope

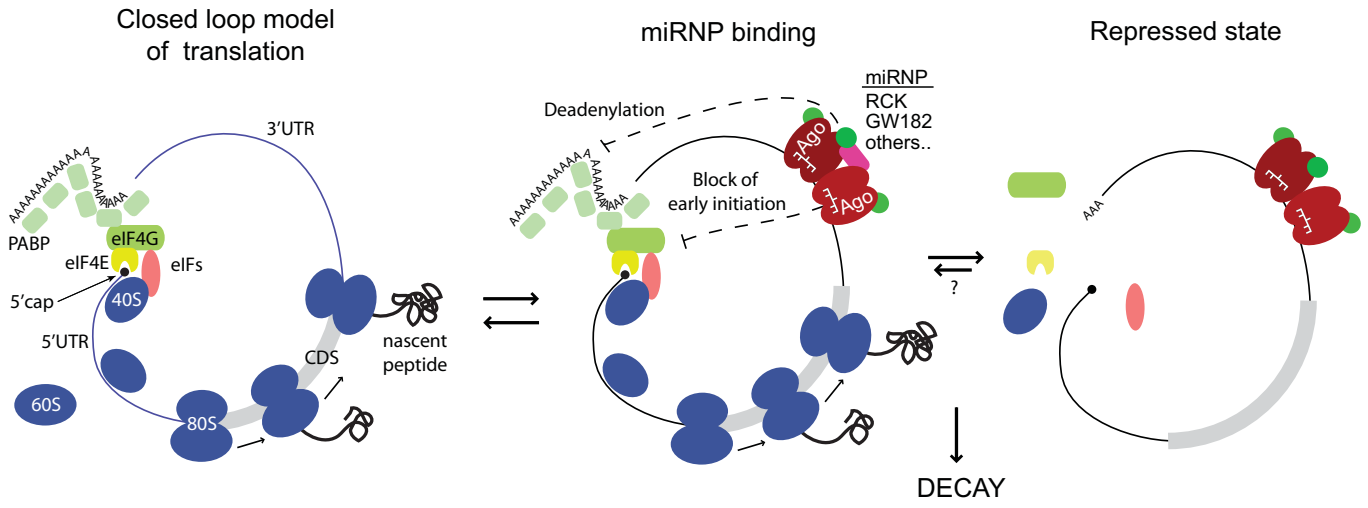

Fig. 1. Components of the mRNA closed loop and translation initiation are targeted by the miRNP to repress translation.
During initiation of translation, the 40S ribosomal subunit is recruited to the 5′ end of the mRNA and scans the 5′ UTR to locate the initiator codon, where it is joined by the 60S subunit to form the 80S ribosome. All steps of initiation require a multitude of accessory factors termed eukaryotic initiation factors (eIFs). This includes the poly(A)-binding protein (PABP), which interacts with the 5′ end of the mRNA through its interaction with eIF4G and forms the basis of the closed loop model. miRNAs guide miRNP complexes to the 3′ UTR of target mRNA, trigger mRNA deadenylation and interfere with initiation complex function. These changes conspire to attenuate translation of mRNA. miRNAs also stimulate mRNA decay by decapping and exonucleolytic decay. Adapted from (24).

SILAC mass spectroscopy combined with transcript profiling (21) or ribosome footprinting (22). Each study concluded that the major measurable effect of miRNA action was the destabilisation of the target mRNA, with observable translational repression generally minor or absent.

So how will understanding the mechanisms of miRNA action on their targets help develop better therapies for cardiac disease? Many studies have shown that miRNAs are key regulators of cardiac disease and modulation of miRNA levels themselves is a core area of future therapeutic intervention. The development of siRNA/miRNAs to target pathogenic mRNAs whose upregulation underlies disease is especially crucial for genes where development of other forms of therapeutic intervention have failed, such as phospholamban, an important regulator of cardiac contractility (5). Given the vagaries of multiple possible mechanisms of miRNA action, which may also depend on cellular context, current therapeutic miRNA designs might not regulate disease genes with the precision we would like. For the same reasons, we cannot assay for off-target effects with total confidence. For example, is it appropriate to only look for changes in mRNA levels, or must we also measure changes in protein levels? We also do not know how robust the mechanism of miRNA action is. For example, will a therapeutic si/miRNA use the same mechanism in a healthy cardiomyocyte versus a hypertrophic one (where rates of protein synthesis are altered; 23) or one in a patient being treated for hypertension? The plasticity of miRNA action is one of the great unknowns in the field and something that will need to be addressed to increase our chances of successfully employing miRNA-based therapeutics. Thus, there are many challenges ahead, but there is also the promise of great reward. After eluding our detection for so long, the discovery of miRNAs has not only brought us greater insight into the complexity of gene expression, but also new therapeutic tools, which will allow us to specifically alter the availability of target mRNAs to the ribosome and treat sufferers of cardiac and other diseases.

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