miRNPs: versatile regulators of gene expression in vertebrate cells

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Abstract
TNFα (tumour necrosis factor α) mRNA bears in its 3′-UTR (untranslated region) a conserved ARE (AU-rich element), a signal that exerts tight post-transcriptional control over the expression of TNFα and other cytokines. We found that the TNFα ARE increases translational efficiency when cell growth is arrested, a physiologically relevant state occurring during inflammation, angiogenesis and monocyte differentiation. Under these conditions, called quiescence, the miRNP (microribonucleoprotein)-associated proteins FXR1 (Fragile X mental retardation-related protein 1) and AGO2 (Argonaute 2), which are usually considered negative regulators, are transformed into effector molecules that bind the ARE to activate translation. We then identified a specific miRNA (microRNA) that directs the association of AGO2 and FXR1 with the ARE during translational up-regulation. Two other well-characterized miRNAs likewise promote translation activation in quiescent or in contact-inhibited cells; yet, they repress translation in proliferating cells in the late S/G2-phase. We conclude that translational regulation by miRNPs oscillates between repression and activation as a function of the cell cycle. The activating role of miRNAs is now being confirmed in the immature Xenopus oocyte, which mimics the quiescent state.

Introduction
miRNAs (microRNAs) constitute a huge new class of non-coding RNAs, numbering in the hundreds of species in mammalian cells. They are tiny (21–22 nt) and act at the post-transcriptional level to modulate gene expression [1–3]. miRNAs originate from longer precursors (pri-miRNAs or primary miRNAs) usually synthesized by RNA polymerase II. They are often encoded within introns. Pri-miRNAs are first cleaved by an RNase III-like enzyme Drosha to ∼70-nt pre-miRNAs (precursor miRNAs), which exit to the cytoplasm. There the miRNA and RNA interference pathways converge, with another RNase III-like enzyme, Dicer, reducing either a pre-miRNA or a completely double-stranded RNA to ∼21-nt fragments. The 21-nt miRNA products are then incorporated into RISC (RNA-induced silencing complex). The core component of RISC is a member of the AGO (Argonaute) family of RNA-binding proteins; in vertebrate cells, this is AGO2. RISC subsequently associates with target sites primarily in the 3′-UTRs (untranslated regions) of mRNAs, directed by RNA–RNA interactions. If complementarity between the 21-nt RNA and the target mRNA is perfect, AGO-directed cleavage ensues and the mRNA is down-regulated. If the interaction is imperfect, but obeys certain ‘seed rules’ [4], then, in proliferating cells, translational repression and, in some cases, deadenylation (leading to mRNA degradation) occur. The molecular mechanism of translational repression has remained controversial [5,6].

AREs (AU-rich elements) are important regulatory signals appearing in the 3′-UTRs of approx. 12% of mammalian mRNAs [7,8]. These mRNAs encode proteins such as oncogenes and growth factors and are tightly regulated by the cell. They are turned on by transcription and then undergo
rapid decay directed by the ARE. Specific ARE-binding proteins attract the exosome to initiate exonucleolytic decay of the poly(A) tail that leads to mRNA degradation. However, there have been reports that, under altered conditions, such as adherence of immune cells, ARE sequences can instead stabilize or enhance translation of an mRNA [9–11].

We set out to investigate how the profile of proteins binding to AREs in mRNAs changes during conditions leading to translational up-regulation. This study led to the surprising discovery that miRNAs can switch from translational repression to activation depending on the cell cycle [12–14]. Our findings have implications both for understanding the full range of roles played by miRNAs in development, differentiation and disease processes in vertebrate cells, and for the design of miRNA-based therapeutics.

mRNA association of FXR1 (Fragile X mental retardation-related protein 1) and AGO2 is essential for ARE-mediated translation up-regulation

In order to isolate and identify proteins binding to AREs under conditions that result in translational up-regulation, the TNFα (tumour necrosis factor α) ARE, a 3′-nt sequence from the highly conserved 3′-UTR of this cytokine mRNA, was inserted into vectors encoding luciferase (Figures 1A and 1B) that could be transfected into standard laboratory human cell lines such as HeLa, HEK (human embryonic kidney)-293 or the monocyte cell line THP1. We discovered >5-fold stimulated production of luciferase from the TNFα ARE-containing reporter upon serum starvation of cultured mammalian cells, conditions that up-regulate cytokine production [14] (Figure 1C, -Semen, compared with asynchronously growing cells, Asyn). Serum starvation causes cells to withdraw from the cell cycle and accumulate in the G0/G1-phase, according to flow cytometric analysis. This is not a stress condition, but rather leads to a cell cycle fate designated quiescence, from which cells can resume cycling upon readddition of serum [15,16]. To assess translational efficiency, we routinely normalized the luciferase output of the reporter to mRNA levels, as analysed by RNase mapping. Thus translation efficiencies exclusively compare changes at the protein level while any effects of mRNA decay occasioned by the presence of the ARE are cancelled out.

A small RNA aptamer, designated S1 because it binds streptavidin [17], was then inserted into the luciferase/TNFα ARE reporters. Extracts from cells transfected with this or a control 3′-UTR reporter were prepared from cycling cells or cells after serum starvation. Before cell lysis, the cells were fixed with formaldehyde in order to prevent rearsortment of bound proteins, as had been documented previously for ARE-binding proteins [18]. After isolation on streptavidin beads, elution with biotin and heat reversal of the formaldehyde cross-links (Figure 2) [14], the protein profiles revealed several differences between the reporters isolated from cycling compared with serum-starved cells. Analysis by MS identified one such band to be FXR1. FXR1 was initially described as a translational regulator that associates with 60S ribosomal subunits [19], and FXR1-knockout mice have more recently been shown to exhibit cytokine deregulation [20]. FXR1 exists in at least seven spliced isoforms; iso-a is present in the human cell lines used in our analyses [21,22]. FXR1 was then shown to be essential for translation up-regulation by siRNA (small interfering RNA) knockdown and by tethering FXR1-iso-a via a λN protein fusion to luciferase reporters containing five copies of the λB box-binding site for the N protein instead of the ARE in the 3′-UTR [14]. Because FXR1 had been reported previously to be associated with RISC, AGO2 was also tested for its contribution to translation up-regulation by both siRNA knockdown and λN-protein-tethering experiments. These experiments revealed that AGO2, in contrast with its well-documented role in translational repression by RISC, stimulates translation efficiency in serum-starved cells [14]. Indeed, when AGO2 was first reported in the literature in 1988 [23], it was found in a fraction that stimulated translation initiation and was implicated as a stimulatory protein [23,24].
Both AGO2 and FXR1 are necessary; tethering one after knockdown of the other does not result in translation up-regulation in serum-starved cells [12].

**A miRNA, miR369-3p, is essential for TNFα ARE-directed translation up-regulation**

Because FXR1 and AGO2 are RISC components, it seemed likely that a miRNA might be the specificity determinant in the binding of these proteins to the TNFα ARE. Five human miRNA candidates were identified by bioinformatics as exhibiting appropriate complementarity to the ARE, as well as conservation across species where the TNFα ARE sequence is conserved. One of these, miR369-3p, withstood the following tests of validity as a critical component of the TNFα ARE RNP (ribonucleoprotein) [12]. It exhibits complementarity to two stretches of seven nucleotides, separated by four nucleotides, within the 34-nt ARE sequence (Figure 1A).

First, knockdown of miR369-3p was accomplished by targeting its pre-miRNA with an siRNA complementary to the loop sequence of the precursor. This approach has the advantage that such a knockdown can subsequently be rescued by addback of synthetic miR369-3p. Whereas knockdown of pre-miR369-3p abrogated luciferase up-regulation, high levels of translation of the TNFα ARE luciferase reporter were restored by addition of synthetic miR369-3p [12].

Secondly, block substitutions within one or the other target seed sequences for miR369-3p in the TNFα ARE inhibit translation up-regulation of the luciferase reporter. Up-regulation can be restored by addition of synthetic miR369-3p bearing compensatory changes. This argues that base-pairing between miR369-3p and its target sites in the TNFα ARE is essential for miRNA-mediated translation stimulation [12].

Thirdly, the presence of miR369-3p in the RNP assembled on the TNFα ARE can be detected after S1 aptamer-dependent isolation, but only from serum-starved and not proliferating cells. As expected, miR369-3p disappears after siRNA knockdown of pre-miR369-3p, but can be restored by addition of synthetic miR369-3p to the transfected serum-starved cells [12].

**miR369-3p switches from activation to repression during the cell cycle**

The state of cultured cells that is generated by serum starvation is termed quiescence and is often called G0/G1 arrest but is distinct from the G1 phase of the cell cycle. Quiescence, by definition, is a reversible state, from which cells can return to proliferation. There are other irreversible cell fates in which cells have withdrawn from the cell cycle, such as terminal differentiation, apoptosis and senescence. These are different as determined by gene expression profiling [15,16] and may or may not exhibit up-regulation of translation by miRNAs comparable with that observed during quiescence.

Our finding of only mild translational repression by miR369-3p in asynchronous cycling cells and the variable 2–10-fold down-regulation reported by other investigators [5,6] prompted us to ask whether the activity of miRNPs (microribonucleoproteins) might vary as a function of the cell cycle. We synchronized cultured cells by subjecting them first to serum starvation and then restoring serum and following progress through the cell cycle by flow cytometric analysis. We observed that there is indeed a period of optimal translational repression by miRNPs that occurs late in S-phase, close to the G2 boundary (Figure 1C, Snc) [12,13].

We then proceeded to compare the requirement for miR369-3p for translational repression compared with translational activation by knocking down pre-miR369-3p. We found that both the activation observed in quiescence and the repression that is optimal in late S-phase can be reversed by eliminating miR369-3p and recovered by adding back synthetic miR369-3p [12].

Interestingly, reporter pullouts via the S1 aptamer revealed a difference in protein composition between the activating compared with repressing RNP formed on the TNFα ARE. Namely, AGO2 can be detected both during quiescence and in late S-phase, whereas FXR1 is present in the RNP only under the serum-starved conditions. We conclude that
FXR1 is neither required for nor a component of the repressive RISC, but is essential for translational activation by AGO2 [12]. Apart from this particular difference in protein composition, there may be many other changes, perhaps driven by cell-cycle-dependent modification states of constituent RISC proteins that lead to the switch from a repressive miRNP to an activating miRNP.

**Other miRNAs can also switch from translational repression to activation depending on the cell cycle**

We reasoned that if miR369-3p can act in either a repressive or activating manner as a function of the cell cycle, the same may be true of other miRNAs. We selected two well-studied systems for analysis [25–27]. In both cases, we were able to reproduce the behaviour of miR369-3p and its target sites in the TNFα ARE [12].

The first system was that developed in P. Sharp’s laboratory, where a completely artificial miRNA cccr4 targets a reporter containing four copies of the CX target site in its 3′-UTR. Here, the miRNA is present only if co-transfected with the CX reporter into cultured cells [25,26]. The second was a system from D. Bartel’s laboratory, where the 3′-UTR of the HMG12 (high-mobility group A2) mRNA possesses seven potential target sites for the let-7 miRNA. Point mutations in some of these target sites had been shown to be rescued by compensatory mutations in let-7 [27]. We observed for both systems that, under quiescent conditions, the presence of the miRNA stimulated the translational efficiency of the reporter >5-fold. In asynchronously proliferating cells, >2-fold effects of the matching miRNA were observed. However, in cells synchronized in late S/G2-phase of the cell cycle, the appropriate miRNA conferred >5-fold repression of translation [12,13].

We also investigated NIH 3T3 cells subjected to contact inhibition, another condition that drives cells into the quiescent state [16]. With both of the above reporters, as well as for the TNFα ARE, we observed stimulation of translation. On the other hand, we verified that, when complementarity between the target sites in the reporter 3′-UTR and the miRNA is perfect, down-regulation of expression ensues in both quiescent and proliferating cells. We conclude that the outcome of targeting an mRNA with an siRNA is not altered by the cell cycle [13].

**The immature Xenopus laevis oocyte mimics quiescent human cultured cells**

Despite the fact that the results reported above were obtained in several human cell lines, we decided to investigate other cell systems that might mimic the quiescent state. Conclusions based on cultured human cell lines pose several worries: (i) immortalized cells lines might not reflect the true biology underlying miRNA action; (ii) introducing reporters by transfection is both difficult and of low efficiency; (iii) serum starvation might elicit cell stress, despite the lack of appearance of stress granules and other markers of the stressed state; and (iv) the conditions leading to quiescence are difficult to reproduce.

In our pursuit of a natural quiescent system that would facilitate our investigation of miRNA-mediated translation activation, we are currently undertaking miRNA functional analyses in the *Xenopus laevis* oocyte system, which mimics the quiescent non-proliferative state. We hypothesize that such a quiescent-like system should recreate the necessary conditions for miRNA-mediated translation activation. In support of this hypothesis, data from R. Carthew’s laboratory on *Drosophila* oocyte development suggested that similar stages of early development demonstrate regulated miRNA function and poor repression, whereas robust miRNA-mediated repression occurred during the later stages of development in the dividing embryo [28]. *Xenopus laevis* is excellent for post-transcriptional studies and has been utilized extensively to define multiple post-transcriptional mechanisms of gene expression and their regulation [29]. This system offers a natural quiescent-like state with effective reporter and small RNA delivery by injection, ease of nuclear compared with cytoplasmic separation and well-controlled biochemical analyses.

**Prospects for determining mechanism**

miRNAs cause repression via an AGO2 complex that involves a scaffolding protein called GW182; both proteins are often observed as foci in proliferating cells [30]. Interestingly, GW182 is cell-cycle-regulated, with protein levels dramatically reduced and the foci minimized in quiescent cells [31,32], correlating with a loss of translation repression. This cell-cycle-regulated alteration in the AGO2-associated complex constitutes in part the switch to translation activation by miRNAs. The translation activation complex involves the same miRNA and AGO2, but contains a new partner protein, FXR1, and probably other modifications and factors induced upon quiescence [12,14].

At least three distinct mechanisms of translational regulation by miRNAs have been proposed for repression, namely initiation, elongation and co-translational protein degradation [5,6]. All three mechanisms are predicted to be involved in mediating repression. We are currently investigating whether similar mechanisms of translation regulation are involved in mediating translation activation via the quiescence-induced modified AGO2-miRNP complex in the mammalian and *Xenopus* oocyte systems.

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**References**


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