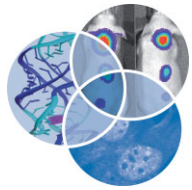
Advanced Review



Posttranscriptional Upregulation by MicroRNAs

Shobha Vasudevan*

MicroRNAs are small non-coding RNA guide molecules that regulate gene expression via association with effector complexes and sequence-specific recognition of target sites on other RNAs; misregulated microRNA expression and functions are linked to a variety of tumors, developmental disorders, and immune disease. MicroRNAs have primarily been demonstrated to mediate posttranscriptional downregulation of expression; translational repression, and deadenylation-dependent decay of messages through partially complementary microRNA target sites in mRNA untranslated regions (UTRs). However, an emerging assortment of studies, discussed in this review, reveal that microRNAs and their associated protein complexes (microribonucleoproteins or microRNPs) can additionally function to posttranscriptionally stimulate gene expression by direct and indirect mechanisms. These reports indicate that microRNA-mediated effects can be selective, regulated by the RNA sequence context, and associated with RNP factors and cellular conditions. Like repression, translation upregulation by microRNAs has been observed to range from fine-tuning effects to significant alterations in expression. These studies uncover remarkable, new abilities of microRNAs and associated microRNPs in gene expression control and underscore the importance of regulation, in *cis* and *trans*, in directing appropriate microRNP responses. © 2011 John Wiley & Sons, Ltd.

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INTRODUCTION

Manipulation of gene expression at the mRNA level, subsequent to the onset of transcription, enables cells with a means to rapidly or transiently change gene expression profiles in response to intracellular changes or extracellular cues.[1,2] Posttranscriptional regulatory mechanisms enhance or reduce gene expression and include: increased mRNA levels, relocalization of mRNAs/mRNPs, mRNP modification, and alteration of the translation machinery to induce or repress the expression of specific mRNAs under distinct conditions.[3,4] In eukaryotes, repression is more common while posttranscriptional upregulation has been observed in specific cell types such as developing germ cells and with distinct transcripts, factors, and conditions. These activation elements are essentially regulatory elements that retain the ability to repress and activate expression in response to distinct cues.[4–8] The cytoplasmic polyadenylation element (CPE) exemplifies a regulatory element that represses translation of specific messages in immature oocytes but activates the same upon maturation of the oocytes.[5,9,10] Importantly, posttranscriptional mechanisms, in particular, RNA stability and translation control mechanisms can be manipulated by non-coding RNAs and RNPs to mediate gene expression responses to signaling cues and altered states.

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REGULATORY RNAS

Gene expression can be controlled posttranscriptionally by regulatory RNAs in conjunction with associated effector proteins to elicit specific expression outcomes. Such RNA sequences act in *trans* as control non-coding RNAs such as small RNAs in bacteria or microRNAs. In *cis*, target microRNA sites or mRNA regulatory sequences and structures recruit *trans*-acting regulatory RNAs and RNPs and can direct processing, localization, stability, and translation of the mRNA in response to intra- and extracellular signals.[4–8]

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Trans-Acting Regulatory RNAs

Small RNAs in Prokaryotes

In bacteria, small RNAs (sRNAs) can alter expression in either direction.[11–13] sRNA regulation of translation often involves the role of RNA-binding proteins, such as HfQ that enables bacteria to adapt to environmental cues. sRNAs in bacteria act to stimulate or inhibit translation usually by base pairing to their targets and by influencing the activities of proteins. sRNAs employ direct mechanisms, targeting the 5′- or 3′-untranslated region (UTR) to alter RNA processing, translation, or mRNA stability, as well as indirect mechanisms, preventing the degradation of an activating sRNA or trapping repressive sRNAs to alleviate repression,[14] similar to some of the newly uncovered functions of microRNAs.

MicroRNAs in Eukaryotes

MicroRNAs are specialized small non-coding 20- to 24-nt RNAs that function as RNA regulators primarily via sequence-specific recognition to guide associated effector RNP complexes and modulate posttranscriptional gene expression.[15] Their aberrant expression or function is associated with profound clinical consequences including tumorigenesis, immune, and developmental disorders.[16–18] MicroRNAs usually recruit a critical effector protein, Argonaute (AGO or eukaryotic initiation factor 2C, eIF2C) to form the functional microRNA associated protein complex (microribonucleoprotein or microRNP). MicroRNAs and thereby microRNPs recognize target RNAs through a pattern of base pairing, which can dictate the functional outcome. Perfect base pairing of the microRNA to its target can lead to mRNA cleavage and decay,[15] while partial base pairing to target sites can lead to mRNA deadenylation[19,20] and translation regulation,[3,21–24] primarily mRNA translation silencing or as discussed in this review, to upregulated expression in response to specific cellular conditions, sequences, and cofactors. Upregulated posttranscriptional expression can be mediated directly by distinct microRNPs, and is then called activation (Figure 1(a)), or indirectly, as a consequence of regulatory effects on microRNA-mediated repression, and then called relief of repression (Figure 1(b)).

CIS-Acting Regulatory RNAs

Target mRNA Specificity

MicroRNAs are predicted bioinformatically to target over a third of the genome[25] but not all predicted mRNAs are subject to regulation in any given cell and environment. Many examples demonstrate that UTRs usually are alternatively polyadenylated to include or preclude essential sites or have additional sequences, which when occupied in a regulated manner, can prevent or permit microRNA activity.[8,26–28] In muscle differentiation, miR145 upregulated myocardin, while miR143 and miR145 itself downregulated other substrates.[29] KLF4 mRNA was upregulated by miR206 in confluent cells and noncancerous cells but was downregulated by miR206 or miR344 in proliferating cancer cells.[30] Therefore, microRNA-mediated upregulation is target specific, dependent on the specific conditions experienced by distinct target mRNAs. The presence of additional mRNA UTR sequences that interfere/synergize with microRNA functions is expected to dictate the final expression outcome. The additional UTR factors recruited, are subject to modulation, such as tissue-specific expression of Dead end 1 and thereby miR430 target mRNAs[8,27] or cell-cycle regulation of Pum1's ability to alter microRNA accessibility to p27 mRNA,[31] adding yet another avenue for regulated expression.

AU-Rich Elements

The AU-rich element (ARE) was discovered as a decay element.[32–34] Additional posttranscriptional roles, repression and upregulation of translation, were further ascribed to the ARE.[1,35–39] The ARE-/U-rich binding protein, HuR, and its related family of ELAV proteins, demonstrated the case as stability factors that influenced not only RNA processing, export and decay but also translation repression and activation.[39–45] Interestingly, both AREs and microRNPs affect common functions of deadenylation and translation, possibly employing similar mechanisms.[46]

Several studies demonstrate interconnections between microRNAs and AREs.[46] Several microRNA targets are predicted (about 75%) or have been demonstrated to be within AU-rich sequences.[47–51] MicroRNPs co-purify ARE-associated proteins; fragile X mental retardation related protein 1 (FXR1-iso-a), which associates with tumor necrosis factor (TNF)α ARE and PAI-RBP1, which associates with PAI mRNA ARE.[52–54] Finally, AREs and their binding factors have been demonstrated to be associated with and affect the functions of microRNPs. TNFα mRNA can be degraded by TTP and miR16, in association with the microRNP,[55] and is translationally activated in quiescent conditions by microRNAs targeting the ARE.[56] HuR can relieve or recruit microRNP-mediated repression[26,44,45,57–59] while microRNAs compete with TTP to increase mRNA stability,[60,61] suggesting coordinated regulation of mRNA expression by AREs and microRNAs.

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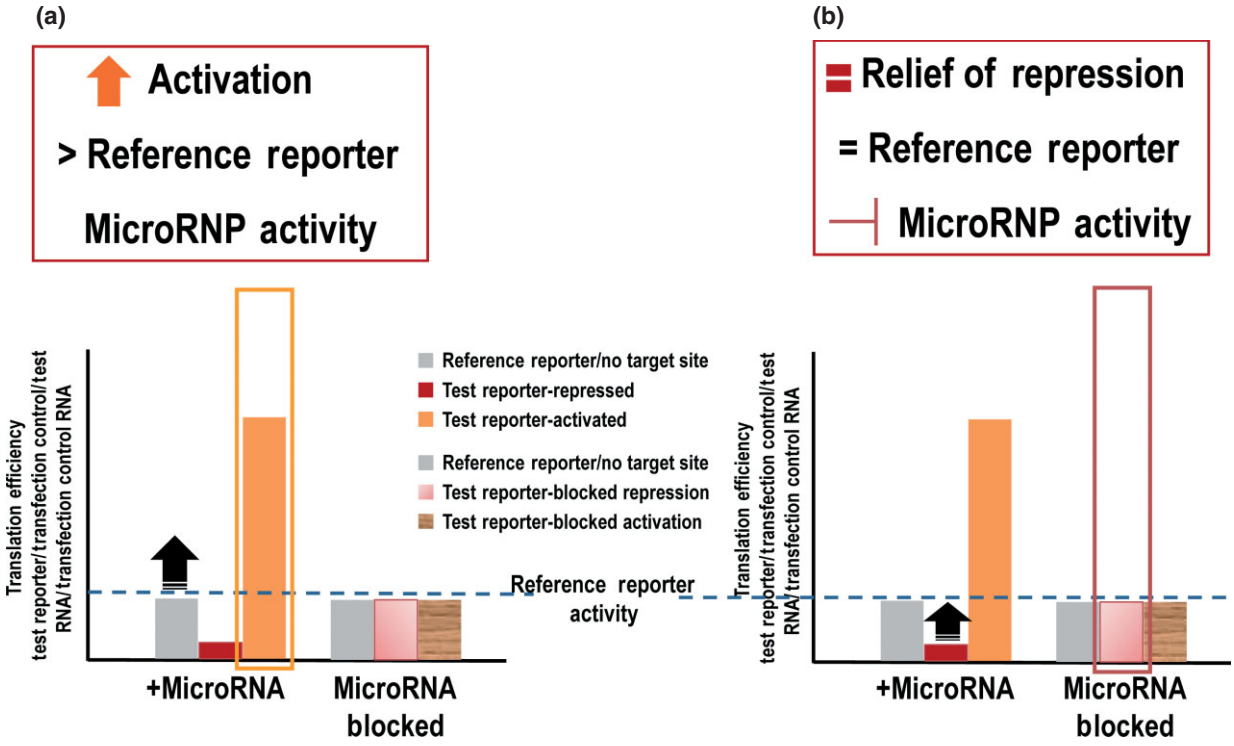

FIGURE 1 | Posttranscriptional upregulation by microRNAs/microRNPs. The expression of a reporter bearing the test target site untranslated region (UTR) (Firefly luciferase with test UTR) is normalized to the expression of a transfection control reporter (Renilla luciferase) to exclude the effects of transfection efficiency and obtain the test-UTR-specific translation ratio. The ratio is further normalized against their RNA levels to obtain translation efficiency.[20] The reference reporter translation efficiency (first and fourth gray bars of each graph) reads out the translation efficiency in the absence of the microRNA target site and provides basal translation levels (dashed blue line). Upregulated expression by microRNAs and microRNPs is observed as direct (activation) or indirect (relief of repression): (a) Activation: In comparison to the basal reference reporter translation efficiency (dashed blue line marking the translation of the first and fourth gray bars of each graph) as shown by a nontargeted or reference reporter, a targeted test reporter can be activated (orange bar with the change from basal reference reporter translation shown by the wide black arrow) when the translation efficiency is greater than that of the reference reporter translation (gray bars, dashed blue line). (b) Relief of repression: The test reporter translation can be repressed (red bar) where its translation is less than that of the reference reporter translation (gray bars, dashed blue line). When the regulatory effect of repression is abrogated, translation efficiency is increased (increase depicted by the wide black arrow) from repressed (red bar) to basal levels (pink bar comparable to the gray bars and dashed blue line depicting the reference reporter translation) because of the blocked repression. When the regulatory effect of activation (orange bar) is abrogated, the increased translation is lost and brought down to basal levels from activated levels (brown bar comparable to the gray bars and dashed blue line depicting the reference reporter translation) because of the blocked activation.

ASSOCIATED FACTORS

eIF2C2

The central factor in microRNA functional outcomes in mammalian cells is eIF2C2 or AGO2. AGO2 targets mRNAs that are partially complementary to microRNAs for deadenylation and translational regulation.[15] AGO2/eIF2C2 was initially purified as co-eIF2a, a translation-enhancing activity from rabbit reticulocyte lysate, which stabilized the ternary complex in the presence of mRNA at low concentrations and co-purified with eIF2 (a critical translation initiation factor that forms the ternary complex with initiator methionyl tRNA, an important intermediate that recruits the tRNA to ribosomes).[21,62,63] These results remain unexplored and the precise functional role of co-eIF2a or eIF2C2 has not been examined in detail;[21] the translation activation observed in recent literature may reflect this upregulatory activity of eIF2C2.

FXR1-iso-a/dFXR1

Fragile-X mental retardation related protein 1 (FXR1) is similar in protein structure to the FMR1 family of KH domain RNA-binding proteins;[64–68] however, the mouse knockout does not show an FMR1-related phenotype and instead demonstrates muscle loss and neonatal death associated with cytokine deregulation.[69,70] FXR1 and its Drosophila orthologue, dFXR1/dFMR1, were

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found to interact with Argonaute in microRNPs as well as with Argonaute-related family members, P-element-induced wimpy testis (PIWI), in MIWI/PIWI and MILI complexes.[52,71,72] FXR1 is not required for downregulation by microRNAs and FXR1-iso-a, which activates translation upon overexpression unlike other FXR1 isoforms and FMR1, is associated with AGO2.[73] FXR1-iso-a was demonstrated to be required for AGO2/microRNA-mediated translation activation of specific transcripts in quiescent cells,[73] suggesting that FXR1-iso-a association in distinct conditions contributes to altered gene expression by the microRNP. Other such factors may also mediate upregulation.

MICRORNA/MICRORNP-MEDIATED UPREGULATED EXPRESSION

Translation upregulation by microRNAs has been observed as a result of two possible outcomes: activation by the direct action of microRNAs/microRNPs (Figure 1(a)) and relief of repression (Figure 1(b)), where the action of a repressive microRNA or microRNP is abrogated. When the relative translation of the test UTR/target site reporter (normalized to a transfection control) or its translation efficiency[20] (further normalized for RNA levels) is greater than the relative translation or translation efficiency of a reference reporter (mutated at or lacking the key test UTR site, normalized similarly) expressed from the same promoter in a parallel experiment, then translation is activated or upregulated indicating that the tested site and microRNA stimulate translation, increasing it over and beyond general translation represented by the reference reporter (Figure 1(a), orange bar compared to gray bar). Relief of repression is the loss of repression by the microRNA, where the regulatory effect or reduction of relative translation of the test reporter below the basal or reference reporter translation (Figure 1(b), red bar compared to gray bar) is alleviated,[26] leading to the test UTR reporter demonstrating translation similar to that of the reference reporter (Figure 1(b), red bar compared to the pink bar, which is similar to the gray bar) and acting like a transcript devoid of microRNA/test UTR repression effects. It should be noted that translation activation would be greater than the reference reporter and is therefore distinct from relief of repression (Figures 1(a) and (b), orange bar compared with the gray bar compared to the pink bar). The translation state when the microRNA is depleted would define whether the microRNA was inducing activation (in which case there would be a loss of stimulated expression and translation would be reduced to that of the reference reporter; Figures 1(a) and (b), activation depicted by the orange bar becomes reduced to that of the brown bar, comparable to the gray bar) or whether the microRNA/RNP was relieved of repressive function (in which case there would be no change as absence of the microRNA would cause the same effect as relief; Figure 1(b), relief of repression depicted by the pink bar, comparable to the gray bar). Biologically, both activation and relief of repression would result in an increase in the relative levels of the protein produced.

The discovery of a potential, although yet unexplored, translation stimulation role of eIF2C2[21,62,63] was further augmented by the elucidation of mRNA translation activation roles of the related PIWI family protein, Aubergine, in the Drosophila germline[74–76] and of the related PIWI proteins in Drosophila and mouse germlines.[77–80] One of the earliest demonstrations that RNAi and potentially microRNA activity may be regulated was observed in early oocyte development in Drosophila.[75,81] A demonstration of hepatitis C virus (HCV) RNA upregulation by miR122 at the RNA level[82] was one of the first indications that microRNAs potentially have silencing independent functions, while transcription at specific promoter regions was demonstrated to be upregulated by small RNAs.[83–86] This was followed by studies demonstrating alleviation of microRNP repression of distinct messages under specific cellular conditions.[26,87] Our study revealed that eIF2C2/AGO2 can activate translation of specific mRNAs in quiescent G0 cells.[56,73,88] Subsequent studies summarized below affirmed that microRNPs have the potential to activate translation directly. Additionally, other studies demonstrate indirect alleviation of repression.

ACTIVATION BY MICRORNAS

Activation of Specific mRNAs Regulated by Cellular Conditions: Effect of Quiescence-Like Conditions

Quiescence (G0) is a unique adaptive response in a population of dividing cells which enables an advantageous escape from harsh situations that lead to permanent arrest; instead, the cell is suspended reversibly in an assortment of G0 states where it can develop and respond adequately to more favorable conditions by returning to the cell cycle.[89] Such reversibly arrested cells are, in contrast to general assumptions, quite active metabolically, requiring the expression of very specific genes to maintain the state, resist harsh stimuli as well as respond to altered conditions.[90,91] These are likely achieved by posttranscriptional

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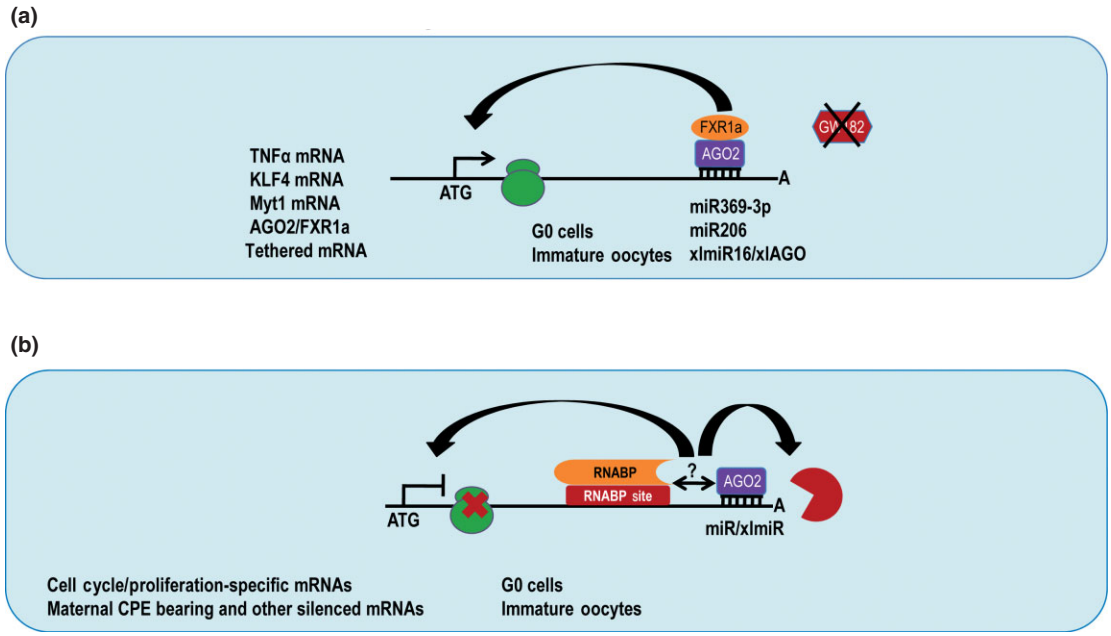

FIGURE 2 | Posttranscriptional regulation in quiescent cells (G0 mammalian cells and G0-like immature oocytes). G0 conditions not only demonstrate selective activation of G0 expressed mRNAs/microRNAs that are required for G0-related functions but also display transcriptional and posttranscriptional downregulation[113] especially of cell-cycle factors and other such undesired genes,[89,91] suggesting that other mRNAs, microRNAs, and regulatory RNA-binding proteins downregulate gene expression of cell-cycle factors[88]; both positive and negative regulation may collectively enable inhibition of the cell cycle and maintenance of the G0 state.[89,91] (a) Translation upregulation of G0-specific mRNAs/ microRNAs. GW182 is an essential component of the repressive microRNP complex regulated in G0.[105–107] In conditions where GW182 interaction with AGO2 is restricted (oocytes, G0 cells, dAGO2), repression is abrogated.[73,108,110–112] Under G0 conditions, FXR1-iso-a (FXR1a) can interact with the microRNP and alter its function to enable activation of specific, natural target mRNAs and of AGO2 or FXR1-iso-a tethered reporters. Activation of translation in quiescent conditions is observed with specific mRNAs such as tumor necrosis factor α (TNFα) and KLF4 in G0 mammalian cells, and Myt1 in immature *Xenopus laevis* oocytes mediated by specific microRNAs, miR369-3p, miR206, and xlmiR16 respectively.[30,56,92] (b) Downregulation of cell-cycle mRNAs, maternal CPE-bearing and other silenced mRNAs. Posttranscriptional downregulation by mechanisms including deadenylation[113] is observed with cell-cycle genes in G0 mammalian cells[89,91] and with maternal mRNAs that are temporarily silenced in immature oocytes such as CPE-bearing mRNAs in *Xenopus laevis* oocytes.[9,10] These mechanisms may be mediated by UTR-RNA-binding regulatory proteins as observed in oocytes[9,10] as well as potentially, by microRNA-dependent mechanisms with or without collaboration with adjoining RNA-binding protein sequences (RNABP site) and factors.

mechanisms[28,89,91] that may include translation upregulation by microRNAs[73,88,92] (Figures 2(a), (b), and 3(a)).

Translation upregulation by microRNAs was observed to be transient and appears to be restricted to specific G0 states and not in G1 arrest or other states of the cell cycle;[30,56,73,88,92] therefore, activation mediated by microRNAs in mammalian cell lines in G0 is less frequently observed than repression. Quiescence is also influenced by cell-to-cell contact, where replating proliferating cells at high density inhibited entry into quiescence and translation activation.[89,93,94] In concurrence, Hwang et al., demonstrated that increasing cell-to-cell contact via increased density of replated proliferating cultures enabled increased repression by microRNAs.[95] A block to cell division by multiple means leads to cell death, G1 arrest or senescence rather than quiescence in cells lacking quiescence-entry capacity, which, in the absence of specific tests and markers to distinguish G0 from G1 arrest and senescence, can misleadingly suggest G0.[89,93,94,96,97]

Altered MicroRNP Components

In quiescent G0 cells, the levels of an essential AGO2-associated component of the repressive microRNP, GW182,[98–104] a component of GW bodies, that contributes to microRNA-mediated deadenylation and repression, decreases, is altered in its interaction with AG0[105–108] and is marked by a decrease in GW bodies,[105–107] although the bodies are not essential for repression.[109] Failure to maintain GW182 interaction with AGO2 leads to a loss of repression[108,110,111] and can be associated with activation.[112] Activation in G0 could be mediated by tethering AGO2 or FXR1-iso-a to a reporter (Figure 2(a)), suggesting that recruitment of

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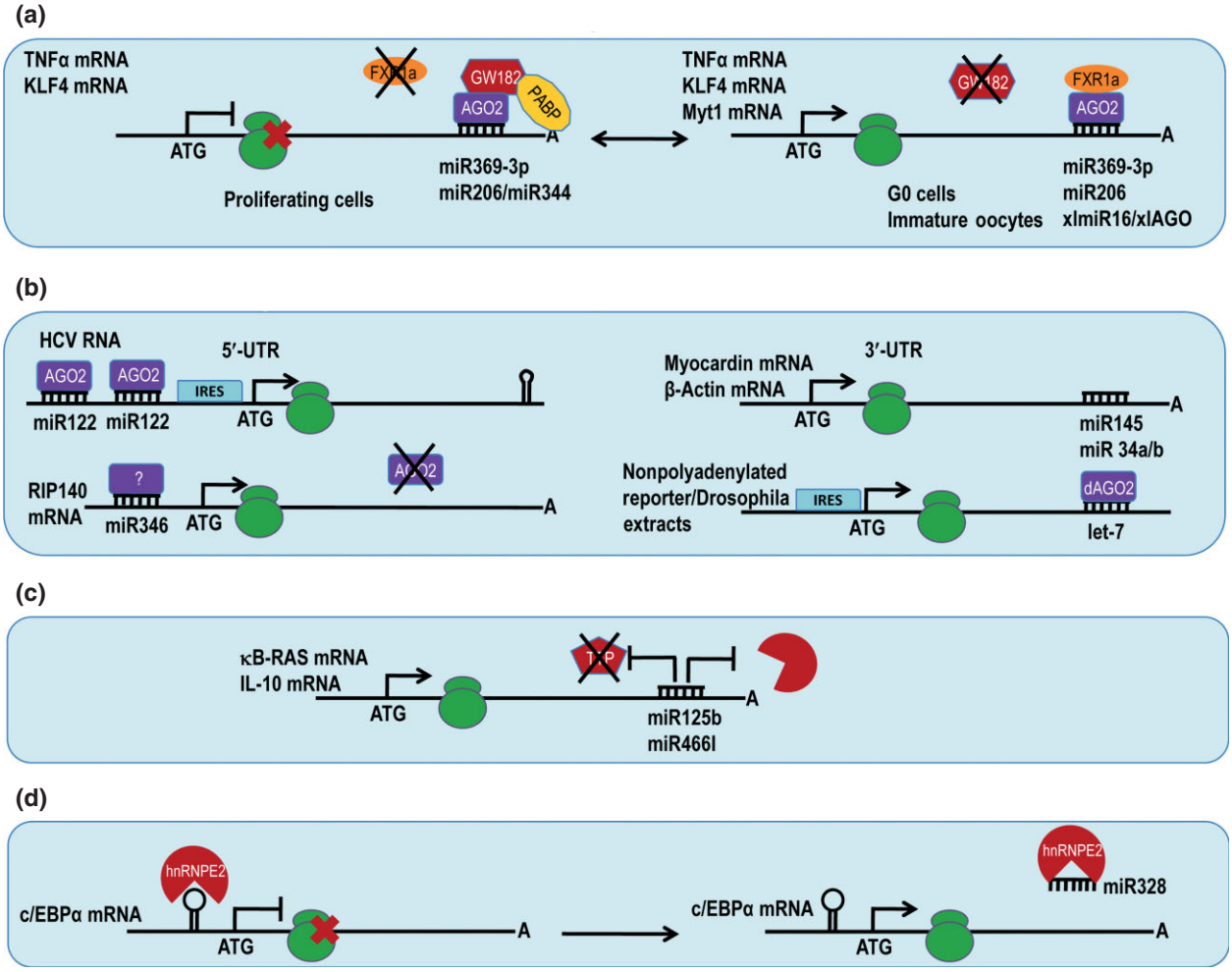

FIGURE 3 | Activation of gene expression by microRNAs. Examples of microRNAs and target mRNAs with target sites in the 5′- or 3′-UTRs (untranslated regions) that demonstrate upregulated expression due to microRNA-mediated activation. (a) Increased translation in response to cellular conditions. miR369-3p and miR206 bind the 3′-UTRs of and upregulate translation of TNFα and KLF4 mRNAs respectively in quiescent mammalian cell lines.[30,56] FXR1-iso-a (FXR1a) and AGO2 are required for translation activation in G0 and in immature *Xenopus laevis* oocytes and are associated with the TNFα mRNA in G0 cells. XlmiR16 binds the 3′-UTR and upregulates translation of Myt1 mRNA in immature *Xenopus laevis* oocytes.[92] In proliferating cells, GW182 and AGO2 associate with target mRNAs and mediate repression. (b) Specific transcripts, microRNAs, and factors. Other specific microRNAs as well as specific factors can activate distinct transcripts with target sites in either the 5′-UTR or the 3′-UTR. 5′-UTR: Liver specific miR122 stimulates translation of HCV RNA through direct binding to two target sites in the 5′-UTR.[134] miR346 interacts with the 5′-UTR of RIP140 and upregulates translation in mouse brain tissue and p19 cells independent of AGO2.[142] 3′-UTR: Binding of miR145 to the 3′-UTR of myocardin mRNA increases its expression during smooth muscle development.[29] Mmu-miR34a/34b-5p binds the 3′-UTR and upregulates translation of an alternatively polyadenylated variant of β-actin mRNA in mouse neuronal cells.[143] Drosophila dAGO2 activates translation of m7G-capped or A-capped IRES-containing 3′-UTR target reporters lacking poly(A) tails in a Drosophila extract system.[112] (c) Increased mRNA stability and translation. miR125b binding to the 3′-UTR of κB-Ras2 mRNA and miR466l binding to interleukin (IL)-10 mRNA mediates increased mRNA stability in human macrophages.[60,61] (d) MicroRNA-mediated decoy. miR328 binds hnRNP-E2 (hnRNPE2) and prevents it from repressing c/EBPα mRNA translation.[145]

an altered microRNP in conditions where interaction of AGO2 with GW182 is decreased mediates activation.

TNFα mRNA and miR369-3p

We found that microRNAs recruit a modified microRNP comprising AGO2 and a specific isoform of FXR1, FXR1-iso-a, to mediate translation activation of specific mRNAs such as TNFα in quiescent G0 cells by miR369-3p (Figures 2(a) and 3(a)), which is induced in G0 in certain cell lines.[56,73] Deadenylation is increased in quiescent mammalian cells,[113] suggestive of a distinctive mechanism of translation of poly(A) tail shortened mRNAs, consistent with some

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of the studies on microRNA-mediated translation upregulation.[112,114]

KLF4 mRNA and miR206 in Quiescent and Non-Transformed Cells

miR206 was demonstrated to cause repression of KLF4 expression in breast cancer cells but not in normal immortalized MCF10A cells. Interestingly, as described above in G0 cells, this effect was driven by the absence of proliferation and by quiescence in confluent RK3E cells, where miR206 levels increased and caused upregulated translation in comparison to cycling cells, where miR344 levels increased instead and caused repression of KLF4 mRNA and 3′UTR reporters[30] (Figures 2(a) and 3(b)). These studies demonstrate that under quiescent conditions, specific G0-modified mRNPs/microRNPs are capable of functioning as translation upregulatory complexes (Figures 2(a) and 3(a)).

Activation and Downregulation in G0

G0 conditions also display transcriptional and post-transcriptional downregulation[113] especially of cell-cycle protein factors and other such undesired genes,[89,91] suggesting that other mRNAs, microRNAs, and regulatory RNA-binding proteins downregulate gene expression of cell-cycle factors, which would enable inhibition of the cell cycle and maintenance of the G0 state (Figure 2(b)). Given the nature of the targets and microRNAs observed, activation in G0 appears restricted specifically to certain G0 expressed mRNAs/microRNAs that would be physiologically required for G0-related functions (Figures 2(a) and 3(a)). These results suggest that microRNAs induced in specific G0 cellular conditions, miR369-3p in the case of TNFα mRNA and miR206 in the case of KLF4 mRNA, may be recruited into distinct, altered microRNPs that promote activation of specific G0 expressed mRNAs, required for the G0 state (Figures 2(a) and 3(a)), while downregulation ensues on preexisting cell-cycle-related gene mRNAs (Figure 2(b)). Given that newly synthesized AGO is required for microRNP function in trypanosomes[115] and that free, unbound AG02 is limiting in the mammalian cell,[116] it may be possible that G0 conditions induce distinct microRNA/mRNA species that would be bound by new AGO complexes, associated with different cofactors that replace the reduction of GW182 interaction in G0[56,73,105,107] to mediate these effects (Figures 2(a) and (b)).

Oocyte MicroRNP Regulation

Proteomic analysis of Dicer depletion in oocytes revealed limited loss of downregulation,[75,81] suggesting that repression by microRNAs may be regulated at the early stages of development, emerging in subsequent stages of egg maturation. Recent studies demonstrated that mouse oocytes lacked GW182 colocalization with AGO2 and while downregulation of perfect complementary target sites was maintained, repression by microRNAs was greatly alleviated[108,111,117] although microRNPs are present.[118] These features that are in common with G0 conditions indicate the potential for selective translation activation.

Translation activation has been well observed in the developing germline, which are non-proliferating quiescent-like cells, yet undergo growth in size, changes, and maintenance,[6,7,79,119–123] using regulated posttranscriptional mechanisms including selective translation (Figures 2(a), (b) and 3(a)). Unlike mouse oocytes and human AGO2, the limiting Argonaute in oocytes of *Xenopus laevis* is distinct in its behavior and lacked the usual RNAi-mediated cleavage activity,[124] indicating a specialized role. Oocyte immaturity is in part regulated by the cAMP pathway,[125–131] which also influences quiescence in mammalian cells.[93,97] Our study uncovered that in response to cAMP regulation in immature oocytes, AGO recruits xlmiR16 and promotes the expression of Myt1, a specific mRNA that would need to be expressed to maintain immaturity[92] (Figures 2(a), 3(a)). Consistently, upon maturation, Myt1 expression is no longer required and is abrogated and replaced by CPE-regulated expression of other maternal mRNAs required upon maturation[9,10] (Figure 2(b)).

Specific mRNAs, microRNAs, and Factors

HCV RNA Translation

HCV tropism for the liver was demonstrated to be in part dependent on miR122, a microRNA abundant in the liver that enabled increased levels of the HCV RNA through base pairing at two 5′-UTR miR122 sites.[82,132,133] New investigations using monocistronic reporters bearing the natural UTRs of HCV revealed that the 5′-UTR miR122 sites also caused translation upregulation[134] (Figure 3(b)). Relocation of the HCV miR122 target sites from the 5′-UTR to the 3′-UTR led to repression; however, microRNP/AGO2 tethering at the 5′-end also permits repression in mammalian cells,[135] and activation is observed via 3′-UTR sites in quiescent cells and in extracts.[73,112] This suggests that collaboration of the miR122 sites with adjoining UTR sequences and/or the overall RNP structure is conducive to activate expression in the 5′-UTR as supported by mutational analysis[114] but may inadvertently interfere with activation upon relocation to the 3′-UTR.[134] The variable region of the HCV

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3′-UTR influenced translation efficiency via the 5′-UTR miR122 target sites, suggesting an important interconnected role between the 5′-UTR and 3′-UTR to effect translation activation.[134,136] Upon miR122 base pairing with the 5′-UTR target site, the HCV mRNA demonstrated increased association with 40S subunits, increased 48S complex incorporation and at later stages, enhanced formation of polysomes[134,136] in a mechanism involving AGO2.[137] The HCV 3′-UTR lacks a poly(A) tail, which appears to be an important feature common to some of the studies of translation activation by microRNAs.[92,112,114,134,136] Similar to the above studies (Figures 2(a) and 3(a)), HCV translation by miR122 is also influenced by G0 synchronization of the cells (C. Fehr and M. Niepmann, personal communication), potentially indicating common effects of G0 on the microRNP complex.

5′-Terminal OligoPyrimidine (TOP) Tract mRNAs

5′-Terminal oligopyrimidine (TOP) tract mRNAs that often encode ribosomal protein and other protein-synthesis-related factor mRNAs, harbor a TOP tract that renders these mRNAs translationally repressed upon arrest at various points in the cell cycle and upon nutritional deprivation.[138,139] miR10a binds such target ribosomal protein mRNAs immediately downstream of the TOP sequence at non-canonical microRNA target sites, alleviates TOP-mediated repression and stimulates translation in a rapamycin/mTOR sensitive manner during amino acid starvation, following anisomycin treatment or overexpression of a mutant activated RAS; treatments that activate TOP mRNA translation.[140] The TOP sequence appears to be required for the activation mediated by miR10a.

Drosophila Extract/Drosophila AGO2 (dAGO2)

The nature of the double strand influences whether the small RNA is recruited to dAGO2 or dAGO1 respectively in Drosophila. dAGO1 is an inefficient nuclease but can mediate translation repression; in particular, repression requiring GW182 and mediated after cap recognition.[112,141] dAGO2 functions as an RNAi nuclease apart from demonstrating cap dependent translation repression with poly(A)+ mRNA in an oocyte extract translation system but does not interact with GW182.[141] Remarkably, only dAGO2 mediates translation activation of poly(A)- mRNA in the extract system (Figure 3(b)), suggesting that this microRNP/mRNP complex required a poly(A)-status for translation activation.[112] Activation is also observed in quiescent mammalian cells[56] and in immature oocytes[92] where mRNAs generally have shortened poly(A) tails[5,113] and with HCV RNA, where the mRNA is not polyadenylated and as in the Drosophila extract system, is internal ribosome entry site (IRES) driven and lacks a cap.[114,134,136]

miR346

In brain and p19 cells, miR346 upregulates expression of a specialized splice variant of RIP140 mRNA via its 5′-UTR. Intriguingly, upregulation was also observed upon knockdown of AGO2,[142] indicating that another RNP complex or RNA structural alterations by the microRNA may mediate upregulation (Figure 3(b)).

miR34a/b-5

The neuronal tissue specific, alternatively cleaved and polyadenylated longer form of β-actin mRNA harbors a miR34a/b-5 target site that specifically upregulated translation without increasing mRNA levels at neuronal synapses[143] (Figure 3(b)).

miR223

miR223 increases along with Glut4 expression in type 2 diabetic patient samples of the insulin resistant heart. miR223 increase in neonatal rat ventricular myocytes, cardiomyocytes, and cell lines leads to Glut4 mRNA translation upregulation without altering mRNA levels, describing another specific microRNA targeting a clinically important mRNA in cardiomyocytes and potentially exacerbating the disease state in the insulin resistant heart.[144]

miR145

miR145 promotes the quiescent phenotype of smooth muscle cells and smooth muscle differentiation via regulated expression of some of its targets including upregulated expression of myocardin (Figure 3(b)), a component of the molecular switch for the vascular smooth muscle development, which bears miR145 target sites in its 3′-UTR.[29] Repression was mediated by other microRNAs in the same cells and miR145 represses other targets, suggesting that the myocardin 3′-UTR, which is sufficient to replicate the same effect in COS7 cells, bears all the necessary signals to mediate upregulation.

Increased RNA Levels and Expression by Competition with Decay Factors

The ARE decay pathway is often interconnected with microRNA regulation, where microRNA effects are mediated[55,57,73] or abrogated[26,59] due to the action of ARE-binding factors. In turn, microRNAs can

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also mediate upregulation indirectly by competing with or influencing ARE-binding decay factors leading to stabilization by increased mRNA levels[60,61] (Figure 3(c)). MicroRNAs stabilize specific target transcripts by preventing association of the ARE decay factor, TTP: miR466l upregulates IL-10 mRNA levels and miR125b enhances the stability of κB-Ras2 mRNA by preventing TTP binding to the ARE, thereby preventing degradation by TTP and enhancing their expression in macrophages[60,61] (Figure 3(c)).

MicroRNA-Mediated Decoy of Repressive Proteins

Unlike traditional relief of repression, where a microRNA is prevented from associating with its targets and functioning (Figure 1(b), pink bar compared to red bar), a distinct microRNA has recently been demonstrated to also prevent repressive factors from accessing their targets, thus activating expression, similar to sRNAs in bacteria. In blast crisis chronic myelogenous leukemia (CML-BC), miR328 levels decrease via the MAPK pathway. Restoration of miR328 expression rescues differentiation and impairs survival of leukemic blasts. miR328 was demonstrated to decoy away a repressive protein, hnRNP E2 from its target mRNA, C/EBPα, whose expression was then upregulated in a seed sequence independent manner[145] (Figure 3(d)) and promoted differentiation. However, the same microRNA repressed another target mRNA encoding the survival factor for leukemic blasts, PIM1, further contributing to differentiation.[145,146] These results suggest that microRNAs can exert dual mechanisms to lead to a common biological outcome: repressing specific targets via seed sequence mRNA target recognition while blocking repressive proteins from functioning and thereby causing upregulated expression of other targets (Figure 3(d)).

RELIEF OF REPRESSION

Relief of Repression by Target mRNA Manipulation

Abrogation of microRNA-controlled translation repression in mammalian cells can be mediated by several mechanisms by RNA-binding proteins that bind the mRNA and influence the target site, mRNA localization, or mRNA structure, and thereby microRNA access or function. Bhattacharyya et al., first reported that a 3′-UTR U-rich sequence that binds HuR upon stress, interferes with microRNA repression upstream mediated by miR122 on the CAT-1 mRNA[26] in response to amino acid starvation and other forms of cellular stresses, leading to relocalization of the mRNA/mRNP from repressive GW/P bodies in the cytoplasm to polysomes (Figure 4(a)). Similarly, miR134-mediated repression of Limk1 mRNA in the dendritic spines is alleviated in response to extracellular stimuli involving the TOR pathway.[87,147,148] HuR can also compete for the microRNA target site negating microRNA repression by competing directly as in the case of RhoB mRNA and of TOP2A mRNA where miR19 and miR548c-3p respectively are prevented access to the target site by HuR binding to the mRNA.[58,59] However, these functions of HuR are regulated and microRNA/mRNA specific[149] as HuR has also been demonstrated to function in concert with microRNAs to promote repression.[57]

Another RNA-binding protein, Dead end 1 (DND1), alleviates repression and downregulation by binding to specific mRNA 3′-UTRs via U-rich regions, thereby preventing microRNAs from binding their target sites[8,27] (Figure 4(a)). In contrast, the UTR-binding Pumilio 1 (Pum1,[150] binds specific mRNAs such as p27KIP1 in a cell-cycle regulated manner and opens an RNA stem loop structure that obscures a microRNA binding site and thereby promotes microRNA access and repression of p27 mRNA exclusively in cycling cells but not in arrested cells.[31,151] Pum1 is phosphorylated in cycling cells to enable its ability to bind RNA while Pum1 RNA binding and overall stability is reduced in arrested cells. The inability of Pum1 to bind in arrested cells permits the RNA stem loop structure of p27 mRNA 3′-UTR to block microRNA access and thereby cause relief of repression.[31,151] DND1 is also regulated: DND1 prevents miR430-mediated repression of Nanos1 and TDRD7 mRNAs in primordial germ cells in zebrafish; however, miR430, in the developing embryo, where DND1 is absent, is essential for deadenylation and removal of maternal transcripts. Therefore, alleviation of repression is further regulated and restricted to specific tissues and stages of the cell cycle via the regulation of expression and function of the relieving mediators.

Relief of Repression by Decoy

Relief of Repression by Decoy Non-Coding RNAs

Pseudogene transcripts and non-coding RNAs with target site sequences can compete with mRNAs for microRNA binding and result in alleviation of microRNA targets.[152–154] PTEN, as well as its pseudogene, PTENP1, are tumor suppressors targeted by miR19b and miR20a; however, PTENP1 functions as a tumor suppressor by acting as a decoy for

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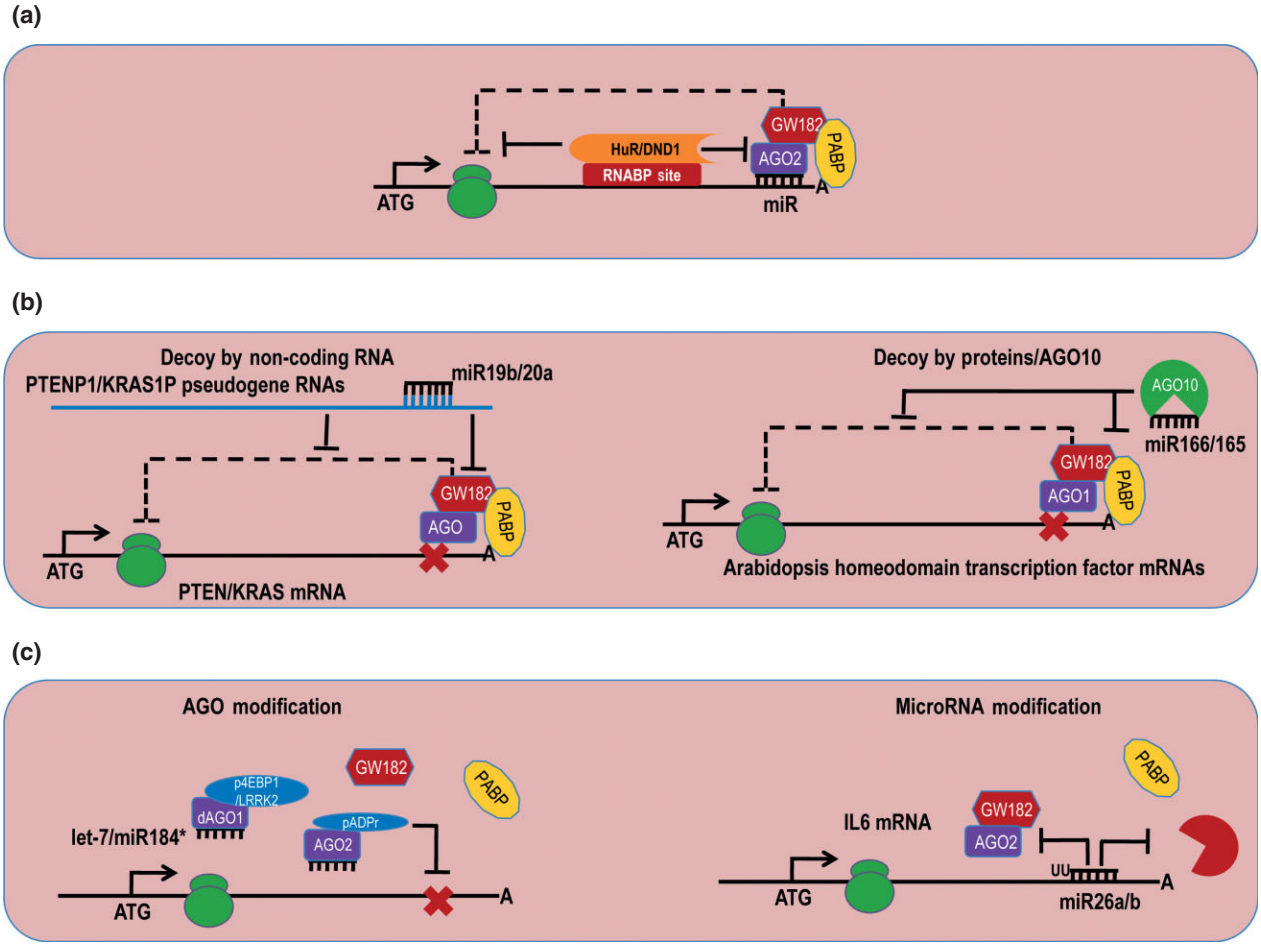

FIGURE 4 | Relief of repression of gene expression by microRNAs. Examples of microRNAs and specific target mRNAs demonstrating relief of microRNA-mediated repression. (a) Relief of repression by target mRNA manipulation: influence of RNA-binding proteins on the target site. Specific UTR-RNA-binding, regulatory proteins, such as HuR and DND1, can bind upstream or downstream of microRNA target sites in a regulated manner and influence the target site-microRNP interaction, function, or alter the localization of the RNP, leading to alleviation of repression by the microRNP.[26,27] (b) Relief of repression by decoy. Specific non-coding RNAs such as pseudogene transcripts can relieve repression by decoying away distinct microRNAs via base pairing and functioning as decoy targets while specific factors can relieve repression by functioning as decoy AGO proteins. Non-coding RNA: Non-coding RNAs such as pseudogene transcripts PTENP1 and KRAS1P (depicted by a blue line base pairing with the microRNA), which bear similar sites to that found on the target PTEN and KRAS mRNAs are capable of binding and preventing microRNAs, miR19b, and miR20a from accessing their transcripts.[152] Decoy by proteins/AGO10: Decoy proteins such as AGO10 in Arabidopsis is specifically expressed in shoot apical meristem to decoy miR166/165 away from the repressive AGO1 microRNP, thereby preventing repression of their targets, homeodomain leucine zipper transcription factor mRNAs, resulting in maintenance of undifferentiated shoot apical meristem.[155] (c) Relief of repression by microRNP modification. The microRNP complex effector protein, AGO, as well as the microRNA may be modified to abrogate repression and thereby, permit expression. Stress-induced modification of AGO: AGO proteins can be modified by poly-ADP ribose (pADPr) as a stress response to conditions like amino acid starvation, glucose starvation, and anisomycin, leading to relocalization of AGO2 to the cytoplasm and decreased ability to associate with target sites and cause cleavage or repression.[158] Mutant active LRRK2 in familial and sporadic Parkinson's disease and age-related neuronal degeneration in Drosophila causes phosphorylation of 4E-BP1 (p4EBP1), which associates more strongly with dAGO1 and hAGO2 and abrogates specific microRNA (let-7 and miR184*)-mediated repression.[159] Uridylation of microRNAs: MicroRNAs modified at the 3′-end, especially by uridylation, can lead to either altered stability of the microRNA or as in the case of miR26a/b, abrogate repression and mediate expression of target mRNAs such as IL-6 mRNA.[180]

these microRNAs to relieve and promote expression of PTEN mRNA levels and protein[152] (Figure 4(b)). Other pseudogene targets such as KRAS1P 3′-UTR also lead to increased levels of its counterpart gene, KRAS.[152] Therefore, non-coding RNAs such as pseudogene transcripts[152] and viral transcripts[153] can act as decoys or sponges[154] to sequester microRNAs and promote expression (Figure 4(b)).

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Relief of Repression by a Decoy Argonaute Protein, AGO10

A unique example of relief of repression in Arabidopsis involves AGO10, a specific Argonaute that recruits miR166/165 by recognizing the distinctive secondary structure of its duplex form and decoys it away from AGO1, a primary microRNA effector of repression in plants. This enables expression of the microRNA targets (Figure 4(b)), homeodomain leucine zipper transcription factors, and thereby maintenance of undifferentiated cells of the shoot apical meristem.[155] These results suggest that specialized AGO proteins may promote rather than repress expression by sequestering microRNAs from repression-effecting microRNPs.

Relief of Repression by MicroRNP Modification

Relief of Repression by Stress-Induced AGO Modification by Poly-ADP Ribose

Relief of repression by microRNAs as a stress response had been observed previously along with relocalization of target mRNAs from P bodies[26] to the cytoplasm. The formation of stress bodies and relocalization of AGO from P bodies to a lesser extent to stress granules was also observed;[156,157] however, quantitatively, most AGO relocated to the cytoplasm where stress-induced relief of repression was observed as a result of poly-ADP ribose modifications of AGO, leading to decreased repression and cleavage by microRNAs[158] (Figure 4(c)).

Relief of Repression by Stress-Induced Modification of AGO by Pathogenic (Kinase Active) LRRK2

Gain of function mutations in leucine-rich repeat kinase 2 (pathogenic LRRK2, associated with familial and sporadic Parkinson's disease and age-dependent degeneration of dopaminergic neurons) in Drosophila caused loss of specific microRNA repression (let-7 and miR184*) because of its association with dAGO1 and its subsequent association with the LRRK2 kinase substrate, phosphorylated 4E-BP1. LRRK2 phosphorylates 4E-BP1 and a dominant phospho-4E-BP1 mutant was sufficient to associate strongly with hAGO2 and antagonize repression by let-7 in human cell lines (Figure 4(c)). LRRK2 also appears to cause decreased dAGO1 levels in aging fly brains.[159] This modification of microRNP function and relief of repression is not only tissue- and stress/aging-specific but also selective for particular microRNAs/microRNPs, suggesting that individual microRNAs may associate with distinct microRNP complexes.[160] Consistently, overexpression of 4E-BP was demonstrated to be neuroprotective in the LRRK2 model and its role is important in the aging brain.[161,162] How phospho-4E-BP1 associates with dAGO1 but not with dAGO2 and alleviates specific microRNA-mediated repression remains to be investigated.

Relief of Repression by MicroRNA Uridylation

3′-end modification of microRNAs is now reported to be widespread due to high throughput sequencing analyses although the precise roles of these modifications have not been delineated.[163–169] Uridylation has traditionally been associated with increased instability,[165,167,170–177] while adenylation has been demonstrated to cause increased stabilization of the microRNA.[178,179] Uridylation of specific microRNAs, miR26a and miR26b, lead to the alteration of microRNA functions; relieving repression and promoting expression of a specific cytokine target mRNA, IL-6[180] (Figure 4(c)). How the modified microRNA leads to a loss of repression and increased specific expression remains to be further investigated.

MECHANISTIC FEATURES AMONG EXAMPLES OF MICRORNA-MEDIATED ACTIVATION

MicroRNAs or siRNAs have been observed to predominantly cause downregulation, either as repressed translation, deadenylation, or cleavage of the mRNA.[181] Activation has been observed in a growing array of studies with specific mRNAs by distinct microRNAs and in particular cellular conditions, suggesting that activation by microRNAs is a specialized, regulated pathway. Exploring the molecular similarities and differences of translation activation among distinct cases of activation (Figures 2 and 3) would significantly expand our understanding of the mechanistic features and functions of microRNPs.

GW182 Interaction and MicroRNP Modification

An important feature of translation activation appears to be the reduction of GW182 levels[105,107] and its interaction with AGO2.[73,88,108,110–112] AGO2 interaction with GW182 was demonstrated to be essential for translation repression.[98–104] Abrogating GW182 or its interaction with AGO2 leads to a loss or relief of repression[110] or to activation as in the case of dAGO2 with unadenylated reporters[112,141] and its reduction did not affect activation by HCV as

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significantly as reduction of AGO2,[114] suggesting that the interaction with GW182 is restricted to mediate translation activation.[73,92] GW182 bodies decrease and GW182 and its interaction with AGO appear reduced in quiescent-like G0 mammalian cells or oocytes,[105,108,111] where repression is reduced and translation activation is observed.[73,92] In the absence or alteration of the repressive AGO-GW182 interaction in G0, specific mRNAs may instead recruit a translation activating complex, such as AGO–FXR1-iso-a as in the case of TNFα and in oocytes, which relocalizes to polysomes in G0.[56,73,88] Regulation by G0 cAMP/p21-activated kinase (PAK) kinase signals may give FXR1-iso-a an advantage[182] in quiescent cells and lead to recruitment of the activating AGO–FXR1-iso-a complex[56,73,88,92] when AGO–GW182 interaction is altered.[105,108,111] Additionally, specific mRNA features such as the lack of a cap or a poly(A) tail as in the case of HCV RNA[114,134,136] and in regulation by dAGO2,[112] may cause preferential recruitment of an activation complex over the repressive AGO–GW182 by such distinct mRNAs.

mRNA Features-Role of the Cap and Poly(A) Tail

MicroRNAs also mediate deadenylation of their messages[19,20] and addition of a poly(A) tail in *trans* to nuclease-treated reticulocyte lysate extracts enables microRNA-mediated translation repression in these lysates,[183] while ribosome profiling suggested primarily deadenylation in the case of three microRNAs.[184] Deadenylation occurs subsequent to translation inhibition and in other cases, is insufficient to account for the translation repression effect, which is also observed on non-polyadenylated transcripts.[20,185–187] GW182, a core component of the repressive complex,[98,99,103,104,188] was recently demonstrated to bind poly(A) binding protein, PABP, and further recruit CAF1-CCR4 deadenylase; these associations were required for microRNA-mediated mRNA silencing.[100–102,185,189–191] In contrast, in several of the above examples of translation activation, the RNP complex fails to interact with GW182[73,108,112,141] and lacks a poly(A) tail[112] or have short poly(A) tails due to general poly(A) shortening.[113] Additionally, translation upregulation with the HCV mRNA and miR122 was primarily observed with its natural 3′-UTR that precludes a poly(A) tail on the mRNA and in the absence of a 5′-cap while addition of a poly(A) further prevented activation of capped HCV mRNA reporters.[114] Activation by dAGO2 in a Drosophila extract system required the use of non-polyadenylated reporters and reporters with a poly(A) tail precluded observation of activation in oocytes.[92] These observations suggest that these two features, reduced GW182 interaction and altered PABP/poly(A), represent a potential, common mechanism that in part transforms the mRNP/microRNP into a translation activation complex. These data indicate that the activation of specific mRNAs may involve distinct interactions with the translation machinery via altering the role of the poly(A) tail/PABP that remains to be elucidated.

Specific Interactions with Components of the Protein Synthesis Machinery

MicroRNAs have been demonstrated to repress translation by several mechanisms including translation initiation, post-initiation, and nascent peptide turnover.[23,24,192] AGO and PIWI proteins associate with Murine VASA homolog or MVH, a homolog of VASA, which functions to activate translation of specific germ-cell mRNAs through interactions with the general translation initiation factor, eIF5B,[193–197] enabling 60S joining in translation initiation.[3,198,199] Repressed microRNP targets were shown to be associated with 40S subunits,[200] and Let-7 microRNA-mediated functions are affected by eIF3.[201] EIF3 is an essential translation factor that associates with the 40S subunit of the ribosome and enables its association with the ternary complex and with mRNA, and can stabilize the ternary complex in the presence of the mRNA.[198,199,202] Although requiring further investigation, EIF2C was previously purified in association with eIF3, isolated as co-eIF-2A and co-eIF-2C components respectively, of a co-eIF2 complex associated with eIF2.[62,63] The closely related MILI was recently demonstrated to be associated with eIF3a and stimulates translation.[79] It remains to be investigated whether these associations regulate general ribosome recruitment or indicate a role for specific translation activation. These interactions do not exclude effects on subsequent steps in protein synthesis as observed with microRNP-mediated repression.[23,24,192] Finally, many of the examples of microRNA-mediated upregulation occur with transcripts and cellular conditions that involve specialized translation features: IRES and a lack of a poly(A) tail with HCV or dAGO2 in Drosophila extracts, a 5′ TOP sequence and the mTOR pathway, short poly (A) mRNAs in conditions where general translation is redirected to specific mRNA translation as in oocytes and G0 cells. These features suggest that activation involves alternative translation mechanisms that are adapted by the microRNA pathway for specific mRNAs to elicit precise gene expression to fulfill the biological requirements of these distinct conditions.

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CONCLUSION

Posttranscriptional upregulation by microRNPs has only recently begun to be examined with new revelations about microRNP functions. An emerging concept from these observations is that microRNAs may act in concert with particular mRNP complexes and in response to specific cellular conditions to elicit distinct gene expression outcomes. A comparison of their mechanisms and common requirements as well as an investigation of regulatory switches that mediate between repression and stimulation will reveal greater insights into microRNP functions in gene expression control.

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