



## Minireview

# Regulation of mammalian microRNA processing and function by cellular signaling and subcellular localization

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## ABSTRACT

For many microRNAs, in many normal tissues and in cancer cells, the cellular levels of mature microRNAs are not simply determined by transcription of microRNA genes. This mini-review will discuss how microRNA biogenesis and function can be regulated by various nuclear and cytoplasmic processing events, including emerging evidence that microRNA pathway components can be selectively regulated by control of their subcellular localization and by modifications that occur during dynamic cellular signaling. Finally, I will briefly summarize studies of microRNAs in synaptic fractions of adult mouse forebrain, which may serve as a model for other cell types as well.

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## 1. Introduction

The purpose of this brief mini-review is to discuss some of the current gaps in research on the cellular regulation of microRNA processing and function—particularly those questions that do not appear to be currently under study by many investigators.

The general sequence of microRNA biogenesis and function has been well characterized [e.g., see reviews in refs. [1–4]]. In mammals, the genes encoding microRNAs are transcribed by pol II as primary transcripts, or pri-miRs, which appear to be induced and regulated by transcription factors in a fashion that is similar, if not identical, to that of conventional mRNAs (though a few microRNA genes may be transcribed by pol III [5]). The primary transcripts are processed in the nucleus by the RNase III enzyme Drosha working with co-factors (DGCR8 and RNA helicase) to form microRNA precursors, or pre-miRs, ~70–110 nt in length (though some intronic pri-miRs bypass Drosha processing and are spliced out of the surrounding introns [6]). MicroRNA precursors have characteristic stem-loop secondary structures and are exported via exportin 5 to the cytoplasm.

The microRNA precursors are processed further within the cytoplasm by a different RNase III enzyme, dicer, and its co-factors (PACT and TRBP) to form small dsRNAs ~22 nt in length. The two strands become separated and one strand is incorporated into the RNA Induced Silencing Complex (RISC). The core component of RISC is an

Argonaute homologue (in mammals, this is called eIF2c; four eIF2c isoforms are expressed and all four are associated with RISC, though only eIF2c2 has “slicer” activity directed against dsRNAs). In turn, microRNA-loaded RISC binds to target mRNAs and regulates their translation by one of several different mechanisms [7]. Until recently, it was thought that microRNAs always inhibit translation, but recent evidence suggests that under some conditions, microRNAs may actually stimulate translation of the target mRNAs [8], see also [9,10]. Also, until recently, it was thought that microRNAs targeted only cytoplasmic messenger RNAs; however, at least one microRNA is transported actively into the nucleus [11] and it appears that microRNAs may target cytoplasmic noncoding RNAs, including repeat-derived RNAs [12–14].

## 2. Regulation of cellular microRNA levels by nuclear events: RNA editing, Drosha processing and nuclear export of pri-miRs and pre-miRs

A large and growing literature has demonstrated that for many individual microRNAs, the levels of primary transcripts, precursors and mature microRNAs may show little, if any, correlation within a given tissue or cell type. These observations strongly suggest that the levels of mature microRNAs are not simply determined by transcription of microRNA genes; rather, one or more steps in the processing pathway can be critical for regulating the final microRNA levels that are expressed within a cell. (These observations are also consistent with the possibility that pri-miRs or pre-miRs may have direct biological functions, though there is currently no experimental evidence for this.)

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At least three different regulated steps in nuclear microRNA processing have been documented experimentally:

- a) In some cases, site-specific RNA editing of individual pri-miRs and/or pre-miRs occurs in a tissue-specific pattern [15,16]. The edited pri-miRs cannot be cleaved by Drosha [16] and the pre-miRs cannot be cleaved further by dicer [15], which therefore blocks the production of mature microRNAs in certain tissues.
- b) In other cases, certain pri-miRs are transported out of the nucleus into the cytoplasm [17,18]. Drosha is generally presumed to be a nuclear protein, so if cytoplasmic pri-miRs fail to be processed further, they may be recognized as aberrant and destroyed without creating pre-miRs [18] (note, however, that it has not been excluded that pri-miRs may have independent biological functions in the cytoplasm, nor that Drosha can also be expressed in the cytoplasm under certain conditions as well).
- c) In some cases, pri-miRs are not processed by Drosha and remain in the nucleus [19,20].
- d) Finally, in some cases, pre-miRs are formed, but they appear to remain within the nucleus without being transported into the cytoplasm [21,22]. These pre-miRs are not subjected to RNA editing and the basis for the block remains unclear.

These nuclear mechanisms are specific in two senses: they may be restricted to specific cell types, and may affect individual microRNAs but not others within the same cell. They have been described both in normal tissues and in cancer cells. However, no one has yet reported whether nuclear processing steps can be regulated by cellular events occurring in real-time (cf. below).

### 3. Regulation of cellular microRNA levels by subcellular localization of microRNA pathway components

There is also growing evidence that microRNA levels or localization can be regulated by cytoplasmic processing steps:

- a) Little is known regarding the normal half-life of microRNA precursors once in the cytoplasm. However, several studies have reported that microRNA precursors are associated with proteins, including fragile X mental retardation protein (FMRP) and Argonaute homologues [23,24], which may provide a basis for their transport within cells and may regulate their interaction with dicer. FMRP interacts with multiple components of the microRNA machinery including dicer, RISC, pre-miRs and mature microRNAs, and at least some of its functions in regulating protein synthesis and dendritic spine development appear to be mediated by microRNAs [25,26]. Note that mature microRNA levels are grossly normal within brains of neonatal mice lacking functional FMRP [27]. This does not exclude a role for FMRP in processing pre-miRs, however, since FMRP homologues FXR1P and FXR2P also bind pre-miRs [28] and are expressed in brain even in FMRP-null mice.
- b) Obernosterer et al. [29] have shown that a particular microRNA, mir-138, expresses pre-miRs that are processed into mature microRNA selectively in brain tissue, due to the presence of a cytoplasmic inhibitor in non-neural cells that prevents cleavage by dicer [30]. This inhibitor is specific for mir-138 and did not affect another microRNA tested. It is not known whether cytoplasmic inhibitors play a more widespread role in regulating the interaction of dicer with pre-miRs in other situations.

- c) Both Argonaute homologues and dicer have been reported to be associated with ER and Golgi membranes in various cell types [31–33], which could conceivably facilitate the local formation or action of microRNAs in their vicinity.
- d) There is also experimental evidence that dicer and Argonaute homologues are associated with cytoskeletal elements within mammalian cells, and that cellular activation can lead to the liberation of these elements into the cytoplasm [34] (see below). Genetic evidence in *C. elegans* has shown that mutations in various cytoskeletal proteins can alter cellular microRNA levels, though no direct interactions of microRNAs or pre-miRs with the cytoskeleton have been reported in that species [35].
- e) Finally, Argonaute homologues are associated with a number of proteins and RNAs that are not obviously related to RISC function (e.g., gemins [36,37] and mitochondrial transfer RNA [38]). Possibly these may represent entirely different functions of Argonaute homologues, or may represent complexes which sequester Argonaute and thus inhibit RISC formation.

Once dicer has cleaved the pre-miR into a short dsRNA hybrid, and one strand (i.e., the mature microRNA) has been incorporated into RISC the mature microRNA may potentially regulate its target mRNAs. However, while little is known about the turnover rate of RISC that is bound to its targets, literally nothing is known regarding the fate or turnover of loaded RISC that is NOT bound to a target mRNA. Conceivably lack of suitable target mRNAs may cause an accelerated loss of the corresponding microRNAs [39]. In post-mitotic cells, the time course of RISC repression seems to last at least several weeks [40], suggesting that RISC is extremely stable, although it is not clear whether this refers to target-bound or unbound RISC. The observation that levels of mature microRNAs are typically 20–50 times higher than the corresponding steady-state levels of the pre-miR [41] also implies that mature microRNAs are very stable within cells. It is not currently known whether the turnover of microRNAs or RISC can be regulated by cellular events.

### 4. Regulation of the microRNA pathway by cellular signaling

Relatively little attention has been focused to date on the dynamic, physiological, real-time regulation of microRNAs within cells in response to cellular signaling, but this is likely to be a major arena for future investigation.

#### 4.1. Proteases

At least two studies have reported that neuronal stimulation leading to intracellular calcium fluxes can activate proteases that cleave microRNA pathway components. In the case of adult mouse forebrain neurons, Lugli et al. [34] found that activation of calpain leads to the liberation of dicer bound to the postsynaptic density and to the activation of its RNase III enzymatic activity. In contrast, in *Drosophila* mushroom body neurons, Ashraf et al. [42] found that calcium activates proteasomal degradation of armitage, a RNA helicase that is part of RISC. Further research is needed to learn whether the effects of synaptic stimulation in a neuron overall lead to repression of translation, de-repression, or both. In this regard, the case of FMRP may be instructive: Synaptic stimulation causes an initial, transient burst of translation of FMRP [43] followed rapidly by loss of FMRP back to basal levels [44,45] that may reflect, at least in part, degradation by proteasomes [45].

#### 4.2. Phosphorylation

In some respects, the interaction of microRNAs with cellular kinases and phosphatases has been well studied. MicroRNAs

prominently regulate target mRNAs that encode signaling proteins. Conversely, kinases and phosphatases regulate transcription factors, which in turn are responsible for activating microRNA gene transcription (e.g., p53, NF $\kappa$ B, CREB, c-Myc, DAF-16/FOXO, etc.). However, to my knowledge, no studies have examined whether phosphorylation of microRNA pathway components provides a dynamic means of regulating overall microRNA function in living cells, even though numerous individual microRNA pathway components have been shown to be modified by phosphorylation. For example:

- a) p68 RNA helicase is a co-factor of Drosha in pri-miR processing [46] and is required for strand separation following dicer cleavage [47]; the ability of p68 to bind and unwind RNA is regulated by phosphorylation events [48,49].
- b) Unphosphorylated FMRP is associated with actively translating polyribosomes while a fraction of phosphorylated FMRP is associated with apparently stalled polyribosomes [50].
- c) PACT and TRBP are co-factors of dicer [51] that are activated via phosphorylation by stimuli that elicit an interferon response.
- d) The nuclear phosphatase PPM1G/PP2C $\gamma$  interacts with and dephosphorylates Gemin3, at least within the SMN complex [52].

Phosphorylation is likely to be a global regulator of cellular microRNA levels. For example, it is tempting to speculate that the facultative shift of microRNAs from repression to activation of protein translation may have its basis in phosphorylation and/or arginine methylation of one or more microRNA pathway-associated components such as FXR1P or HuR [8–10].

## 5. Regulation of microRNAs in synaptic fractions of adult mouse forebrain: a model for other cell types?

An increasing body of evidence indicates that synaptic plasticity and dendritic spine growth depend upon regulation of specific protein synthesis near or within dendritic spines, and emerging evidence suggests that microRNAs play roles in these processes [reviewed in ref. [53]]. For example, in mouse, a specific microRNA, mir-134, regulates a specific dendritic mRNA, LIMK1, which is implicated in growth of dendritic spines [54]. Our studies of the microRNA pathway in synaptic fractions of adult mouse forebrain [34,55] have identified a number of regulatory steps that, to date, have not been investigated in other systems. The key findings are as follows:

- a) Dicer, the RNase III enzyme that cleaves pre-miRs into mature microRNAs, is expressed within dendritic spines and is highly enriched at postsynaptic densities (PSDs). The RISC core component, the Argonaute homologue eIF2c, is also expressed within spines and PSDs [34,55].
- b) Dicer associated with isolated PSDs lacks RNase III activity. However, the activity is cryptic, since upon treatment with the calcium-dependent protease calpain, dicer is liberated from PSDs in both full-length form and as partial proteolytic fragments, and its enzyme activity is activated. The RISC component eIF2c is also liberated from PSDs in full-length form by calpain [34].
- c) Treatment of a synaptic fraction (synaptoneurosomes) with calcium, or NMDA stimulation of acute hippocampal slices, causes the mobilization of full-length dicer and the appearance of discrete dicer fragments in a calcium- and calpain-dependent manner [34].

These experiments and others led us to propose a model in which neuronal activity at excitatory synapses allows in calcium, which

activates calpain, which liberates and activates dicer and eIF2c from PSDs. Dicer would then act upon locally present microRNA precursors to form mature microRNAs, which would become incorporated into RISC and bind to mRNA targets on nearby polyribosomes [34].

More recently, my laboratory has confirmed that a majority of mature microRNAs expressed in adult mouse forebrain are detected within synaptic fractions at levels that are similar to, and in some cases significantly greater than, the levels found in total forebrain homogenate [55]. Similarly, the corresponding pre-miRs are detectably expressed in synaptic fractions at levels that are similar to the levels found in total forebrain homogenate. Within synaptic fractions, mature microRNAs are predominantly found in the cytoplasm whereas the pre-miRs are predominantly associated with PSDs. There was a positive relationship between the extent of synaptic enrichment of a given mature microRNA and its corresponding pre-miR, consistent with the proposal that the mature microRNAs within synaptic fractions derive, at least in part, from local cleavage of pre-miRs [55].

Dendritic spines relate to excitatory synapses made upon pyramidal neurons, and the postsynaptic density (PSD) is admittedly a specialized cytoskeletal structure. However, in a pilot study (Lugli and Smalheiser, unpublished) we treated 293T cells to IFN- $\gamma$  (50 ng/ml) for 2 min and observed a very large mobilization of full-length dicer (i.e., dicer was partitioned into the soluble fraction in greater amounts after IFN-treated cells were lysed with non-ionic detergent Triton X-100). A discrete dicer fragment also appeared in response to IFN treatment (data not shown). This strongly suggests that regulation of the microRNA pathway by cellular signaling and subcellular localization is not specific to neurons but is likely to occur in a wide range of non-neural cells and in response to a wide variety of stimuli.

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