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REVIEW

Gold glitters everywhere: nucleus microRNAs and their functions

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Abstract **As a highly conserved class of endogenous small (~22 nucleotides) non-coding RNAs, microRNAs (miRNAs) regulate a broad spectrum of biological processes. Increasing evidences suggested that miRNAs generally regulated gene expression at the posttranscriptional stage via inhibiting the translational process or degrading mRNA. Recent studies have also revealed that there is extensive amount of miRNA, as well as miRNA function-related proteins, in the cell nucleus. Although the molecular basis underneath the biogenesis and function of nucleus miRNAs remains largely unknown, the presence of various miRNAs and miRNA function-related proteins in the nucleus strongly argue that miRNAs may execute their role throughout the whole gene expression pathway. Here we review the recent advances in the researches about the nucleus miRNAs, including the biosynthesis pathways, biological functions and potential regulation machinery of nucleus miRNAs.**

Keywords nucleus miRNA, nucleus RISC, Argonaute family, Exportins, nucleus-cytoplasm shutting, gene regulatory network

Introduction

MicroRNAs (miRNAs) are short, endogenous RNA molecules that regulate target gene expression at posttranscriptional level through either translational repression or mRNA degradation (Ambros, 2004; Bartel, 2004). Ever since its discovery, miRNA has been viewed as a gold mine for nearly every field of modern biology. However, recent developments have also revealed that there is extensive amount of miRNA, as well as miRNA/RNAi function-related proteins, in the cell nucleus (Hwang et al., 2007; Marcon et al., 2008; Politz et al., 2009). The presence of miRNAs in the nucleus is especially interesting because there have been accumulating evidences showing that siRNA as well as RNAi-related pathways can participate in gene expression at the genome level (Berezhna et al., 2006). However, works seeking the basic mechanism for nucleus miRNAs are still at the very beginning. Here we review the recent advances in knowledge of the nucleus miRNA, including their biosynthesis pathways, biological functions and potential regulation machinery. Based on the findings from recent studies, we further propose a model for an Argonaute protein-based miRNA induced functional complex (RIFC) that executes miRNA function in different subcellular compartment (Fig. 1).

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Identification of miRNA in the nucleus

Most of the previously studied animal miRNAs are predominantly located at cytoplasm where they execute their functions via inhibiting translation of proteins or degrading mRNA (Ambros, 2004; Bartel, 2004). However, since different RNAi-mediated pathways that operate in the nucleus have been previously established, a question is raised whether endogenous miRNAs can exist in the nucleus and function there as well.

As expected, for a miRNA to have any role in the nucleus, this miRNA must maintain a considerable expression level there. The first miRNA being identified in cell nucleus fraction is miR-21 (Meister et al., 2004a, 2004b). Using RNA extracted from purified HeLa cell nucleus and quantitative Northern blotting assays, Meister and colleagues found that about 20% of the total mature miR-21 was located in the nucleus. This phenomenon was confirmed by another group (Politz et al., 2009) using microarray and TaqMan probe-based qRT-PCR assays in L6 rat myoblasts. Although the nucleus miR-21 showed a relatively weak signal in further

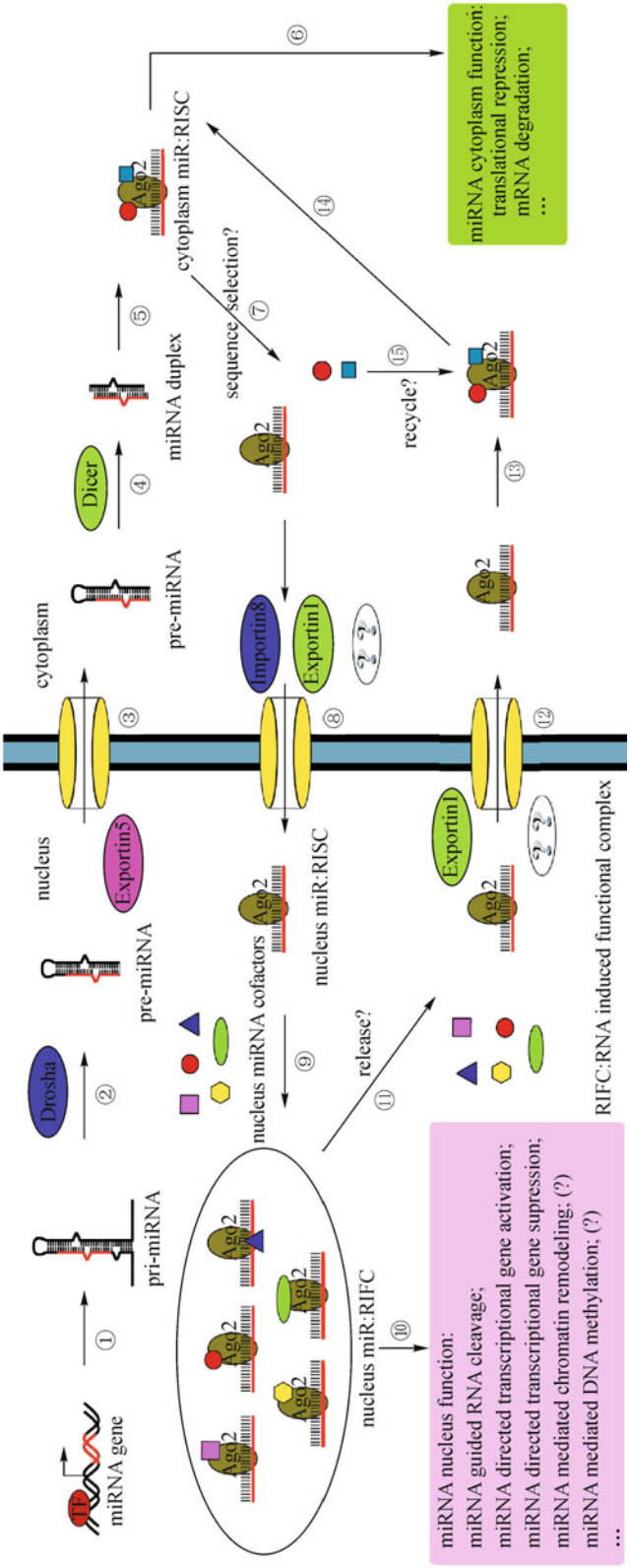

Figure 1 Predicted trafficking pathways and potential functions of nucleus miRNAs.

LNA (locked nucleic acids) *in situ* hybridization assay, it might be due to its interaction with some other molecules that blocks its hybridization as the author suggested. Since miR-21 was indentified both in human and rat cells, this phenomenon might be common throughout the mammalian world.

While Meister and colleagues indentified nucleus miR-21 as an unexpected by-product in their experiments, Robb and colleagues (2005) were the first to show that mature miRNAs may have functions in nucleus. In their study, endogenous miRNAs, at least let-7, were present in the cell nucleus. Furthermore, nucleus Argonaute protein for RNA-induced silencing complex (RISC), another prerequisite for nucleus miRNA functional apparatus, was also found in the nucleus. After transfection with myc-tagged constructs, recombined-Argonaute 1 (AGO1) and AGO2 were clearly detected in both the nuclear and cytoplasmic extracts.

Since all those works described nucleus miR-21 or let-7 were based on Northern blotting assay using RNA extracted from purified cell nucleus, one may challenge these results by arguing that the nucleus fraction could be contaminated by cytoplasm remnants. The first direct evidence for the existence of miRNA in nucleus came from *in situ* hybridization of miR-206 in a rat myogenic cell line (Politz et al., 2006). Politz et al. (2006) found that miR-206 is distributed throughout cytoplasm but also concentrated in the nucleus. More specifically, they demonstrated that miR-206 co-localized with 28S rRNA in the granular component (GC) of the nucleolus. However, it is not likely that miR-206 can directly target rRNA transcripts as most of cytoplasmic miRNAs since there is no 7- or 8-nt sequence elements complementary to the 5′ seed sequence of miR-206 in rat 28S, 5.8S, or 5S rRNAs. Identification of miR-206 in nucleus have also been confirmed by other investigators (Marcon et al., 2008; Politz et al., 2009) employing similar strategies.

When most of other groups applied miRNA *in situ* hybridization in the fixed cells, Földes-Papp and colleagues (2009) were the first to report the expression of miR-122 in liver cells and give an unequivocal proof for the relocation of cytoplasm-assembled single-stranded miRNA-122 into cell nucleus. The authors applied superquencher molecular beacons (SQMB) (Földes-Papp et al., 2009) for the detection of single-stranded miRNA-122 in living cells by hybridization without immobilization. They found the binding of fluorescent SQMB with its miR-122 target, which initially occurred in the cytoplasm, was elevated in the nucleus of human single hepatocytes in a time-dependent manner.

One of the most important works in this field came from Hwang and colleagues in 2007. Combining with Northern blotting, RT-PCR, *in situ* hybridization and co-immunoprecipitation assays, Hwang and colleagues systematically demonstrated that one single miRNA, miR-29b, could be imported into cell nucleus. More importantly, they elucidated for the first time one possible biologic machinery for miRNA nucleus import. Although appears to be rarely used by other mammalian miRNAs, Hwang et al. (2007) demonstrated that, at least for miR-29b, it is a distinctive hexanucleotide 3′ terminal motif (AGUGUU) that distinguishes miR-29b from its family member miR-29a and guides its nucleus import. The nucleus import for miR-29b was confirmed by other two groups (Jeffries et al., 2009; Liao et al., 2010), employing tagged sequences transfection and deep sequencing, respectively. Jeffries and colleagues (2009) also extended the important observations from miR-29b to another miRNA, miR-373-3p, which had a similar *cis*-regulatory motif AGUGCUU on its mature sequence but only at the 5′ terminal (No. 3 to No. 9). Their results showed interestingly that miR-373-3p with AGUGCUU could penetrate into nuclei whereas miR-373-5p without this motif could not.

Cell nuclear have a dramatic change during meiosis and mitosis. Since miRNA may also present in the nucleus of meiosis and mitosis cells, one important issue is whether the expression pattern of nucleus miRNAs would change during these events. The answer is yes, at least during spermatogenesis. Employing high throughput miRNA microarray assay, Marcon and colleagues (2008) indentified a nucleus miRNA signature at a genome wide level during spermatogenesis. In addition to confirm previous results from other groups that miR-206 and miR-320 were in nucleus, they further showed several other miRNAs, including miR-24, miR-214, and miR-202, were also located in nuclei of both spermatocytes and Sertoli cells. Interestingly, most of nucleus miRNA in spermatocytes were localized in the XY-associated dense body, the sex chromatin, the chromosome cores and the telomere, which may further suggest their function in chromatin remodeling.

High throughput technology indeed offers researchers a great opportunity to analyze miRNA expression profile at genome wide level. By applying such technology as microarray and deep sequencing, researchers indentified nucleus miRNA signature throughout different species: miR-1 and miR-351 in rat (L6 myoblasts) (Politz et al., 2009), miR-32, miR-148a and miR-148b in human (nasopharyngeal carcinoma (NPC) 5-8F cell line) (Liao et al., 2010), miR-709 and miR-30e in mouse liver (Tang et al., unpublished data). The most intriguing outcome is, however, although the levels of the miRNAs in nucleus are varied among different tissues/cell lines, these miRNAs can expressed both in nucleus and cytoplasm, and no single miRNA that is exclusive for nucleus has been identified. This result indicates that mature miRNAs are generously transported from cytoplasm into nucleus and that the import of miRNA into the nucleus may be a general phenomenon in the mammalian world.

Possible models for miRNA nucleus-cytoplasm shuttling

Since it is clear that miRNAs can exist in cell nucleus, two

questions came out as nature consequence: a) where did these nucleus miRNAs come from, and b) what is the mechanism underneath this phenomenon?

Most mammalian miRNAs have to mature in the cytoplasm where Dicer, a required miRNA processing enzyme, located. Although recent studies suggest that Dicer proteins are also shuttled between nucleus and cytoplasm in fission yeast (Emmerth et al., 2010), which might argue that mammalian miRNA maturation can occur in nucleus through a non-classic cytoplasmic Dicer-independent pathway, it is generally believed that mature miRNAs in nucleus are imported from cytoplasm. This review will mainly focus on the nucleus import machinery for cytoplasmic miRNAs.

Selection strategy for nucleus miRNAs

First we discuss the recognition strategy used by miRNAs that are imported into nucleus. Since the concept of nucleus miRNA itself was only proposed in very recent years, there is very little work emphasizing on its regulative pathway and some of them were simply deducted from transporting machinery of nucleus RNAi/siRNA import pathways.

By directly visualizing the localization of small interfering RNA (siRNA) in live human cells, Berezhna and colleagues (2006) showed that siRNA could either selectively localize in the cytoplasm or transport into the nucleus, depending on where the target RNA expressed. Deduced from this point, there could have a shuttling equilibrium between nucleus and cytoplasm for some or most miRNAs. Increase of target RNA in nucleus could shift this equilibrium to the nuclear import side, leading to upregulation of nucleus expression for this miRNA.

Hwang and colleagues (2007) conducted deep study and demonstrated that, at least for human miR-29b, *cis*-element at its 3′ terminal can direct nuclear enrichment of this miRNA. Unfortunately, further studies suggested that this 3′ terminal motif (AGNGUN for miR-29b, where N is any nucleotide) seemed to be rarely used by other mammalian miRNAs for nuclear localization (Jeffries et al., 2010; Liao et al., 2010). Nevertheless, their work provides the basic principle for searching conserved *cis*-regulation motif that guided miRNA nucleus importation. So far, another 5′ terminal AGUGCUU motif has also been found to guide nucleus importation of miR-373-3p.

Protein factors required for nucleus miRNA importing apparatus

For a miRNA to execute its function, its association with RNA-induced silencing complex (RISC) is critical. The key components for RISC, AGO1 and AGO2, were also found in nucleus and associated with nucleus miRNAs, implicating that RISC itself may have a role in mediating miRNA shuttling between cytoplasm and nucleus or between different subcellular compartments. As expected, human AGO2 and *C. elegans* NRDE-3, both members of the Argonaute family, have been found to be responsible for siRNA nuclear localization (Guang et al., 2008; Ohrt et al., 2008). In a model proposed by Ohrt and colleagues (2008), cytoplasmic co-factors for RISC are dissociated from hAgo2 protein, then form nucleus RISC through which they translocate into nucleus and bring miRNA strand with it.

Karyopherins are a group of proteins involved in transporting molecules through the pores of the nuclear envelope (Lund et al., 2004; Shibata et al., 2006). For Drosha-processed microRNAs (miRNAs) to be exported from the nucleus to the cytoplasm in mammalian cells, Exportin-5, a member of karyopherin family, serves as a major carrier (Okada et al., 2009). After nuclear processing, the pre-miRNA is exported into the cytoplasm by Exportin-5 (XPO5) in a Ran-GTP dependent manner. In addition to Exportin-5, CRM1 (XPO-1, Exportin-1) also serves as a transporter for miRNA nuclear-cytoplasmic shuttling. Castanotto et al. (2009) reported that inhibition of CRM1 by Leptomycin B results in nuclear accumulation of miRNA guide sequences. Like Exportin-5, CRM1 also co-immunoprecipitates with Argonautes family proteins including AGO1 and AGO2. Weinmann et al. (2009) showed evidences that Importin-8 interacts with human Argonaute proteins in cytoplasm and may have additional functions in importation of nuclear miRNA:RISC. Whether other members of Karyopherin family could be recruited during miRNA:RISC nucleus import remains as an unknown but interesting issue. Although Giw1p only associated with siRNA pathway in Tetrahymena, Noto et al. (2010) provided an alternative direction in searching core factors for miRNA nucleus import, as well as the canonic cytoplasmic miRNA pathways. Following this direction, any factors that have small RNA binding ability might be a candidate as a carrier for miRNA nucleus import, especially members of the Argonaute protein family.

Various functions for nucleus miRNAs

The concept that miRNA can be imported into the nucleus and have various function there has been gradually accepted following the pioneer works by several research groups (Hwang et al., 2007; Jeffries et al., 2010; Liao et al., 2010). Since RNA was located majorly in cytoplasm while the cell nucleus got the priority for genomic DNA storage, it was reasonable to assume that miRNA in the nucleus could participate gene regulation via directly binding genomic DNA. Several articles have discussed this hypothesis from different aspects.

RNAi-directed DNA methylation (RdDM)

In plants, dsRNAs targeted to CpG islands within a promoter can induce RNA-directed DNA methylation (Wassenegger et al., 1994). Employing the synthetic siRNAs targeting CpG

islands of an E-cadherin promoter, Kawasaki and colleagues (2004) demonstrated for the first time that the synthetic siRNAs specifically induced promoter DNA methylation and histone H3 lysine 9 methylation in human, which further repressed correspond gene expression at the transcriptional level. Although not been validated in mammalian cells, the Argonaute proteins AGO4 and AGO1 are required in de novo methylation or maintenance of RdDM in plants.

RNAi/miRNA-mediated chromatin remodeling

RNAi-mediated heterochromatin assembly has been reported in animals (Fukagawa et al., 2004; Pal-Bhadra et al., 2004), plants (Onodera et al., 2005), and fission yeast (Volpe et al., 2002). Fukagawa and colleagues (2004) provided the first evidence that Dicer-related RNA interference machinery is involved in the formation of the heterochromatin structure in chicken–human hybrid DT40 cell line. Loss of Dicer activity would result in premature sister chromatid separation due to abnormalities in the localization of two heterochromatin proteins: Rad21 and BubR1.

Interestingly, studying spermatogenesis process, Marcon and colleagues (2008) indentified a specific miRNA signature that most of nucleus miRNAs in spermatocytes were localized in the XY-associated dense body, the sex chromatin, the chromosome cores and the telomere. All of these components or structures contain highly repetitive DNA sequences and are required for heterochromatin formation during meiosis. Their results suggested a possible role for nucleus miRNAs in heterochromatin formation at least during meiosis.

Furthermore, when Castanotto and colleagues (2009) first discovered the ability for CRM1 (XPO-1, Exportin-1) in guiding the nuclear-cytoplasmic shuttling of the miRNA guide sequences, they also detected that CRM1 coimmunoprecipitated with Topo2α, EzH2 (the methyltransferase part of the polycomb repressive complexes), which also suggested a role of nucleus miRNAs in chromatin remodeling.

Of course, it should be noted that the two pathways discussed above, RdDM and RNAi/miRNA-mediated chromatin remodeling, were validated only by synthetic siRNA and whether these processes occurs similarly in endogenous miRNA pathway remains to be determined.

Nucleus miRNA-guided target RNA cleavage

Meister and colleagues (2004b) showed the first evidence that nucleus miR-21 could guide the cleavage of target RNA, suggesting a role of nucleus miRNA in RNA processing. Employing similar strategy for let-7, Robb and colleagues (2005) confirmed and extended the effect of nucleus miRNAs on target RNA cleavage. Interestingly, although the concentration of let-7 was considerably lower in the nucleus than it in the cytoplasm compartment, nucleus let-7 showed a stronger capability in cleaving fully complementary RNA (~86% and 60% efficiency in nucleus and cytoplasm, respectively).

Transcriptional gene activation and silencing

As early as 2006, Li and colleagues reported unexpected phenomenon that synthetic 21-nt dsRNAs targeting promoter regions of human genes E-cadherin, p21, and VEGF caused a long-lasting induction of these genes. This finding suggests that small nucleus dsRNAs can directly activate gene expression. Place and colleagues (2008) further identified that two genes, E-cadherin and CSDC2, could be directly induced by miR-373 at transcriptional level via binding highly complementary target sides within their promoters. This is an important discovery since it has challenged the classic miRNA working model in which miRNAs work only as posttranscriptional regulators. Another intriguing phenomenon is that not all genes with promoter sequences complementary to miR-373 were induced in response to miR-373, suggesting promoter environment, as well as other cellular status, are also important for miRNA guiding gene activation (Janowski et al., 2007). This observation also resembles the character for transcriptional factor-guided regulation.

As expected, the researchers have also reported the direct role of miRNA in gene transcriptional silencing in the nucleus. By searching miRNA target sites proximal to known gene transcription start sites in the human genome, Kim and colleagues (2008) found that miR-320, a conserved miRNA, is encoded within the promoter region of the cell cycle gene *POLR3D* in the antisense orientation. This finding suggests that this mature miRNA can perfectly match the promoter DNA in sequence. In other word, if miR-320 is expressed and imported into the nucleus, it can perfectly match the sequence of target promoter DNA and thus silence the gene expression. Interestingly, the authors also detected a miR-320-dependent enrichment of AGO1 at the *POLR3D* promoter, which suggested that AGO1 was acting as the effector protein for microRNA induced gene silencing. On the other hand, Janowski and colleagues (2006) showed that AGO1- and AGO2-mediated synthetic antigene RNAs (agRNAs) induced gene transcriptional inhibition in nucleus, suggesting that miRNA might also recruit its cytoplasm pathway for its transcriptional gene silencing function in nucleus.

Conclusion and perspectives

In this brief review, we followed up the research progress on nucleus miRNAs and concluded that miRNAs are important gene expression regulators at not only posttranscriptional level but also transcriptional level. Canonical miRNAs biogenesis pathway involve processing of primary microRNA transcripts by Drosha; exporting pre-miRNA from the nucleus to cytoplasm by Exportin-5, cleaving pre-miRNA into their mature forms by Dicer and finally incorporating mature miRNA into effector complexes (RISC) that mediate gene silencing activities(Ambros, 2004; Bartel, 2004; Winter

et al., 2009). However, recent studies suggested some miRNAs can be naturally imported into cell nucleus where they may directly regulate gene expression. As a piece of real gold, miRNA can be shining at wherever it exists, including the cell nucleus, and that is what we emphasized at the very beginning of this issue.

Inferring from previous research in this field, as well as parallel works in nucleus RNAi field, we have also discussed several possible models for nucleus miRNA import, including the recognition strategy embedded in miRNA sequence and the nucleus import machinery recruited in this pathway. Finally, we described the potential functions of nucleus miRNAs, including miRNA/RNAi-directed DNA methylation, miRNA/RNAi-mediated chromatin remodeling, miRNA-guided target RNA cleavage, transcriptional gene activation and silencing.

It should be noticed that members of Argonaute protein family often take an important role in miRNA guided processes, such as AGO2 in translational repression or mRNA degradation; AGO1 in chromatin remodeling; AGO1 and AGO2 in target RNA cleavage in nucleus and transcriptional gene silencing; AGO4 and AGO1 in RNA directed DNA methylation. More importantly, AGO2 was reported to guide the nucleus import in both miRNA and siRNA pathways.

To summarize all these previous discoveries, we propose the following model for mammalian miRNA functional apparatus in both cytoplasm and cell nucleus (Fig. 1). After canonical biogenesis pathway from nucleus to cytoplasm (I to V), mature miRNA was incorporated into cytoplasm miR: RISC complex which may further execute its cytoplasm function (VI); however, some of these cytoplasm RISC could be dissociated and release its cytoplasm cofactors with its miRNA:AGO protein (here we showed AGO2 as an example) core remaining intact (VII). Those released cofactors could also be recycled and reenter the cytoplasm miRNA pathway (XV). Under the help of Importin-8, Exportin1 as well as other carrier factors, the miRNA:AGO core could be imported into cell nucleus (VIII), where they can recruit other nucleus cofactors and compose the so called "RNA induced functional complex (RIFC)" (IX) which will further guide miRNA nucleus functions (X). RIFC in nucleus can also be dissociated and release the miRNA:AGO core (XI), which will be exported into cytoplasm via Exportin-1 and other cofactors (XII). Finally, the exported miRNA:AGO core could incorporated with cytoplasm RISC cofactors and reenter the cytoplasm miRNA pathway (XIII, XIV) thus complete the miRNA circulation.

Although a general picture of miRNA pathways in the nucleus has emerged, many questions still remain to be answered. To clarify the biogenesis and biologic function of nucleus miRNAs, the future study should emphasize on: 1) the compartments where miRNA maturation occurs; 2) the mechanism or key factors by which miRNA nucleus-cytoplasm shuttling is regulated; 3) selection strategy for the nucleus-predominated miRNA and common elements for miRNA reorganization; and 4) role of nucleus miRNAs and the molecular basis underneath.

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