Regulation of miRNA Processing and miRNA Mediated Gene Repression in Cancer

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Abstract: The majority of human protein-coding genes are predicted to be targets of miRNA-mediated post-transcriptional regulation. The widespread influence of miRNAs is illustrated by their essential roles in all biological processes. Regulated miRNA expression is essential for maintaining cellular differentiation; therefore alterations in miRNA expression patterns are associated with several diseases, including various cancers. High-throughput sequencing technologies revealed low level expressing miRNA isoforms, termed isomiRs. IsomiRs may differ in sequence, length, target preference and expression patterns from their parental miRNA and can arise from differences in miRNA biosynthesis, RNA editing, or SNPs inherent to the miRNA gene. The association between isomiR expression and disease progression is largely unknown. Misregulated miRNA expression is thought to contribute to the formation and/or progression of cancer. However, due to the diversity of targeted transcripts, miRNAs can function as both tumor-suppressor genes and oncogenes as defined by cellular context. Despite this, miRNA profiling studies concluded that the differential expression of particular miRNAs in diseased tissue could aid the diagnosis and treatment of some cancers.

Keywords: Cancer, gene regulation, isomiR, miRNA, miRNA editing, SNP.

INTRODUCTION TO MIRNAS

MicroRNAs (miRNAs) are a family of endogenous RNAs that predominantly regulate the post-transcriptional stages of gene expression. They are single stranded, non-coding RNAs, approximately 21 nucleotides in length. miRNAs were originally identified to be essential for developmental timing [1], however they have subsequently been implicated in the regulation of key biological processes including cell growth, cell differentiation and apoptosis (reviewed in [2]). Within mammalian genomes, miRNAs are predicted to regulate approximately 60% of all coding genes, which illustrates their extensive influence on gene expression [3].

As miRNA-mediated regulation has a considerable impact on numerous pathways, both their function and expression are spatially and developmentally controlled. The miRNA transcriptome is essential for defining and maintaining cellular differentiation and identity. Consequently, compromised miRNA-mediated translational control, for example due to the misregulation of miRNA expression, can result in various diseases including several types of cancer (reviewed in [4-6]).

MIRNA BIOGENESIS

In the human genome, most miRNA coding genes are found either within the introns of protein-coding genes or in non-coding messenger RNAs (mRNAs). A small number of miRNA genes have also been found within the exons of non-coding transcripts [7]. Due to their genomic loci, it is likely that intragenic miRNAs (those encoded within protein-coding genes) share regulatory elements with their host genes [8]. In contrast, independent promoters control the expression of intergenic miRNAs [9].

Human miRNA genes are predominately isolated within the genome, however some are grouped together forming clusters. Clustered miRNAs are transcribed as a single multicistronic primary transcript [10, 11], which helps to coordinate the simultaneous regulation of multiple miRNAs. It is common for miRNAs within a cluster to display high sequence homology, and therefore have common mRNA targets [12, 13]. Clusters containing miRNAs with differing target specificities have also been identified and are thought to regulate genes within a particular pathway or protein complex [14, 15].

The location of the miRNA genes may help explain the prevalence of altered miRNA expression in disease as approximately half of annotated human miRNAs are encoded within fragile regions of chromosomes, i.e. areas of the genome known to be associated with human cancers, suggesting that altered miRNA expression has a role in cancer progression [16].

TRANSCRIPTION AND PROCESSING OF MIRNAS

Similar to protein coding genes, most miRNA genes are transcribed by RNA polymerase II forming a capped and polyadenylated RNA precursor or primary transcript (pri-miRNA) (Fig. 1). Transcription of pri-miRNAs is regulated by transcription factors, many of which function only in specific permissive cellular environments. Stress-signals, that
are indicative of cancer cells, can regulate the production of particular pri-miRNAs by controlling the functionality of certain transcription factors. For example the tumor suppressor protein p53 [17, 18] and the oncogenic protein c-myc [19] both act to regulate the transcription of miRNA genes with known functions in cancer pathogenesis.

Pri-miRNAs fold into a characteristic stem-loop structure that is recognised by proteins involved in miRNA processing [20]. Within the nucleus, Drosha, an RNase III enzyme, and DGCR8 (DiGeorge syndrome critical region protein 8), a double stranded RNA binding protein, bind to form the microprocessor complex. The microprocessor cleaves the pri-miRNA to form a precursor (pre)-miRNA. The pre-miRNA is an approximately 70 nucleotide molecule which forms an imperfect hairpin [21]. Pre-miRNAs enter the cytoplasm via the nucleocytoplasmic transport factor Exportin-5 (XPO-5) where further maturation steps occur [22, 23].

Cytoplasmic cleavage of the pre-miRNA hairpin is mediated by Dicer, an RNase III enzyme, which generates an imperfect miRNA/miRNA* duplex that includes a two nucleotide 3´ overhang [24-26]. The guide strand of the RNA duplex, identified as the strand with the weakest base pairing at its 5´ terminus, is then preferentially incorporated into an Argonaute (Ago) protein [27, 28]. Argonautes are effector proteins required for all miRNA-mediated gene regulation, and an Ago loaded with a miRNA forms the minimal RNA Induced Silencing Complex (RISC) [29, 30].

Alternative miRNA biogenesis pathways have also been identified. For example a minority of miRNAs called mirtrons, coded within the introns of transcripts, are not subjected to Drosha-mediated cleavage before entering the processing pathway [31]. Furthermore, a Dicer independent processing pathway has also been discovered which requires Argonaute-mediated cleavage of the precursor hairpin [32-35].

Each step of the miRNA biogenesis pathway is highly controlled and several proteins implicated in disease, including tumor suppressor and oncogenic proteins, are known to function in regulating this process. These proteins interfere with the processing of all miRNAs or they facilitate and/or inhibit the production of a set of miRNAs. There are also auxiliary protein factors that are specifically regulation the processing of individual miRNAs (reviewed in [36, 37]).

In addition to biogenesis, miRNA half-life and decay is also a controlled process and many different pathways have been found to play a role in miRNA homeostasis [38, 39]. These pathways involve small RNA-degrading nucleases [40], exoribonucleases [41] or decapping scavenger enzymes [42].

**MIRNA TARGET RECOGNITION AND FACTORS THAT MODULATE MIRNA ACTION**

Mature miRNAs function to guide the RISC to complementary target mRNAs, resulting in the repressed translation of the transcript and/or cleavage of target mRNA. As one miRNA can potentially bind to multiple targets that encode proteins with different functions, a single miRNA can be implicated in diverse cellular pathways and processes.

miRNAs bind to cognate mRNAs with imperfect complementarity. In most cases, the minimal base pairing between a miRNA and the target is determined by a 'seed' sequence which is positioned at the 5´ end of the miRNA (nucleotides 2-8) [43-45]. The 3´ proximal nucleotides of the miRNA can potentially enhance target recognition, for example if the seed matching isn’t exact or if the sequence isn’t positioned in an optimal AU-rich context [46, 47].

Thus far, the evidence suggests that the majority of miRNA binding sites implicated in translational repression are located in the 3´ untranslated region (UTR) of the mRNA (reviewed in [2, 48, 49]). However, miRNAs can also bind to target sites found in the 5´ UTR and coding regions of transcripts [50-52].

The majority of experimentally confirmed mRNA targets contain multiple miRNA binding sites, and closely adjacent miRNA binding sites allows for regulation to occur in a cooperative manner [53, 54]. This cooperativity occurs irrespective of the heterogeneous population of miRNAs that may bind a single 3´ UTR [46].

There are several factors which can influence the probability of a miRNA finding its target, including the accessibility of the target mRNA. For example, transcript secondary structure [55] and neighbouring or overlapping RNA-binding motifs could regulate the availability of miRNA binding sites and can therefore modulate the effectiveness of regulation [56-58].

Furthermore, the length of the 3´ UTR of a target can modulate the influence of miRNA-mediated repression. For example, in cancer cells mRNA isoforms with truncated 3´ UTRs, produced from alternative polyadenylation, are highly expressed. The shorter transcripts display differences in function such as altered stability and protein production. These differences are thought to be attributable, in part, to the loss of miRNA-mediated gene regulation [59].

**MECHANISMS OF MIRNA-MEDIATED GENE REGULATION**

miRNAs mediate gene regulation via several, non-mutually exclusive mechanisms that target protein synthesis at multiple post-transcriptional stages of gene expression [60, 61]. Many studies conclude that miRNA-mediated inhibition targets the initiation step of translation [62-65], while others indicate the elongation step is affected [66-69]. Additionally, miRNA-mediated mechanisms can promote mRNA deadenylation, degradation and/or mRNA sequestration [70, 71].

These diverse mechanisms are thought to differ due to wide range of potential RISC protein binding partners that act in response to a dynamic cellular environment.

**ISOMIRS**

Recent advances in sequencing technology has led to the identification of novel miRNAs and the discovery that miRNAs can be heterogeneous in nature varying both in length and sequence [72] (Fig. 1, inset). These miRNA isoforms, or isomiRs [73], interact with both the RISC and translating polysomes, suggesting they bind to target mRNAs [74, 75]. Many of these recently observed miRNA variants are produced at very low levels and display functional redundancies. However some isomiRs display unique expression
**Fig. (1). miRNA biosynthesis and isomiR formation.** Canonical maturation of mammalian miRNAs begins with the miRNA-containing gene being transcribed to form the primary transcript (pri-miRNA). This transcription, a function of RNA polymerase II (RNA Pol II), can be regulated by transcription factors implicated in cancer pathogenesis e.g. p53, c-myc. Pri-miRNA is cleaved by the microprocessor complex (Drosha-DGCR8) to form the precursor miRNA (pre-miRNA) which is then exported into the cytoplasm by Exportin-5 (XPO-5). The protein complex Dicer-TRBP cleaves the pre-miRNA to form the miRNA/miRNA* duplex. One strand of this duplex, the guide strand, is then loaded into an Argonaute protein to form the RNA-Induced Silencing Complex (RISC). The mature miRNA guides the RISC to mRNA targets, resulting in the translation repression of the bound transcripts. Alternative miRNA biosynthesis pathways includes the production of mirtrons, miRNAs that derive from short intronic hairpins, produced from precursors formed by the splicing machinery.

**Inset.** IsomiRs describe miRNA variants that are produced from a common miRNA gene. Typical sources of isomiRs include multiple Dicer cleavage sites present within the pre-miRNA hairpin. Additionally, isomiRs are formed via post-transcriptional modifications of the mature miRNA, such as the addition or removal of nucleotides, or changing internal nucleotides via RNA editing.

Patterns which can vary in response to biological stimuli [76-78], or may be indicative of differentiation or disease status of a cell [79, 80], suggesting that isomiR production is dynamically regulated. Although the influence of isomiRs on miRNA-mediated regulation is as yet uncertain, evidence suggests that isomiRs can influence the incorporation of miRNAs into the RISC, miRNA target association, and miRNA stability (reviewed in [81, 82]).
The discovery of miRNA isoforms suggests that one miRNA gene can potentially produce multiple distinct isomiRs. These isoforms can result from variations in the biosynthesis pathway i.e. in the processing of a miRNA precursor molecule. For example pri-miRNAs can be cleaved at multiple sites by Drosha which generates mature miRNAs with differences in their 5’ end [83, 84]. Likewise, the sequence of mature miRNAs is determined by the position of the Dicer cleavage site within the double stranded precursor molecule. However, Dicer can potentially cleave the pre-miRNA at more than one site per precursor molecule, producing multiple miRNAs with distinct seed sequences and target specificity. The location of this cleavage site is influenced by protein binding partners of Dicer [85, 86].

The majority of isomiRs vary in length from the more ubiquitously expressed canonical miRNA. These differences occur either by the subtraction or addition of nucleotides at either the 5’ or 3’ end of the miRNA. For instance isomiRs that precisely reflect the parental sequence can be formed due to imprecise cleavage by either Drosha or Dicer or alternatively, by exonuclease removing nucleotides from the end of the miRNA [87, 88]. IsomiRs that do not precisely reflect the parent sequence may also arise via post-transcriptional modifications, which can add extra nucleotides to the mature miRNA [81]. A prevailing modification to mature miRNA molecules is the polyadenylation or uridylation of the 3’ end [72, 89].

In rare cases the internal sequence of an isomiR can vary, and these are termed polymorphic isomiRs. It is possible that these variations are due to single nucleotide polymorphisms (SNPs) within the miRNA gene, however studies have found a relatively low number of SNPs present in miRNAs, suggesting an evolutionary selective pressure against these variants [90, 91].

RNA Editing in IsomiR Formation

Llorens et al. [92] analyzed isomiR production in response to epidermal growth factor (EGF) stimulation as the EGF signaling pathway is correlated with the increased survival of cancer cells [93]. The miRNAs sequenced displayed high sequence heterogeneity but this was not as a result of EGF-mediated mechanisms, however there were cell specific preferences in the isomiRs expressed [92].

Novel miRNAs can also arise as a result of miRNA editing, a process describing post-transcriptional enzymatic modifications to the RNA, which produces miRNAs with single nucleotide mismatches. Kawahara et al. [94] reported that tissue-specific editing within the seed region of a miRNA results in the predominant expression of the edited isoform, which specifically targets different genes.

The misregulation of miRNA editing contributes to cancer progression. Choudhury et al. [95] found that a characteristic of human glioblastoma cells is a widespread inhibition of miRNA editing. This disruption in mature miRNA seed sequence alterations causes enhanced migration and invasion potential of these cells. A study investigating the role of miRNA editing in metastatic melanoma cells found an RNA editing enzyme was downregulated and consequently alters the miRNA expression profile. However this regulation occurred by targeting miRNA processing and occurred in an RNA-editing independent manner [96]. As yet the importance of misregulated miRNA editing in cancer is not well established.

SNP (SINGLE NUCLEOTIDE POLYMORPHISM) INFLUENCE ON MIRNA MEDIATED GENE REPRESSION

SNPs are the most common form of variation within the human genome [97]. As miRNA-mediated translational repression is dependent on sequence complementarity, it follows that SNPs found in either the miRNA seed regions or in the target site of an mRNA can have a major impact on target identification and subsequent regulation by either hindering effective binding to the target or by the creation of new target sites [4, 98].

Furthermore, the 3’ UTR of a transcript contains numerous regulatory motifs which are required for normal gene expression [99] and many of those motifs which are highly conserved are important to miRNA function [100]. SNPs within these regions could also influence the binding of miRNAs to their targets.

Identifying SNPs that alter miRNA-mediated regulation has been the focus of several studies. Iwai & Naraba [101] found polymorphisms relevant to all stages of miRNA biosynthesis, while Wu et al. [102] found SNPs present in pri-, pre- and mature miRNAs. Moreover, Zeng et al. [103] detected SNPs in the loop sequence of pre-miRNAs which may affect Dicer recognition, and therefore influence the processing of mature miRNAs [104]. Thus SNPs within miRNA genes can potentially affect miRNA expression, maturation and function.

miRNA-related SNPs have been found to correlate to human diseases [105], leading to the formation of databases to catalogue point mutations within miRNAs and their target sites [91, 102, 106], such as miRdSNP [107] and mirSNP [108]. There is a significant association between SNPs found in mature miRNA sequences or in the binding sites of target genes and susceptibility to cancer (reviewed in [4]), which seem specific to particular cancer type [109, 110].

SNPs found within precursor miRNA molecules also correlate to risk of developing disease as shown in recent studies investigating oral cancer [111], lung cancer [112] and chronic lymphocytic leukemia [113].

MIRNA FUNCTION IN CANCER

Established roles of miRNAs include regulating cell growth and tissue differentiation. As both of these processes are deregulated during tumor formation, miRNAs may have a role in the formation and/or progression of cancer (reviewed in [114, 115]).

Lu et al. [116] observed that overall, miRNA expression levels in tumor samples were at lower levels when compared to healthy tissue. This widespread decrease of miRNA expression in cancers is thought to be due to failures in miRNA biosynthesis [117]. Altered miRNA expression can also be the consequence of changes in the levels of proteins involved in the miRNA biosynthesis pathway. While some studies have found a lower level of Dicer and Ago2 (one of the four effector Argonaute proteins in mammalian RISC)
The relationship between decreased miRNA expression and the loss of cellular differentiation displayed by cancer cells indicates that miRNAs can function as tumor-suppressor genes. Conversely, other studies have shown that the expression of specific miRNAs can be increased in tumor cells and therefore these miRNAs are oncogenes [121-123]. As miRNAs have multiple targets, they have the potential to act as both oncogenic and tumor-suppressors. The specific role of the miRNA is determined by cell type and pattern of expression [124]. Volinia et al. [122] observed that the predicted targets of miRNAs in solid tumor samples comprise a significant proportion of cancer-related genes. However, whether the altered miRNA expression observed in cancer cells is a cause or a consequence of tumor formation remains unclear.

A study investigating human breast cancer cells reported that the decreased expression of a specific family of miRNAs promotes epithelial mesenchymal transition (EMT) [125]. EMT is an essential step in development and cancer progression (reviewed in [126]). Within cancer tissue, there is small population of cells termed cancer stem cells (CSCs). Current hypothesis suggest that CSCs are a main contributor to cancer cell production and cancer progression, and are capable of differentiating into the numerous cell types found in tumors (reviewed in [127, 128]). CSC formation and function is regulated by miRNA-mediated gene regulation [129].

miRNA Profiling in Cancer

As miRNA expression is altered in cancer tissue, profiling of miRNAs in tumors samples provides an accurate technique of classifying cancer subtypes, and therefore the differential expression of certain miRNAs could aid the diagnosis and treatment of cancer. Data from several profiling studies has shown that miRNA expression pattern is indicative of the differentiation state and the developmental lineage of the tumor [130] and that relatively few miRNAs were required to accurately classify human cancers [116]. In addition, miRNA expression patterns may provide insight into patient survival, further supporting the diagnostic value of miRNA profiling [19, 131]. Recent advances in miRNA expression profiling and their potential as biomarkers are reviewed in [132, 133].

As altered miRNA expression is characteristic of many diseases states, there is also potential use for miRNA profiling in other diseases such as epilepsy [134], multiple sclerosis [135] and coronary artery disease [136].

CONCLUSION

Only five years after the identification of the first human miRNA, it was shown that their expression pattern could faithfully characterize different types of cancers and predict survival rates. Since then, miRNAs are the focal points of biomarker discovery, not only in cancer but also in many other diseases. This is not surprising since miRNAs are central hubs of post-transcriptional gene regulation, due to their ability to regulate multiple target mRNAs. Therefore, changes in miRNA expression levels and target specificity could dramatically change the cellular outcome of gene expression.

miRNA biosynthesis is a multi-stage, tightly regulated process. Increasing number of studies has identified proteins and revealed molecular mechanisms that regulate different steps of miRNA processing and miRNA target recognition. Many of these proteins and cellular pathways have already been associated with disease development before their connection with miRNA mediated gene regulation was discovered. It is very important to understand and further study the regulatory mechanisms and mutations that affect miRNA synthesis and miRNA mediated gene regulation in order to transform miRNAs from established biomarkers to potential drug targets.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the ARC DP130103027. G.H. is an ARC Future Fellow. We are also grateful to Nham Tran for helpful suggestions.

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