Regulation of microRNA processing in development, differentiation and cancer

Thomas D. Schmittgen*

College of Pharmacy, Ohio State University, Columbus, OH, USA

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Abstract

microRNA (miRNA) is a class of small, noncoding, regulatory RNAs. The ~21 nt mature miRNA is processed from larger precursor molecules following a coordinated series of events. In theory, miRNA processing may be regulated at any of these steps. A growing body of evidence has demonstrated various steps in the miRNA biogenesis process for which regulation occurs. RNA editing of miRNA precursors, SNPs or mutations in the miRNA precursors, regulation by RNA binding proteins, alterations in the levels of key processing proteins, as well as a number of unknown mechanisms contribute to the regulation of miRNA processing. This article reviews the available literature on the regulation of miRNA processing that occurs within normal cells, during development or in diseases such as cancer.

Keywords: microRNA biogenesis ● microRNA processing

Introduction

microRNAs (miRNAs) are a class of small, regulatory, noncoding RNAs. miRNA was discovered in C. elegans in 1993 by the Ambros and Ruvkun laboratories [1, 2]. The founding members of this class of RNAs include lin-4 and let-7. In 2001, interest in the field peaked when hundreds of miRNAs were discovered in Drosophila, C. elegans and mammals [3–5]. Over the past 7 years, numerous miRNAs have been discovered in many species including 678 in human, 472 in mouse and 154 in C. elegans [6]. miRNA represents a new class of gene regulation; it has been estimated that miRNA regulates up to one-third of human genes [7]. A number of excellent reviews on various aspects of miRNA have been published including overviews [8, 9], miRNA processing [10], post-transcriptional regulation of miRNA [11] and effect of RNA editing on miRNA [12]. This article reviews the literature on the regulation of miRNA processing that occurs within normal cells, during development or in diseases such as cancer.

microRNA processing and function

The genes encoding miRNAs are located within introns, intergenic regions and within exons [13]. The majority of human miRNA loci are encoded within intronic regions [14]. Although some exceptions exist [15], miRNA genes are transcribed by RNA polymerase II [16]. The primary transcript that results from the RNA polymerase II transcription is termed the primary miRNA (pri-miRNA) (Fig. 1). pri-miRNA contains a hairpin structure from...
which the mature miRNA is processed. Like mRNAs, the pri-
mRNA is polyadenylated, contains a 5' cap structure and may
undergo splicing. The pri-miRNA is processed in the nucleus by a
complex that includes the RNase III enzyme Drosha and the dou-
ble stranded RNA binding domain protein DGCR8 to produce the
pre-miRNA [17]. The complex contains a yardstick to measure the
hairpin and to allow precise processing of the pri-miRNA [18].
Processing of the pri-miRNA appears to proceed prior to splicing
[14]. The pre-miRNA contains the 2 nt 3' overhang that is charac-
teristic of Dicer processing of siRNA. The pre-miRNA is transported
to the cytoplasm by Exportin 5, a Ran-GTP-dependent mechanism
[19]. Once inside the cytoplasm, the pre-miRNA is subsequently
loaded into a complex that includes the RNase III enzyme Dicer
and TRBP/Loquacious. This complex cleaves the loop from the
pre-miRNA to produce a double-stranded complex composed of
the miRNA and miRNA*. The miRNA* strand is typically degraded
and the mature miRNA strand, along with the Argonaute protein
Ago 2, is further assembled into a ribonucleoprotein complex
known as RISC (RNA-induced silencing complex).

RISC will then scan cellular mRNA in an attempt to locate the
mRNA's target. miRNAs function by binding through imperfect
complimentarity to conserved sequences within the untranslated
region (UTR) of mRNAs. Although most work has focused on
miRNA binding to the 3' UTR, miRNAs have also been shown to
bind to the 5' UTR [20]. Nucleotides 2 through 8 on the 5' end of
the mature miRNA, the so-called 5' seed, interact with the reverse
complement sequences on the mRNA. In situations of perfect
complimentarity, the mRNA will be cleaved [21]. In animals, it is
more likely that the miRNA will bind to the mRNA through imper-
fect complimentarity and produce translational repression.
Translational repression proceeds without cleavage of the mRNA
[22]. Translational repression is believed to occur by blocking
translation, often by inhibiting translational initiation [23, 24].
miRNAs have been shown to be sequestered into processing-
body (P-bodies) [25]. The role of P-bodies in target repression
and miRNA biogenesis remain unclear. Knockdown of Drosha and
DGCR8 resulted in a loss of the P-bodies suggesting that they are
a consequence, rather than the cause, of miRNA biogenesis [26].

**Regulation of microRNA processing**

**during development, differentiation**

**and normal cell function**

Conceivably, production of the mature miRNA may be regulated at
multiple steps in the biogenesis process (Fig. 1). Possibilities
include Drosha processing, export from the nucleus, processing
by Dicer or through altered stability of the mature miRNA.
Changes in miRNA processing may be viewed experimentally by
examining the levels of precursor and mature miRNAs on a
Northern blot. An increased or decreased level of mature miRNA
without noticeable changes in the levels of miRNA precursors is
the hallmark of regulated miRNA processing. Such phenomenon
was shown by Ambros et al. in studies of *C. elegans* development
[27]. The precursor to miR-38 was evident during all stages of
development, however the mature miR-38 was present only in the
embryonic stage [27].

Various members of the let-7 family were regulated post-
transcriptionally during neural differentiation of embryocarcinoma
The levels of the precursor forms of the miRNAs were consistent during differentiation, whereas the mature form increased [28]. An increase in the let-7 precursor processing activity in vitro during neural differentiation of embryocarcinoma cells demonstrated that regulation occurred at the Dicer processing step [28]. Similar to [27], Thomson et al. showed that the levels of mature let-7g (but not precursor) miRNAs increased during development of the mouse [29]. A number of miRNAs (let-7g, let-7i-2, miR-24, miR-140, miR-103–1, etc.) were increased at the mature level at days 10.5 and 14.5 of gestation compared to an embryonic stem cell line [29]. However, the levels of pri-miRNAs were consistent throughout this period, demonstrating that regulation occurred at the miRNA processing steps and not transcription.

The answer to this puzzle came recently when it was discovered that the developmentally regulated RNA binding protein Lin-28 inhibits the processing of the primary precursor of the let-7 family members [30, 31]. Lin-28 protein was identified from cellular extracts of P19 cells using a biochemical approach [31]. Lin-28 protein levels decreased during the same time of differentiation as mature let-7 increased. As in the prior studies, there was no change in the precursor form of the let-7. Knockdown of Lin-28 using RNA interference increased the amount of mature let-7 but not miR-15a, miR-16–1, miR-122a or other miRNAs assayed by a micro-array demonstrating the specific nature of this interaction [31]. Lin-28 was shown to selectively bind to the terminal loop of the let-7 precursors and that the loop mediates miRNA processing inhibition [30, 32]. This is of particular importance as single nucleotide polymorphisms (SNPs) or mutations in the loop region of let-7 family members may cause Lin-28 to bind less effectively, resulting in increased levels of mature let-7.

The expression profiles of 142 Xenopus tropicalis miRNAs were studied by Northern blotting [33]. The tissue-specific expression profile of the pri-miRNA was different from that of the mature miRNA. For example, xtr-miR-98 and xtr-let-7i are clustered on a 427 nt DNA fragment and can be assumed to be cotranscribed as a common pri-miRNA. The expression of the primary precursors of xtr-miR-98 and xtr-let-7i was consistent among seven different tissues studied, whereas the expression of the mature xtr-miR-98 and xtr-let-7i differed. Mature xtr-let-7i was present in the heart, whereas xtr-miR-98 was not, demonstrating differential processing of the precursor forms of these two miRNAs. Sometimes the pri-miRNA was present but not the pre- or mature forms (e.g. xtr-miR-215), other times the pri- and pre-miRNAs were present but not the mature miRNA (e.g. xtr-miR-200b), suggesting differential precursor processing in both the nucleus and cytoplasm [33].

Lugli et al. studied the expression of mature and precursor miRNAs in synaptic fractions of the adult mouse forebrain [34]. Both the precursor and mature forms of several miRNAs expressed within synaptic fractions of the adult mouse forebrain were present at levels that were similar to or greater than total forebrain homogenate. Mature miRNAs were predominantly associated with soluble components of the synaptic fractions, whereas miRNA precursors were predominantly associated with post-synaptic densities [34]. Dicer that is present in the post-synaptic densities is enzymatically inactive until conditions arise that cause activation of calpain. These authors propose that mature miRNAs are formed locally within synaptic fractions following synaptic stimulation via processing of miRNA precursors [34].

Obernosterer et al. studied the post-transcriptional regulation of miRNA in the developing mouse and in HeLa cells [35]. They found that many miRNAs are transcribed at the precursor level, however upon differentiation, the ubiquitously expressed precursor miRNAs are processed to mature miRNA in a tissue-specific manner. In situ hybridization revealed that pre-miR-138–2 is present in nearly all of the tissues in the E17 mouse embryos, whereas mature miR-138 was expressed only in neuronal tissues (brain, CNS) and in the foetal liver [35]. HeLa cells are another example of a cell type that expressed the miR-138–2 precursor but not the mature miR-138. Conceivably, inhibition of Exportin 5 transport would prevent the pre-miR-138 from reaching the cytoplasm and be processed to mature. However, pre-miR-138–2 was present in the cytoplasm of HeLa cells effectively ruling out this possibility. HeLa cytoplasmic extracts inhibited pre-miR-138–2 processing by recombinant Dicer but not pre-miR-19a (a miRNA that is normally expressed in HeLa cells) [35]. Therefore, the presence of some unknown inhibitory factor appears to be preventing processing of pre-miR-138–2 in the cytoplasm of HeLa cells. These authors have developed a protocol to assist in the identification of cytoplasmic, inhibitory factors of miRNA processing [36].

The miR-17–92 polycistron is composed of six miRNA precursors (i.e. miR-17, -18a, -19a, -19b-1, -20a and -92–1). Forced expression of the polycistron along with c-myc was tumourigenic, suggesting that this group of miRNAs may function as oncogenes [37]. The splicing regulatory factor heterogeneous nuclear ribonucleoprotein (hnRNP) A1 is required for Drosophila processing of miR-18a from the primary precursor [38]. HnRNP A1 was shown to interact specifically with the miR-18a hairpin and this interaction occurred prior to the processing by Drosha [7]. Using RNA interference knockdown of hnRNP A1, it was shown that miR-18a and not the other five miRNAs on the pri-miRNA required hnRNP A1 for processing. This is the first example of an hnRNP regulating the processing of miRNA. There are at least 28 known human hnRNPs and miRNA–protein complex proteins (mRNPs) [39]. Conceivably, some of these hnRNPs or mRNPs may be involved in regulating the processing of other miRNAs.

Effects of RNA editing on miRNA processing

RNA editing is a process in which adenosine deaminases (ADARs) convert adenosine to inosine on double-stranded RNA (reviewed in [40]). This process will have profound differences when protein-coding mRNA is edited as inosine is translated as guanosine. On double-stranded noncoding RNA, guanosine will base pair with cytidine, thus altering the base pairing specificity and ultimately the three-dimensional structure of the RNA. Therefore, processes such as miRNA processing that relies heavily upon three-dimensional RNA structure would be expected to be affected by RNA
Examples of altered miRNA processing in cancer

Numerous studies have identified widespread alterations in the expression of miRNAs in human cancer (reviewed in [48, 49]). In fact, differential miRNA expression has been identified in all malignancies studied to date. There is no clear trend in the direction of the differentially expressed miRNA in human cancer as the expression is increased or decreased compared to normal tissue. Whether the expression is increased or decreased depends upon the individual miRNA. For example, some miRNAs, e.g. miR-15, -16 [50], miR-143, -145 [51–53], let-7 [54], miR-199a, -199a* [55–57] and 122a [56, 58] are reduced in cancer. Other miRNAs, e.g. miR-21 [59–62], miR-221, -222 [63–65], miR-100 [63] and miR-155 [66] are increased in cancer. At least three different mechanisms have been proposed to explain the alterations in miRNA expression in human cancer. These include (i) miRNAs that are located at cancer associated genomic regions [67, 68], (ii) epigenetic regulation of miRNA expression [69, 70] and (iii) abnormalities in miRNA processing. This review will discuss only the later mechanism.

The initial study demonstrating the relationship between alterations in miRNA processing in cancer was that of Michael et al. [51]. Two miRNAs, miR-143 and miR-145, had reduced expression of the mature form in colorectal adenocarcinoma compared to the matched normal mucosa. Interestingly, the Northern blots showed that the levels of pre-miR-143 and -145 were identical between the tumour and normal tissues. These authors went on to examine the miR-143 and -145 expression in several cancer cell lines and showed that like the tumours, the cell lines had reduced levels of mature miRNA, whereas equivalent levels of the precursors were detectable. Thus, the reduction in mature miR-143 and -145 in colorectal cancer cell lines and tissues is a result of reduced processing of the miRNA precursors.

miR-143 was subsequently shown to target ERK5 [71]. ERK5 is a growth-related mitogen-activated protein kinase. Transfection of a miR-143 and miR-145 precursor oligonucleotides into colorectal carcinoma cell lines reduced cell viability and reduced the levels of ERK5 protein [52]. Furthermore, the genomic loci of the majority of the cancer cell lines were unchanged and alterations in DNA methylation among the cell lines did not occur [52]. miR-143 and -145 was found to be reduced in most of the B-cell malignancies examined, including chronic lymphocytic leukaemias, B-cell lymphomas, Epstein–Barr virus-transformed B-cell lines and Burkitt lymphoma cell lines; these authors did not examine the levels of miR-143 and miR-145 precursors [53]. The results of these studies strongly suggest that miR-143 and -145 function as tumour suppressors and that deregulation in colorectal cancer and B-cell malignancies contributes to the tumourigenesis. This is of particular interest because it would exemplify the first tumour suppressive miRNAs whose expression is regulated by processing of the precursor.

miR-7 inhibits the epidermal growth factor receptor and the Akt pathway [72]. Mature miR-7 was predominately reduced in...
glioiblastoma tumours and in the glioblastoma cell line U87MG [72].
The mature miR-7 is processed from three different primary precursors, miR-7–1, miR-7–2 and miR-7–3. Using a quantitative PCR approach, levels of all three pri-miR-7 isoforms were similar between the glioblastomas and the normal tissues, however the levels of the pre-miR-7 isoforms were reduced in the glioblastomas [72]. The reduction in the miR-7 processing was shown to occur at the pri- to pre-miRNA level and not at the pre-miRNA to mature level and was specific to the U87MG cell line and not to HeLa cells [72]. Deletions or mutations were not responsible for the reduction in miR-7 processing. These results indicate that there is a specific defect in the processing of the pri-miR-7 in glioblastomas.

Altered expression levels of Dicer in cancer

One possibility to explain changes in the expression levels of miRNA in neoplasia is if the expression of the processing enzymes (Dicer and/or Drosha) were altered in cancer. Increased or reduced expression of these enzymes would result in a global increase or decrease in the expression levels of the miRNA, respectively. Data on the potential role of altered Drosha or Dicer expression in cancer is inconsistent. Some studies report an increase in Dicer levels in cancer, some report reduced Dicer expression and some studies report no change. There is likely no one size fits all explanation for altered processing in cancer and each malignancy must be examined on an individual bases with regards to Dicer or Drosha processing.

In their study of reduced mature miR-143 and -145 expression in colorectal carcinoma, Michael et al. did not examine the Dicer expression levels in the neoplastic and normal tissues, so a conclusion to describe their data on the basis of Dicer processing cannot be made at this time [51]. Lu et al. reported predominately reduced expression of multiple miRNAs in several different solid tumours [73]. They did not, though, detect a reduction in Drosha or Dicer mRNA in these tumour specimens and conclude that other mechanisms are responsible for the reduction in miRNA expression [73].

In a study of non-small-cell-lung cancer (NSCLC), it was determined that the expression levels of Dicer but not Drosha were reduced in a fraction of lung tumour tissues and that those patients with reduced Dicer expression had a poorer prognosis [74]. The expression of Drosha as well as other genes on chromosome 5p was increased in cervical cancer [75]. In a tissue micro-array consisting of 232 prostate specimens, Dicer protein was found to be up-regulated in prostatic intraepithelial neoplasia and in 81% of the prostate adenocarcinomas [76]. It was proposed that increased Dicer expression in prostate carcinoma was partially responsible to explain the global increased miRNA expression in prostate cancer that was identified by this group [76]. However, others have concluded that there is a predominate reduced expression of miRNAs in prostate cancer [73, 77, 78], so a conclusion on the contribution of Dicer expression in prostate cancer awaits further study.

Precursor lesions of peripheral adenocarcinoma of the lung (i.e. atypical adenomatous hyperplasia and bronchoalveolar carcinoma) showed increased Dicer protein by immunohistochemistry [79]. The opposite was true for invasive and advanced adenocarcinoma, where Dicer protein was reduced [79]. The later phenomena may be partially explained by deletions in the Dicer locus [79]. This same group of researchers studied the Dicer protein expression in specimens of mucoepidermoid carcinoma (MEC) and correlated the Dicer expression with survival [80]. They determined that Dicer protein expression could either be increased or decreased in MEC. The high-grade, stage-III/IV, margin positive MECs showed predominate increased Dicer expression, whereas the tumour specimens from patients over 55 years of age were predominately reduced [80].

Evidence of post-transcriptional regulation of miRNA expression in cancer

Thomson et al. used published messenger RNA data to approximate the pri-miRNA xpression of those miRNAs that are encoded within introns [29]. Thus, the expression levels of a large number of pri-miRNAs were determined in several tumours and normal tissues without additional profiling. Because the mature miRNA was profiled in this same data set [73], it was possible to establish the correlation between the levels of pri-miRNA and mature miRNA in a number of normal tissues and tumours. Consistent with their data in the developing mouse, the expression of the pri-miRNA was more or less consistent between tumour and normal, whereas the levels of mature miRNA were considerably reduced in the malignancy compared to the normal tissue [29]. These authors conclude that the widespread down-regulation of miRNA in cancer is due to a failure at the Drosha processing step. This failure is not due to reduction in Drosha mRNA expression as the levels of Drosha mRNA in this set of tissues did not change [73] and therefore alternative explanations are necessary.

Perhaps the strongest case for a relationship between reduced mature miRNA expression and cancer comes from the study by Kumar et al. [81]. shRNAs to three regulators of miRNA processing (Drosha, DGC8 and Dicer) were used to dramatically reduce the levels of these genes and thus impair miRNA processing. Transformed cells transfected with the shRNAs showed enhanced transformation properties in vitro and in vivo. These include enhanced colony formation and growth in soft agar. miRNA processing impaired cells injected into nude mice formed tumours that grew both faster and larger than control cells. Protein levels of the oncogenes K-Ras and c-Myc were elevated in the cells with impaired processing compared to control [81]. Since the let-7 family of miRNAs regulate K-Ras [82], this is but one example of the effects of reduced mature miRNA on an oncogene. Unlike the transformed cells, primary cells with experimentally impaired processing did not show the transformed properties and grew slower than wild-type cells. This suggests that reduction in mature miRNA levels is not an initiating event but perhaps an event that maintains the transformation properties.

Lee et al. used real-time PCR assays specific to the miRNA precursors [83, 84] and mature miRNA [85] to expression profile the precursor and mature miRNAs in a number of cancer cell lines,
tumours and normal tissues [86]. Quite often, the miRNA precur-
sor was present but not the mature miRNA. For example, the
brain-specific miR-128a precursor was present in most of the
22 normal tissues at levels of several hundreds of copies per cell,
however the mature miR-128a was expressed only in brain and
skeletal muscle [86]. As expected, plenty of examples of pre-
miRNAs being processed to mature miRNA exist. This, in effect,
rules out the possibility of widespread reduction in the expression
of Drosha or Dicer being responsible for reduced mature miRNA
as one would expect the reduction in processing to be consistent
across all tumours, tissues and cell lines.

The processing of miR-31 was of particular interest [86]. Equiva-
lent miR-31 precursor levels were detectable by real-time PCR and Northern blotting in a number of cancer cell lines. The
mature miRNA was detectable by real-time PCR and Northern blot-
ing in some of the cell lines (e.g. HS766T cells) but not others
(e.g. MCF-7). In situ hybridization of the mature miR-31 showed the
presence of mature miR-31 in the cytoplasm of HS766T cells but
was undetectable in the MCF-7 cells. Detection of the pri- and pre-
miR-31 with RT in situ PCR demonstrated that the miR-31 precu-
sors were present in both the nucleus and cytoplasm of cells that
expressed mature miR-31 (e.g. HS766T) but in cells that lacked
mature miR-31 (e.g. MCF-7); the miRNA precursors were confined
to the nucleolus [86]. Thus, miR-31 is an example of a miRNA that
is processed to the mature form in some cell lines, but in other cell
lines it is processed to the pre-miRNA and is not processed to the
mature miRNA because it is retained in the nucleolus.

A large-scale comparison was made to determine the Pearson
coefficients for correlations between the precursor and mature
miRNA expression. This comparison allowed an approximation of the
degree to which the miRNA precursors are being processed to
mature miRNA. The data demonstrated that the miRNA precursors
were processed to a greater extent in pancreas tumours, whereas in
hepatocellular cancer a greater percentage of the precursor miRNA
were not processed to mature miRNA [86]. In pancreas cancer, there
was a predominately increase in the expression of a number of
miRNAs [63, 64], whereas in hepatocellular carcinoma, there was a
predominant decrease in the miRNA expression [55, 56]. These find-
ings suggest that in pancreatic cancer, the increased expression of
miRNA is regulated at the level of transcription or perhaps post-tran-
scriptionally with no apparent reduction in processing, but in hepa-
tocellular carcinoma the reduced expression of miRNA in the
tumours may result from a reduction in miRNA processing.

Recently, it was shown that the SMAD proteins (SMAD1,
SMAD2 and SMAD3) control processing by Drosha of miR-21 and
miR-199a [87]. Human primary pulmonary artery smooth muscle
cells exposed to TGF-β or bone morphogenic factor (BMP) dis-
played increased expression of the precursor and mature forms of
miR-21 and miR-199a but not the primary precursor forms of these
miRNAs, demonstrating that the increased expression of these
mature miRNAs was due to post-transcriptional regulation. The
post-transcriptional regulation was due to a specific interaction
between the pri-miRNAs, SMAD proteins, p68 and the Drosha com-
plex [87]. The post-transcriptional regulation of pri-miR-21 and pri-
miR-199a is independent of SMAD4, the SMAD required for most
transcriptional responses to TBF-β and BMP. TGF-β signalling was
inhibited in MDA-MB-468 breast cancer cells using a mutated form
of the TGF-β type I receptor. MDA-MB-468 cells with impaired
TGF-β signalling produced reduced levels of both basal and TGF-β-
induced pre- and mature miR-21 but did not alter the pri-miR-21
levels. This study identifies a critic link between the TGF-β family
signalling pathways and miRNA biogenesis and suggests that one
explanation for increased miR-21 expression present in a number of
solid tumours results from increased TGF-β signalling.

Conclusion

This review highlighted the available data that describe situations
where alterations in the levels of mature miRNA may be explained by
alterations in miRNA processing. This phenomenon occurs in termi-
inally differentiated cells, during development and in at least one
human disease, cancer. As reviewed here, several known and
unknown mechanisms are responsible for causing reduced miRNA
processing (Fig. 1). Known mechanisms include binding of hnRNPs
to the pri-miRNA [38], A-to-I editing of the pri-/precursor
miRNA [41–43], mutations on the miRNA precursors that likely affect
processing [45], the presence of inhibitory proteins that regulate
processing [30, 31], altered levels of Dicer and/or Drosha [74, 76],
regulation by the TGF-β family signalling pathways [87] and seques-
tration in different cells types [34]. Because processing proteins (e.g.
Drosha, Dicer, DCGR8, etc.) produce global effects on miRNA pro-
cessing, it is unlikely that situations of altered processing result from
altered levels of these factors. More than likely, regulation of processing
results from miRNA-specific factors that aid or inhibit processing of the
precursors [30–32, 38, 87]. In this way, individual miRNAs may
be regulated without affecting global miRNA expression.

A number of important questions await further study. Do
inhibitory factors besides Lin-28 exist that regulate the processing
of the miRNA precursors? What is the prevalence of SNPs and/or
mutations in the miRNA precursors and how do they affect
processing? Do hnRNPs modulate processing of miRNAs other than
miR-18? Are specific factors present that assist in the intracellular
trafficking of miRNA precursors and could alterations in these factors
account for altered trafficking within the cell (e.g. reduced
cytoplasmic transport). How many miRNAs are regulated post-
transcriptionally by the TBF-β/SMAD signalling pathway and what
other signalling pathways regulate miRNA processing? How preva-
ent is the reduction or induction of miRNA processing in human
cancers? And finally, do other human diseases exist in which alter-
ations in miRNA processing is a major contributor? Clearly, under-
standing the mechanisms responsible for alterations in miRNA
processing will be an active area of research for years to come.

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