Chapter 1

Controlling miRNA Regulation in Disease

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Abstract

Our understanding of the importance of noncoding RNA molecules is steadily growing. One such important class of RNA molecules are microRNAs (miRNAs). These tiny RNAs fulfill important functions in cellular behavior by influencing the protein output levels of a high variety of genes through the regulation of target messenger RNAs. Moreover, miRNAs have been implicated in a wide range of diseases. In pathological conditions, the miRNA expression levels can be altered due to changes in the transcriptional or posttranscriptional regulation of miRNA expression. On the other side, mRNA molecules might be able to escape the regulation by miRNAs. In this review, we give an overview on how miRNA biogenesis can be altered in disease as well as how mRNAs can avoid the regulation by miRNAs. The interplay between these two processes defines the final protein output in a cell, and thus the normal or pathological cellular phenotype.

Key words: microRNA, microRNA biogenesis, microRNA expression regulation, microRNA regulation in disease, microRNA expression in disease

1. Introduction

The discovery that the small RNA lin-4 could regulate the expression of lin-14 in the nematode *Caenorhabditis elegans* (1) nearly 20 years ago was the beginning of a major shift in biological research. It contributed to our current awareness that RNA molecules are not just simple intermediates between DNA and proteins, but also can play important roles in gene regulation. These regulatory functions of RNA molecules can explain, at least in part, how such complex organisms can arise despite that they harbor only a limited set of protein-encoding genes. Although a tremendous progress in the understanding of small RNA-mediated gene regulation has been achieved in recent years, there is still a whole layer of relatively unexplored RNA-mediated mechanisms to be discovered that play a role in fine-tuning cellular behavior.

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After the initial discovery of lin-4, the different classes and number of small regulatory RNAs has expanded dramatically. One important class of such small RNA molecules are microRNAs (miRNAs), of which there are currently several thousand annotated in various species in the publically available database miRBase (2). miRNAs are 20–23 nucleotides long, and are estimated to regulate the expression of as much as one-third of genes in mammals (3). miRNAs can downregulate gene expression by binding to complementary regions within the 3′-untranslated region of mRNAs, thereby effecting the translation or stability of the mRNA. In this chapter, we intend to give an overview on the current knowledge on miRNA biogenesis, their mode of action, and factors that influence miRNA-mediated regulation in normal and disease development.

2. The Biogenesis of an miRNA

In the human genome, miRNAs exist as either individual genes or in clusters. A substantial fraction of miRNA genes are clustered together, which might be related to their evolutionary mechanism for spreading throughout the genome (4). These relatively closely located miRNAs can be transcribed as one polycistronic transcript, giving rise to multiple co-expressed mature miRNAs. Nonclustered miRNA genes, on the other hand, are expressed as individual transcripts and have their own promoters (Fig. 1). Such transcriptional organization of miRNA genes provides for numerous combinations of miRNAs that are individually or simultaneously expressed in a spatiotemporal manner.

Also the genomic context of miRNAs can differ between the individual miRNAs. The genes encoding for miRNAs can be embedded in the intronic region of a protein-coding gene, as well as located in an intergenic region. Those miRNAs that are imbedded within other genes can have two forms of transcriptional regulation. They are either co-transcribed and processed together with the protein-coding gene, or have their own intronic promoters (5) (Fig. 1). Thus, the expression levels of intronic miRNAs do not always have to correlate with the expression levels of the host gene. Also, the independent transcriptional control of miRNAs underscores their role as independent regulators of gene transcription in eukaryotic biology.

MiRNAs are transcribed as long precursor molecules by the RNA polymerase II, and these so-called primary (pri-)miRNAs can be up to several kilobases long. During and after transcription, pri-miRNAs fold back into imperfect double-stranded RNA molecules that are the stepping stones for subsequent processing into the mature miRNAs. The maturation of pri-miRNA transcripts

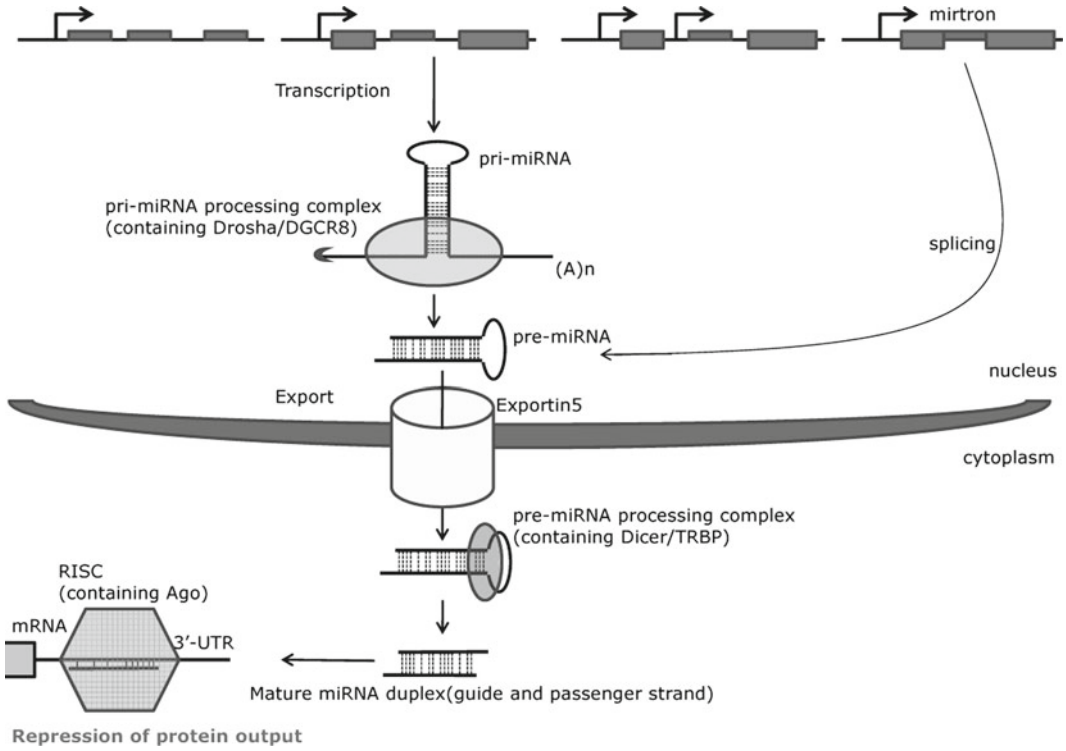

Fig. 1. The miRNA biogenesis pathway. MicroRNAs (*thin boxes*) can be located in intergenic regions or are embedded in the intronic region of a protein-encoding gene (exonic regions are shown here as *thick boxes*). In addition, microRNAs can be co-expressed with the genes in which they are implanted, or they have their own promoter sequence (*arrows*). After transcription, the pri-miRNA sequence is processed by the Drosha complex, the resulting pre-miRNA sequence is exported to the cytoplasm, and subsequently cleaved by the Dicer complex into a mature miRNA sequence. On the other hand, mirtrons are not cleaved by Drosha, but the pre-miRNA sequence is generated through splicing, after which it follows the biogenesis pathway of canonical miRNAs. The mature miRNA sequence is loaded into the RISC complex, which results in a reduction of protein output after binding to the target mRNA. See the main text for more details.

into mature miRNAs involves several consecutive protein-mediated processing steps (6) (Fig. 1). In mammalian systems, the first processing step of the pri-miRNA to precursor pre-miRNA occurs in the nucleus by the microprocessor complex that consists of at least 20 distinct proteins, including the RNAse III enzyme Drosha and the double-stranded RNA-binding enzyme DGCR8. A single pri-miRNA contains a typical stem structure of around 33 bp, and Drosha is guided by DGCR8 to cleave about 11 bp away from this stem-ssRNA junction (7). This cleavage step results in pre-miRNAs that are around 60–80 nt in length and contain a typical 2 nt 3′-overhang due to the Drosha cleavage.

The following processing step takes place in the cytoplasm. Thus, in order to become mature miRNAs, the pre-miRNAs are first transported from the nucleus into the cytoplasm. This transport is mediated by nuclear pore complex exportin5 and RanGTP (8–10),

and the length of the stem loop structure as well as the presence of the 3′-overhang is important for recognition of the pre-miRNA by exportin-5 (9, 11). In the cytoplasm, the pre-miRNAs are cleaved into mature miRNA molecules by the RNAse III enzyme Dicer. This generates the final double-stranded miRNA products of 21–23 nucleotides, containing 2 nucleotide 3′-overhangs on both sides of the molecule. The Dicer protein alone is sufficient for generating the mature miRNAs from pre-miRNAs, but it does interact with a series of other proteins, such as TRBP (12).

In order for an miRNA to be functional in the regulation of gene expression, it has to be incorporated in the multiprotein complex (miRNA-induced silencing complex (miRISC). TRBP can form a bridge between the miRNA processing by Dicer and the ultimate functionality of the miRNA, by forming a complex with Dicer and recruiting Argonaute (Ago), an essential member of the miRISC complex (12). In general, the miRNA strand with the thermodynamically less stable 5′-end is incorporated in the miRISC complex (6) and functions as a template for scanning mRNA 3′-UTRs for sequences complementary to the miRNA. The other strand, referred to as the star or passenger strand is in most cases rapidly degraded.

Strikingly, the miRNA does not have to possess a completely perfect complementary sequence to the 3′-UTR for mediating gene repression. More precisely, the number of perfect matches needed between the miRNA and the target mRNA seems to differ from miRNA to miRNA to mRNA. The only critical sequences for targeting seem to be in the 5′-end of the miRNA, the so-called seed sequence, which comprises nucleotides 2–8, although weaker seed sequences might be compensated through pairing of the 3′-end of the miRNA to the mRNA. This lack of black-and-white well-defined miRNA target recognition rules thus makes it extremely difficult to identify bona fide miRNA targets in silico (13).

3. Post-transcriptional Factors That Modulate miRNA Biogenesis

The biogenesis of miRNAs is subjected to complex regulatory mechanisms. The regulation of miRNA production can provide the cell with a fast-acting response to environmental changes. Also, this effect will be wide-spread, as miRNAs act on many different mRNAs simultaneously. Influencing the efficiency of miRNA biogenesis can therefore be a quick and powerful approach to anticipate to external and internal cellular fluctuations.

As mentioned above, the canonical processing of pri-miRNAs into mature miRNAs occurs in two consecutive steps (Fig. 1). The transcribed pri-miRNAs are cleaved by the Drosha complex into pre-miRNAs, of which subsequently mature miRNAs are generated by the Dicer-processing complex. The processing efficiency of

subsets of pri-miRNAs into the final mature miRNAs is influenced by a battery of other proteins, generating a complex network of miRNA expression regulation, which we are only just beginning to understand. We discuss several examples below.

During differentiation, there is a steady increase in mature Let-7 g levels. However, the pri-miRNA levels of Let-7 g remain fairly constant (14). This indicates that there is some kind of Drosha processing block for pri-miRNA Let-7 g processing, either due to posttranscriptional modification of Drosha, or due to the influence of other regulatory proteins. One of these regulatory proteins is the tumor suppressor p53, as p53 can enhance the processing efficiency of several miRNAs by interacting with a protein in the Drosha complex (15). Intriguingly, of the miRNAs that were tested in this study, the miRNAs that were influenced by p53 had growth suppressive functions. How p53 selects between different miRNAs is yet unknown, but it directly couples the tumor suppressive network to the process of miRNA biogenesis. Another family of proteins that acts on the level of Drosha cleavage are Smad proteins (16). These proteins recognize and bind to a specific motif present within the stem loop region of certain miRNAs. Binding of the Smad proteins subsequently enhances the docking and cleavage by Drosha.

Thus, both Smad proteins as well as p53 enhance the processing step of pri-miRNAs into pre-miRNAs. On the other side, there are also proteins that can inhibit the processing of pri-miRNAs. Upon binding of the estrogen receptor α to estrogen, the receptor associates with the Drosha complex and inhibits the maturation of a set of miRNAs (17). In addition, depletion of the nuclear factor (NF) NF90 and NF45 results in an accumulation of pri-miRNAs in the cells (18).

A subgroup of miRNAs that skips the Drosha processing step altogether are mirtrons (19–21). These miRNAs are encoded within small intronic regions of transcribed genes. Upon splicing, the intronic region folds into a pre-miRNA hairpin structure. These pre-miRNA hairpin mimics subsequently follow the canonical miRNA biogenesis pathway to mature miRNAs.

The step following Drosha cleavage is the transport of the pre-miRNA into the cytoplasm. However, to what extent miRNA biogenesis is controlled by the efficiency of the pre-miRNA export is still a matter of debate (22). In the cytoplasm, the processing of miRNAs can be controlled by factors that influence the efficiency of Dicer. The protein TRBP is phosphorylated through the MAPK signaling pathway, and phosphorylated TRBP does stabilize the Dicer-processing complex. This stabilization results in an increased miRNA production and miRNA-mediated target silencing (23). Thus, crucial signaling pathways, such as the tumor suppressive p53 pathway and the oncogenic MAPK/Erk pathway, are involved in miRNA biogenesis. This suggests that these cell growth-regulating pathways can also partly exert their effects through regulating miRNA

expression, and that misregulation of miRNA biogenesis can be involved in oncogenesis, as is discussed in more detail below.

There are also proteins that can interfere with both the Drosha- as well as the Dicer-mediated cleavage step. The protein Lin28 binds to a specific sequence present in the loop structure of the miRNA let-7. This binding inhibits both the Drosha and Dicer cleavage step (24–27). Moreover, Lin28 recruits a terminal uridylyl transferase that uridylates pre-let-7, guiding pre-let-7 into a degradation pathway (28). However, protein binding can also have an opposite effect, resulting in an enhanced Drosha and Dicer processing. In the case of the miRNA mir-18a, the protein hnRNP A1 binds both the terminal loop structure as well as the stem loop of the pri-miRNA (29), and knockdown of hnRNP A1 reduces the levels of both pri- and pre-mir-18a (30). Also the protein KSRP can promote the processing of several miRNAs. KSRP is a component of both the Drosha- and Dicer-multiprotein complex, and it binds to the terminal loop region of pri- and pre-miRNAs (31). Binding of this protein can subsequently result in the recruitment or correct positioning of the Drosha- or Dicer-complex.

The above-described proteins directly interact with proteins of the miRNA-biogenesis machinery. Another family of proteins that has a completely different mode of action is the ADARs. These proteins can alter the primary sequence of double-stranded RNA molecules, such as pri-miRNAs (32–36), through the hydrolytic deamination of adenosines. This results in the conversion of an adenosine into an inosine, and the inosine behaves similar to guanosine. This A-to-I editing of pri-miRNAs can alter the processing efficiency of Drosha and/or Dicer and is expected to occur in roughly 16% of all pri-miRNA molecules (37). Most interestingly, editing also occurs in the seed region of miRNAs. The editing at these positions can alter the target recognition of the miRNA, with the edited version targeting another set of genes than the unedited version (34). Although difficult to assess (38), the editing of the mature miRNA sequence seems to be limited and the main effect of miRNA editing therefore appears to be the alteration of miRNA biogenesis.

The final generated mature miRNA is incorporated into the miRISC complex and its ultimate half-life is dependent upon several factors (39). The miRNA is protected against decay through incorporation into the Argonautes, and ectopic expression of the Ago proteins results in increased miRNA levels (39, 40). However, for example in neurons there is still a rapid turnover of miRNAs with a half life of approximately 1 h (41). In the nematode *C. elegans*, the degradation of the exposed mature single-stranded miRNAs is mediated by the 5′-3′ exonuclease XRN-2 (42). This protein seems to have an intriguing double function, as it can also mediate the release of the miRNA from the Ago-complex if the miRNA is not interacting with its target molecule. This regulatory mechanism

might be important for the rapid transitions seen in miRNA expression profiles.

Also posttranscriptional modification of the miRNA could increase its half-life. However, this seems to be a much more common phenomenon in plants rather than in animals. Although deep-sequencing studies indicated that mammalian miRNAs frequently contain untemplated adenine or uracil additions (43), a functional effect has so far only been demonstrated for miR-122, where the addition of an adenine at the 3′-end enhances its stability (44).

There is thus a whole array of regulatory proteins that influence the homeostasis of mature miRNAs, and more proteins and regulatory mechanisms are likely yet to be discovered (22, 39). The interplay between all these proteins will generate a broad spectrum of miRNA expression levels, which cannot be deducted solely from their initial transcription levels. Another pending question is how precisely miRNAs subsequently regulate the repression of gene expression. As is discussed in more detail below, also here there might be more (regulatory) roads that are leading to Rome.

4. miRNA-Mediated Regulation of Gene Repression

In animal systems, miRNAs can regulate gene expression through either the inhibition of protein translation or initiation of mRNA degradation (6). This is a major difference with the plant kingdom, where miRNAs form highly complementary Watson-Crick base pairing with the target mRNA and subsequently solely induce mRNA cleavage (45). Nonetheless, in animals there seems to be a correlation with the extent of base-pairing between the miRNA and its target, and subsequent cleavage or translational repression (46).

In mammals, there are four members of the Argonaute family (AGO1-4) (47), of which only AGO2 has mRNA cleavage ("slicer") activity and is also the only AGO protein that functions in the RNA interference (RNAi) pathway by cleaving the mRNA in the center of the mRNA-siRNA duplex (47, 48). Interestingly, there is also an miRNA mir-451, whose maturation is directly regulated by the slicer activity of AGO2. Instead of Dicer, this pre-miRNA is cleaved by AGO2 and likely subsequently polyuridylated and trimmed into the mature miR-451 sequence (49). There is thus a close interplay between miRNA production and activity. Besides the slicer activity of AGO2, miRISCs containing either AGO2 or other AGO proteins can cause a reduction in the protein output without directly diminishing the mRNA levels. In these instances, reduction in protein expression is caused by interfering with the translational machinery.

There are currently several working models that describe the repression of translation after binding of the miRISC complex to the mRNA molecule. Broadly, these mechanisms can be divided into inhibition before or after the initiation of translation. Inhibition of translation at the initiation step can occur through interference with the protein assembly on the mRNA molecule, prior to translation. In brief, proteins that bind to either the 5′- cap or the 3′-polyA tail of the same RNA molecule interact with each other, bringing the two ends of the RNA molecule together. This circularization stimulates the initiation of translation. In the case of miRNA-mediated repression, AGO2 might interact with the 5′-cap of the RNA molecule, competing with the binding of the regular translation initiation factors (6,50). In addition, miRNA-mediated repression can result in the shortening of the 3′-polyA tail through deadenylation (51–54), which might cause the disruption of the circularization of the RNA molecule. Thus, inhibiting the translation by miRNAs can be caused by targeting either of both the 5′- or 3′-ends of the RNA molecule.

As with many new rapidly evolving research fields, there is not yet one solid answer to the question of how miRNAs regulate the inhibition of protein translation; besides inhibiting the translational machinery at the initiation step, there is also evidence that miRNAs can repress gene expression during the elongation of translation. An intriguing example is the study from Petersen et al. (55) that showed that short RNA-induced repression can also occur in the downstream open reading frame of a bi-cistronic reporter containing and IRES. The translation of this downstream RNA molecule is thus independent of the 5′-cap structure. Moreover, other data indicated that translating ribosomes do assemble but might prematurely drop-off again. It is very well feasible that miRNAs can act as inhibitors of translation on both the level of translation initiation as well as translation elongation. However, the importance of either mechanism is still fuel for an ongoing scientific debate.

Besides mRNA cleavage or the inhibition of the translational machinery, other mechanisms that reduce the protein output can play a role as well. miRNAs can induce the deadenylation of the mRNA polyA tail, leading to de-capping and subsequent degradation of the RNA molecule through 5′ → 3′ exonucleolytic activity (52–54, 56). So there seem to be many different mechanisms by which a miRNA can cause a reduction in the protein output. At this moment, it is not yet clear what determines whether the RNA is destabilized or if there will be inhibition of translation. Also, it is unclear whether RNA deadenylation results from translational repression, or whether this is a primary event. A recent study from Guo et al. (57) demonstrated that over 80% of the decreased protein production was assigned to a decrease in mRNA levels, thus supporting the mRNA destabilization model.

5. miRNA Regulation in Disease

miRNAs play an important regulatory role in many crucial cellular pathways, such as development, differentiation, and apoptosis. However, knockdown of specific miRNAs in flies and worms in many cases did not reveal crucial viable or developmental defects (58–60). But when environmental conditions changed, the defects due to knockdown of certain miRNAs became apparent (61). This indicates that miRNAs can play a role in maintaining homeostasis in a cell, and there is a loss of fine-tuning when conditions are altered. It is therefore not surprising that an altered miRNA-mediated regulation can contribute to pathological conditions, such as cancer, cardiovascular disease, and many others. This altered regulation of the miRNA target can be the result of changed miRNA levels, but also because the mRNA target might simply escape miRNA-controlled regulation (Fig. 2).

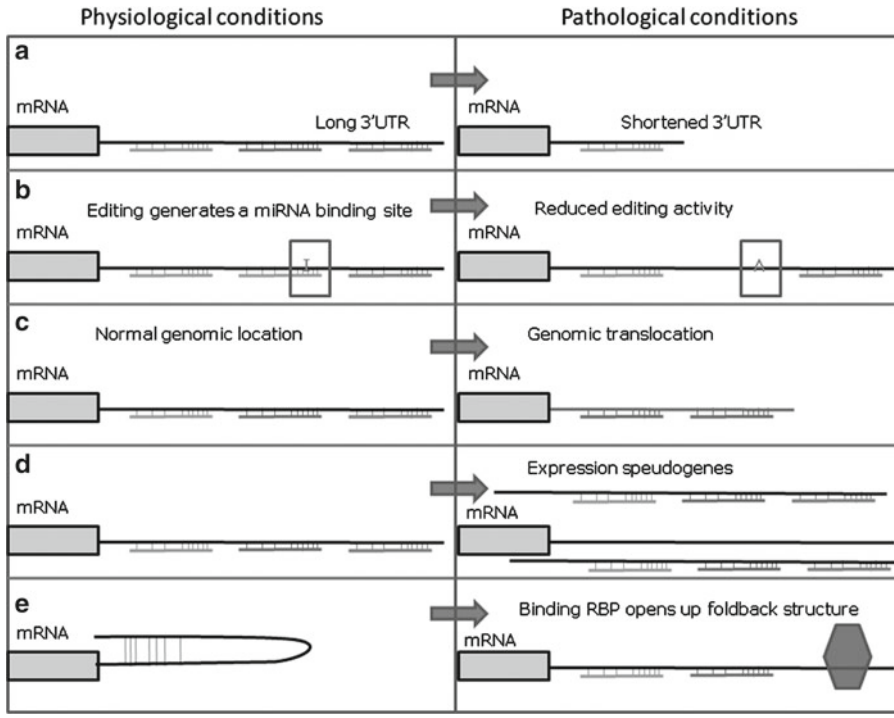

Fig. 2. Preventing the regulation by miRNAs (**a**) Shortening of the 3′-UTR results in less binding sites for miRNAs. (**b**) Decreased A-to-I RNA editing activity causes an increased prevalence of an adenosine over an inosine, which subsequently causes the loss of an miRNA binding site. (**c**) Genomic translocation leads to another 3′-UTR sequence and consequently other miRNA-binding sites. (**d**) The expression of a pseudogene captures the miRNAs away from the original mRNA target sequence. (**e**) Binding of an RNA-binding protein opens up the structure of the 3′-UTR and makes it more accessible for the miRNA to target the mRNA.

6. Escaping miRNA-Mediated Regulation

The interaction between the miRNA and the target mRNA occurs predominantly in the 3′-UTRs of the mRNA molecule. This results either in inhibition of translation or RNA degradation, as outlined above. Thus, a way for mRNAs to escape the regulation by miRNAs is to shorten the length of the 3′-UTR by alternative polyadenylation (Fig. 2a). This intriguing possibility was first investigated by Sandberg and coworkers (62), who demonstrated the widespread shortening of 3′-UTRs upon activating/proliferating stimuli in a variety of immune cells. To further extrapolate their results, they also investigated a wide variety of cell lines. The length of the 3′-UTR was consistently lower in cell lines in comparison to the tissues from which they originated, further indicating that the length of the 3′-UTR was associated with the cellular proliferation potential. Using a luciferase-based system, a longer 3′-UTR was shown to decrease protein output in comparison to the short 3′-UTR sequence, likely due to the inhibition of translation or RNA stability. Finally, they suggest a model in which the shortening of the 3′-UTR region results in reducing the potential for targeting by antiproliferative factors, such as certain families of miRNAs (62).

In another key study, the shortening of the 3′-UTRs is linked to oncogenic transformation rather than proliferation per se (63). Mayr and Bartel show that cancer cell lines compared to nontransformed cell lines have shortened 3′-UTRs, despite a similar proliferation potential. These shorter mRNA forms are more stable and characteristically produce about ten times more protein. In the case of oncogenes, shortening of the 3′-UTR by alternative polyadenylation can result in an increased protein production and consequent cellular oncogenic transformation. The increased protein output could at least in part be explained by escaping miRNA-mediated regulation (63). These observations suggest that mRNAs can escape miRNA-mediated control due to a shortened 3′-UTR, and that this alternative polyadenylation could contribute to the oncogenic potential of tumor cells.

Another intriguing mechanism by which mRNAs can escape the control of miRNAs in disease is an alteration in RNA editing activity (Fig. 2b). As mentioned above, A-to-I RNA editing converts an adenosine into an inosine, which is subsequently interpreted as guanosine. RNA editing is a widespread phenomenon, and, for example, in brain tissues it is estimated to occur once in every 17,000 nt (64). Most editing events take place in the untranslated regions, including 3′-UTRs. A study investigating the interplay between editing and miRNA regulation demonstrated that mRNA editing seems to avoid miRNA seed sequences (65). However, several sites were found where editing abolished an miRNA binding site, or generated a new miRNA recognition site.

The editing efficiency at an individual position can range from <2% to 90% (66), and its activity is altered in cancer and a variety of other diseases (67). This generates a complex interplay between two important posttranscriptional mechanisms to fine-tune gene regulation in normal and diseased conditions.

A more permanent methodology to eliminate miRNA-binding sites is the acquisition of genomic mutations in the 3′-UTR during tumor development (Fig. 2c). One such example is the oncogene Hmga2 (68), in which a chromosomal translocation causes disruption of the 3′-UTR. The Hmga2 3′-UTR contains several binding sites for the tumor-suppressive miRNA let-7, and disruption of these binding sites can lead to higher levels of Hmga2 and consequent oncogenic transformation. Thus, not only mutations in the open reading frame of specific proteins can result in pathological conditions, but also genomic alterations in the 3′-UTR that disrupt or create miRNA-binding sites, can play a role in disease development.

More subtle genomic alterations can occur through single nucleotide polymorphisms (SNPs). When located in the 3′-UTR of a gene, these SNPs can either disrupt or create miRNA binding sites. SNPs are allele-specific, and measuring the differences in allele-specific mRNA expression levels indeed revealed that genes carrying an SNP-generated miRNA-binding site were repressed in those tissues where the miRNA was expressed as well (69). As two unrelated humans have a distinct set of SNPs, many genes will be expressed in an individual-specific manner and can subsequently also underline differences in disease risk and clinical outcome.

The final protein levels from a certain transcript depend both on the number of miRNA molecules and the number of mRNA molecules present in a certain cell. However, if the miRNA binds to other RNA molecules, the original target mRNA is thus derepressed and translation can take place. This function can be fulfilled by pseudogenes, such as PTENP1 and KRASP1, which can modulate the protein production of respectively the tumor-suppressor PTEN and the oncogene KRAS, by functioning as a decoy for miRNA binding (Fig. 2d). Intriguingly, in certain cancer types, the pseudogene PTENP1 has indeed a loss of genomic copy number, whereas the KRAS1P locus is amplified in a variety of human tumors, which is consistent with their tumor-suppressive and oncogenic roles (70). As pseudogenes are nearly as numerous as protein-coding genes in the transcriptome, many more examples might exist, attributing a novel role for pseudogenes in regulating the expression of coding genes.

Finally, another way for an mRNA molecule to escape the regulation by miRNAs is to hide the miRNA-binding site for the binding of the miRNA (71) (Fig. 2e). The miRNAs mir-221 and mir-222 are required in cancer to inhibit the expression of p27 and subsequently stimulate proliferation. Intriguingly, the levels of these

miRNAs and p27 mRNA are not significantly altered in quiescent and cycling cells. However, in cycling cells the RNA-binding proteins PUM1 and PUM2 are activated and bind to the 3′-UTR of p27. It is this binding that opens up the secondary structure of the p27 3′-UTR and subsequently causes the miRNAs to bind and repress p27 protein expression (71). There is thus an extensive interplay between the control of protein expression by miRNAs and the many factors that influence the overall availability of miRNA-binding sites. The balance between this availability, as well as mRNA and miRNA levels ultimately define the protein output of a certain transcript, and if this balance shifts it might contribute to the development of disease. Besides the alteration of factors influencing the availability of miRNA-binding sites in pathological conditions, the expression levels of miRNAs themselves are also often altered in disease states. This can be due to differences in expression levels and also due to factors influencing the processing of miRNAs, as discussed below.

7. Altered miRNA Expression Levels in Pathological Conditions

At present, there are hundreds of research papers describing a role for miRNAs in pathological conditions, and the number of miRNA publications is steadily growing each year (72). This is not surprising, as miRNAs can influence the expression levels of many proteins that are involved in cell development, homeostasis, and disease. The strength of a single miRNA lies predominantly in the fact that it can influence the expression of an array of proteins. Thus, often the inhibitory effect of one miRNA on one protein is only marginal, but it is the cumulative effect of one miRNA on many proteins that results in the final cellular behavioral response. Changes in the expression level of a single miRNA can thus be an important factor in severe pathological conditions.

Among the first miRNAs that were suggested to play a direct role in tumor biology were mir-15a and mir-16-1. They were originally found in a genomic area that is often deleted in chronic lymphocytic leukemia (CLL), and these miRNAs were shown to be lost in about 70% of developing CLL (73). Strikingly, many other miRNA genes are located in cancer-associated genomic regions or fragile areas as well (74). Besides mir-15-1 and mir-16-1, a variety of other miRNAs have now been shown to play a direct role in tumor biology, functioning either as a tumor-suppressors or oncogenes (75).

A striking example of how one miRNA can drastically alter a cellular phenotype is mir-21 (76). This miRNA is overexpressed in a great number of tumor types and induction of mir-21 expression in vivo results in a pre-B malignant lymphoid-like phenotype.

Upon subsequent inactivation of mir-21 expression, this phenotype completely regresses again within a few days, indicating that tumors can become addicted to oncogenic miRNAs and demonstrating the strength of a single miRNA to regulate cellular behavior.

mir-21 was artificially introduced in these cell types, which resulted in the oncogenic transformation. In cancer, however, the expression levels of a wide variety of miRNAs was shown to be reduced (77) to such an extent that clustering of miRNA expression profiles causes tumor tissues to group together, instead of clustering to the tissue from which they derive (77). These miRNA changes can be the consequence of altered miRNA gene transcription levels. Among others, widespread repression of miRNA gene expression can occur via the c-Myc oncogene (78) or hypermethylation of CpG island promoters (79–81). But global changes in the mature miRNA levels can also occur posttranscriptionally, as often the altered levels in mature miRNAs do not correlate with changes in the primary miRNA transcript (14). What is the cause of these altered mature miRNA levels is still relatively unknown, although alterations in mechanisms that control the steady-state levels of miRNAs could at least partly explain these observed wide-spread changes in miRNA expression profiles.

As mentioned above, the tumor-suppressor gene p53 influences the processing of a group of miRNAs. As the protein levels of p53 are reduced in a high number of tumor types, this might alter the expression of these miRNAs as well. The same can hold for other proteins that influence the processing of pri- or pre-miRNAs, such as SMADs, NF90, or ADARs. Differential expression of each of these proteins in diseased tissues can cause an alteration in the expression levels of subsets of miRNAs.

Yet another protein involved in the differential expression of miRNAs in disease is the key miRNA-processing enzyme Dicer. In a variety of cancers, the expression of Dicer was shown to be reduced, including lung cancer, hepatocellular carcinoma, and ovarian cancer (82–85). This reduced expression was in general correlated with a poor clinical outcome. In addition, knock-down of Dicer caused an enhanced proliferation and migration rate of tumor cells, and in mice decreased Dicer expression resulted in a reduced survival rate due to an increased tumor burden (86). Intriguingly, this reduced survival was correlated to a hemizygous instead of a homozygous loss of Dicer, indicating that Dicer functions as a haploinsufficient tumor suppression gene. Also, hemizygous deletion of Dicer in cell lines indeed resulted in reduced steady-state miRNA levels (87). These results indicate that the observed reduced Dicer expression in cancer at least partly contributes to the global reduction in miRNA expression levels that are typical for cancerous tissues (77, 82, 87).

No loss-of-function genetic mutations have been reported for the Dicer gene in tumor tissues, even though it acts as a tumor suppressor

gene and shows reduced protein levels in a variety of tumor types. Reduced Dicer expression might occur due to changes in the transcriptional regulation of Dicer, mediated for example by TAp63 in metastasis (88). However, reduced Dicer expression can also at least partly arise from mutations in the gene encoding for TRBP (89). As discussed above, TRBP is involved in miRNA processing through direct interaction with the endonuclease Dicer. Heterozygous mutations in the TRBP gene are found in several types of cancer, where it causes a reduction in the TRBP protein levels. Reduced TRBP protein levels results in the destabilization of the Dicer protein and thus subsequently reduces Dicer protein levels. Moreover, mutations in TRBP also lead to reduced miRNA levels and this deregulation of miRNA processing caused an enhanced tumor growth (89). Thus, the global inhibition in miRNA expression as seen in cancer does not seem to be a mere consequence of a reduced differentiation state of the cell. Instead, a reduction in the general miRNA processing efficiency seems to have a direct effect on the oncogenic potential of tumor cells, which is also in agreement with the finding that Dicer can act as a tumor suppressor gene.

In general, proteins that are involved in the homeostasis of miRNAs could also influence the miRNA expression levels in diseased conditions, either by inducing or reducing the processing efficiency of subsets of miRNAs or globally altering the miRNA expression levels. These altered miRNA levels can subsequently have an influence on the cellular phenotype, such as an increase in the oncogenic potential of a cell. Pinpointing and influencing the underlying mechanisms that regulate miRNA homeostasis can therefore be relevant for finding novel therapeutic strategies.

8. Concluding Remarks

The biogenesis of an miRNA is still an intensive area of investigation for many research groups. Besides the pri- and pre-miRNA processing complexes there are also many other factors involved that fine-tune the regulation of miRNA expression after transcription. This fine-tuning is highly important, as miRNAs can play important roles in disease development and thus their expression should be tightly controlled. We are still only at the beginning of understanding the regulatory mechanisms that control the biogenesis of an miRNA, and it is expected that other regulatory proteins are yet to be discovered. This enhances our understanding on how miRNA expression can be deregulated in disease and might provide novel therapeutic avenues for controlling the miRNA balance within a cell.

In addition to miRNA expression alterations, some mRNA targets seem to directly escape miRNA-mediated regulation in diseased

conditions. For example, the global shortening of 3′-UTRs as well as the genomic translocation of protein-encoding genes might directly result in increased protein levels due to a loss of miRNA control. For now, it remains to be investigated if this loss of miRNA-mediated control of gene expression directly results in an altered cellular behavior, or whether this is a secondary effect.

Overall, the balance between the miRNA levels as well as the availability of the mRNA target defines the final protein output. It is this balance that is disturbed in many diseases, and might play a more significant role than has been recognized during these last decades. Our understanding of the consequences that small molecules play in normal development and disease is steadily growing and will ultimately change our perspective on cellular behavior. In this respect, the availability of deep-sequencing methods to find novel small RNA species in addition to determining their expression levels and modifications as described in the following chapters, are of great importance for elucidating the effect of small RNAs on the final cellular phenotype.

References

1. Wightman, B., Ha, I., and Ruvkun, G. (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans, *Cell* **75**, 855–862.
2. Kozomara, A., and Griffiths-Jones, S. (2011) miRBase: integrating microRNA annotation and deep-sequencing data, *Nucleic Acids Res* 39, D152–7.
3. Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008) Widespread changes in protein synthesis induced by microRNAs, *Nature* **455**, 58–63.
4. Altuvia, Y., Landgraf, P., Lithwick, G., Elefant, N., Pfeffer, S., Aravin, A., et al. (2005) Clustering and conservation patterns of human microRNAs, *Nucleic Acids Res* **33**, 2697–706.
5. Martinez, N. J., Ow, M. C., Reece-Hoyes, J. S., Barrasa, M. I., Ambros, V. R., and Walhout, A. J. (2008) Genome-scale spatiotemporal analysis of Caenorhabditis elegans microRNA promoter activity, *Genome Res* **18**, 2005–15.
6. Filipowicz, W., Bhattacharyya, S. N., and Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* **9**, 102–14.
7. Han, J., Lee, Y., Yeom, K. H., Nam, J. W., Heo, I., Rhee, J. K., et al. (2006) Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex, *Cell* **125**, 887–901.
8. Bohnsack, M. T., Czaplinski, K., and Gorlich, D. (2004) Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs, *RNA* **10**, 185–91.
9. Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E., and Kutay, U. (2004) Nuclear export of microRNA precursors, *Science* **303**, 95–8.
10. Yi, R., Qin, Y., Macara, I. G., and Cullen, B. R. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs, *Genes Dev* **17**, 3011–16.
11. Zeng, Y., and Cullen, B. R. (2004) Structural requirements for pre-microRNA binding and nuclear export by Exportin 5, *Nucleic Acids Res* **32**, 4776–85.
12. Chendrimada, T. P., Gregory, R. I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., et al. (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing, *Nature* **436,** 740–4.
13. Brodersen, P., and Voinnet, O. (2009) Revisiting the principles of microRNA target recognition and mode of action, *Nat Rev Mol Cell Biol* **10**, 141–8.
14. Thomson, J. M., Newman, M., Parker, J. S., Morin-Kensicki, E. M., Wright, T., and Hammond, S. M. (2006) Extensive post-transcriptional regulation of microRNAs and its implications for cancer, *Genes Dev* **20**, 2202–7.
15. Suzuki, H. I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., and Miyazono, K. (2009)

Modulation of microRNA processing by p53, *Nature* **460**, 529–33.

16. Davis, B. N., Hilyard, A. C., Nguyen, P. H., Lagna, G., and Hata, A. Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha, *Mol Cell* **39**, 373–84.
17. Yamagata, K., Fujiyama, S., Ito, S., Ueda, T., Murata, T., Naitou, M., et al. (2009) Maturation of microRNA is hormonally regulated by a nuclear receptor, *Mol Cell* **36**, 340–7.
18. Sakamoto, S., Aoki, K., Higuchi, T., Todaka, H., Morisawa, K., Tamaki, N., et al. (2009) The NF90-NF45 complex functions as a negative regulator in the microRNA processing pathway, *Mol Cell Biol* **29**, 3754–69.
19. Berezikov, E., Chung, W. J., Willis, J., Cuppen, E., and Lai, E. C. (2007) Mammalian mirtron genes, *Mol Cell* **28**, 328–36.
20. Okamura, K., Hagen, J. W., Duan, H., Tyler, D. M., and Lai, E. C. (2007) The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila, *Cell* **130**, 89–100.
21. Ruby, J. G., Jan, C. H., and Bartel, D. P. (2007) Intronic microRNA precursors that bypass Drosha processing, *Nature* **448**, 83–6.
22. Slezak-Prochazka, I., Durmus, S., Kroesen, B. J., and van den Berg, A. MicroRNAs, macrocontrol: regulation of miRNA processing, *RNA* **16**, 1087–95.
23. Paroo, Z., Ye, X., Chen, S., and Liu, Q. (2009) Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling, *Cell* **139**, 112–22.
24. Newman, M. A., Thomson, J. M., and Hammond, S. M. (2008) Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing, *RNA* **14**, 1539–49.
25. Piskounova, E., Viswanathan, S. R., Janas, M., LaPierre, R. J., Daley, G. Q., Sliz, P., et al. (2008) Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28, *J Biol Chem* **283**, 21310–4.
26. Rybak, A., Fuchs, H., Smirnova, L., Brandt, C., Pohl, E. E., Nitsch, R., et al. (2008) A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment, *Nat Cell Biol* **10**, 987–93.
27. Viswanathan, S. R., Daley, G. Q., and Gregory, R. I. (2008) Selective blockade of microRNA processing by Lin28, *Science* **320**, 97–100.
28. Heo, I., Joo, C., Cho, J., Ha, M., Han, J., and Kim, V. N. (2008) Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA, *Mol Cell* **32**, 276–84.
29. Michlewski, G., Guil, S., Semple, C. A., and Caceres, J. F. (2008) Posttranscriptional regulation of miRNAs harboring conserved terminal loops, *Mol Cell* **32**, 383–93.
30. Guil, S., and Caceres, J. F. (2007) The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a, *Nat Struct Mol Biol* **14**, 591–6.
31. Trabucchi, M., Briata, P., Garcia-Mayoral, M., Haase, A. D., Filipowicz, W., Ramos, et al. (2009) The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs, *Nature* **459**, 1010–4.
32. Blow, M. J., Grocock, R. J., van Dongen, S., Enright, A. J., Dicks, E., Futreal, P. A., et al. (2006) RNA editing of human microRNAs, *Genome Biol* **7**, R27.
33. Kawahara, Y., Zinshteyn, B., Chendrimada, T. P., Shiekhattar, R., and Nishikura, K. (2007) RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer-TRBP complex, *EMBO Rep* **8**, 763–9.
34. Kawahara, Y., Zinshteyn, B., Sethupathy, P., Iizasa, H., Hatzigeorgiou, A. G., and Nishikura, K. (2007) Redirection of silencing targets by adenosine-to-inosine editing of miRNAs, *Science* **315**, 1137–40.
35. Luciano, D. J., Mirsky, H., Vendetti, N. J., and Maas, S. (2004) RNA editing of a miRNA precursor, *RNA* **10**, 1174–7.
36. Yang, W., Chendrimada, T. P., Wang, Q., Higuchi, M., Seeburg, P. H., Shiekhattar, R., et al. (2006) Modulation of microRNA processing and expression through RNA editing by ADAR deaminases, *Nat Struct Mol Biol* **13**, 13–21.
37. Kawahara, Y., Megraw, M., Kreider, E., Iizasa, H., Valente, L., Hatzigeorgiou, A. G., et al. (2008) Frequency and fate of microRNA editing in human brain, *Nucleic Acids Res* **36**, 5270–80.
38. de Hoon, M. J., Taft, R. J., Hashimoto, T., Kanamori-Katayama, M., Kawaji, H., Kawano, M., et al. (2010). Cross-mapping and the identification of editing sites in mature microRNAs in high-throughput sequencing libraries, *Genome Res* **20**, 257–64.
39. Kai, Z. S., and Pasquinelli, A. E. MicroRNA assassins: factors that regulate the disappearance of miRNAs, *Nat Struct Mol Biol* **17**, 5–10.
40. Diederichs, S., and Haber, D. A. (2007) Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression, *Cell* **131**, 1097–108.
41. Krol, J., Busskamp, V., Markiewicz, I., Stadler, M. B., Ribi, S., Richter, J., et al. (2010). Characterizing light-regulated retinal microRNAs reveals rapid turnover as a common property of neuronal microRNAs, *Cell* **141**, 618–31.

42. Chatterjee, S., and Grosshans, H. (2009) Active turnover modulates mature microRNA activity in Caenorhabditis elegans, *Nature* **461**, 546–9.
43. Chiang, H. R., Schoenfeld, L. W., Ruby, J. G., Auyeung, V. C., Spies, N., Baek, D., et al. (2010) Mammalian microRNAs: experimental evaluation of novel and previously annotated genes, *Genes Dev* **24**, 992–1009.
44. Katoh, T., Sakaguchi, Y., Miyauchi, K., Suzuki, T., Kashiwabara, S., and Baba, T. (2009) Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2, *Genes Dev* **23**, 433–8.
45. Jones-Rhoades, M. W., Bartel, D. P., and Bartel, B. (2006) MicroRNAS and their regulatory roles in plants, *Annu Rev Plant Biol* **57**, 19–53.
46. Bartel, D. P. (2009) MicroRNAs: target recognition and regulatory functions, *Cell* **136**, 215–33.
47. Peters, L., and Meister, G. (2007) Argonaute proteins: mediators of RNA silencing, *Mol Cell* **26**, 611–23.
48. Tolia, N. H., and Joshua-Tor, L. (2007) Slicer and the argonautes, *Nat Chem Biol* **3**, 36–43.
49. Cifuentes, D., Xue, H., Taylor, D. W., Patnode, H., Mishima, Y., Cheloufi, S., et al. (2010) A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity, *Science* **328**, 1694–8.
50. Kiriakidou, M., Tan, G. S., Lamprinaki, S., De Planell-Saguer, M., Nelson, P. T., and Mourelatos, Z. (2007) An mRNA m7G cap binding-like motif within human Ago2 represses translation, *Cell* **129,** 1141–51.
51. Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R.,et al. (2005) Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation, *Cell* **122**, 553–63.
52. Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., and Izaurralde, E. (2006) mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes, *Genes Dev* **20**, 1885–98.
53. Giraldez, A. J., Mishima, Y., Rihel, J., Grocock, R. J., Van Dongen, S., Inoue, K.,et al. (2006) Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs, *Science* **312**, 75–9.
54. Wu, L., Fan, J., and Belasco, J. G. (2006) MicroRNAs direct rapid deadenylation of mRNA, *Proc Natl Acad Sci USA* **103**, 4034–9.
55. Petersen, C. P., Bordeleau, M. E., Pelletier, J., and Sharp, P. A. (2006) Short RNAs repress translation after initiation in mammalian cells, *Mol Cell* **21**, 533–42.
56. Eulalio, A., Huntzinger, E., Nishihara, T., Rehwinkel, J., Fauser, M., and Izaurralde, E. (2009) Deadenylation is a widespread effect of miRNA regulation, *RNA* **15**, 21–32.
57. Guo, H., Ingolia, N. T., Weissman, J. S., and Bartel, D. P. Mammalian microRNAs predominantly act to decrease target mRNA levels, *Nature* **466,** 835–40.
58. Bushati, N., and Cohen, S. M. (2007) microRNA functions, *Annu Rev Cell Dev Biol* **23**, 175–205.
59. Leaman, D., Chen, P. Y., Fak, J., Yalcin, A., Pearce, M., Unnerstall, U., et al. (2005) Antisense-mediated depletion reveals essential and specific functions of microRNAs in Drosophila development, *Cell* **121**, 1097–108.
60. Miska, E. A., Alvarez-Saavedra, E., Abbott, A. L., Lau, N. C., Hellman, A. B., McGonagle, S. M., et al. (2007) Most Caenorhabditis elegans microRNAs are individually not essential for development or viability, *PLoS Genet* **3**, e215.
61. Leung, A. K., and Sharp, P. A. MicroRNA functions in stress responses, *Mol Cell* **40**, 205–15.
62. Sandberg, R., Neilson, J. R., Sarma, A., Sharp, P. A., and Burge, C. B. (2008) Proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites, *Science* **320**, 1643–7.
63. Mayr, C., and Bartel, D. P. (2009) Widespread shortening of 3'UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells, *Cell* **138**, 673–84.
64. Paul, M. S., and Bass, B. L. (1998) Inosine exists in mRNA at tissue-specific levels and is most abundant in brain mRNA, *EMBO J* **17**, 1120–7.
65. Liang, H., and Landweber, L. F. (2007) Hypothesis: RNA editing of microRNA target sites in humans? *RNA* **13**, 463–7.
66. Athanasiadis, A., Rich, A., and Maas, S. (2004) Widespread A-to-I RNA editing of Alu-containing mRNAs in the human transcriptome, *PLoS Biol* **2**, e391.
67. Farajollahi, S., and Maas, S. Molecular diversity through RNA editing: a balancing act, *Trends Genet* **26**, 221–30.
68. Mayr, C., Hemann, M. T., and Bartel, D. P. (2007) Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation, *Science* **315**, 1576–9.
69. Kim, J., and Bartel, D. P. (2009) Allelic imbalance sequencing reveals that single-nucleotide polymorphisms frequently alter microRNA-directed repression, *Nat Biotechnol* **27**, 472–7.

70. Poliseno, L., Salmena, L., Zhang, J., Carver, B., Haveman, W. J., and Pandolfi, P. P. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology, *Nature* **465**, 1033–8.
71. Kedde, M., van Kouwenhove, M., Zwart, W., Oude Vrielink, J. A., Elkon, R., and Agami, R. A Pumilio-induced RNA structure switch in p27-3' UTR controls miR-221 and miR-222 accessibility, *Nat Cell Biol* **12**, 1014–20.
72. Hsu, S. D., Lin, F. M., Wu, W. Y., Liang, C., Huang, W. C., Chan, W. L., et al. (2011) miRTarBase: a database curates experimentally validated microRNA-target interactions, *Nucleic Acids Res* **39**, D163-9.
73. Calin, G. A., Dumitru, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., et al. (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia, *Proc Natl Acad Sci USA* **99**, 15524–9.
74. Calin, G. A., Sevignani, C., Dumitru, C. D., Hyslop, T., Noch, E., Yendamuri, S., et al. (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers, *Proc Natl Acad Sci USA* **101**, 2999–3004.
75. Ventura, A., and Jacks, T. (2009) MicroRNAs and cancer: short RNAs go a long way, *Cell* **136**, 586–91.
76. Medina, P. P., Nolde, M., and Slack, F. J. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma, *Nature* **467**, 86–90.
77. Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., Peck, D., et al. (2005) MicroRNA expression profiles classify human cancers, *Nature* **435**, 834–8.
78. Chang, T. C., Yu, D., Lee, Y. S., Wentzel, E. A., Arking, D. E., West, K. M., et al. (2008) Widespread microRNA repression by Myc contributes to tumorigenesis, *Nat Genet* **40**, 43–50.
79. Lujambio, A., Calin, G. A., Villanueva, A., Ropero, S., Sanchez-Cespedes, M., Blanco, D., et al. (2008) A microRNA DNA methylation signature for human cancer metastasis, *Proc Natl Acad Sci USA* **105**, 13556–61.
80. Lujambio, A., Ropero, S., Ballestar, E., Fraga, M. F., Cerrato, C., Setien, F., et al. (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells, *Cancer Res* **67**, 1424–9.
81. Saito, Y., Liang, G., Egger, G., Friedman, J. M., Chuang, J. C., Coetzee, G. A., et al. (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells, *Cancer Cell* **9**, 435–43.
82. Faggad, A., Budczies, J., Tchernitsa, O., Darb-Esfahani, S., Sehouli, J., Muller, B. M., et al. (2010) Prognostic significance of Dicer expression in ovarian cancer-link to global microRNA changes and oestrogen receptor expression, *J Pathol* **220**, 382–91.
83. Karube, Y., Tanaka, H., Osada, H., Tomida, S., Tatematsu, Y., Yanagisawa, K., et al. (2005) Reduced expression of Dicer associated with poor prognosis in lung cancer patients, *Cancer Sci* **96**, 111–5.
84. Merritt, W. M., Lin, Y. G., Han, L. Y., Kamat, A. A., Spannuth, W. A., Schmandt, R., et al. (2008) Dicer, Drosha, and outcomes in patients with ovarian cancer, *N Engl J Med* **359**, 2641–50.
85. Wu, J. F., Shen, W., Liu, N. Z., Zeng, G. L., Yang, M., Zuo, G. Q., et al (2010) Downregulation of Dicer in hepatocellular carcinoma, *Med Oncol.* Apr 20. doi: 10.1007/s12032-010-9520-5.
86. Kumar, M. S., Lu, J., Mercer, K. L., Golub, T. R., and Jacks, T. (2007) Impaired microRNA processing enhances cellular transformation and tumorigenesis, *Nat Genet* **39**, 673–7.
87. Kumar, M. S., Pester, R. E., Chen, C. Y., Lane, K., Chin, C., Lu, J., et al. (2009) Dicer1 functions as a haploinsufficient tumor suppressor, *Genes Dev* **23**, 2700–4.
88. Su, X., Chakravarti, D., Cho, M. S., Liu, L., Gi, Y. J., Lin, Y. L., et al. (2010) TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs, *Nature* **467**, 986–90.
89. Melo, S. A., Ropero, S., Moutinho, C., Aaltonen, L. A., Yamamoto, H., Calin, G. A., et al. (2009) A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function, *Nat Genet* **41**, 365–70.