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Causes and Consequences of microRNA Dysregulation

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Abstract

It is currently well recognized that microRNA deregulation is an hallmark of human cancer, and how an aberrant expression of these tiny regulatory RNA molecules in several cell types is not just a random association, but it plays a causal role in different steps of the tumorigenic process, from the initiation and development to progression toward the acquisition of a metastatic phenotype.

Different regulatory mechanisms can control microRNA expression at a genetic or epigenetic level as well as involving the biogenesis machinery or the recruitment of specific transcription factors. The tumorigenic process implies a substantial alteration of these mechanisms, thus disrupting the equilibrium within the cell and leading to a global change in microRNA expression, with loss of oncosuppressor microRNAs and overexpression of oncomiRNAs.

Here we review the main mechanisms regulating microRNAs, and the consequences of their aberrant expression in cancer, with a glance at the possible implications at a clinical point of view.

Introduction

The interest and the knowledge on microRNA field has increased incredibly fast in the last few years, and it is currently well recognized how these small RNA molecules represent an essential part of the encoding genome, finely tuning gene expression and thus exerting a crucial role in all the most important processes and in different species, including vertebrates (1). microRNAs represent indeed an entire novel level of gene regulation that forced scientists to revise and somehow reorganize their view of the molecular biology.

Since the first discovery, remarkable advances in the understanding of microRNA biology have been made, including: the identification of hundreds of miRNA genes; the dissection of miRNA biogenesis pathways; the identification of numerous miRNA targets and the establishment of principles of target regulation; the study of their biological functions in physiological and pathological conditions.

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microRNA biogenesis can be summarized in two main processing steps, taking place respectively in the nucleus and in the cytoplasm: mainly transcribed by RNA Polimerase II as long primary transcripts characterized by hairpin structures (pri-microRNAs), microRNAs are first processed into the nucleus by RNAse III Drosha into 70–100 nts long pre-microRNAs (2). However, there is alternative miRNA biogenesis pathway, the so-called miRtron pathway, which does not require the Drosha-mediated cleavage, since miRtrons (miRNAs localized within introns of protein-encoding or -non-encoding genes) are directly processed using the splicing machinery to generate pre-miRs (3,4,5).

The short stem plus a ~2-nt 3′ overhang of the originated precursor molecules are recognized by the Ran-GTP-dependent transporter Exportin 5, which mediates the translocation to the cytoplasm (6). Here the second cropping process (dicing) takes place, performed by the RNAse III enzyme Dicer (Dicer 1 in flies) associated to TRBP (TAR RNA-binding protein) or PACT (also known as PRKRA), and Argonaute (AGO1-4), which cleave the miRNA precursor hairpin generating a transitory miRNA/miRNA* duplex (approximately 22 nucleotides long), which includes the mature miRNA guide and the complementary passenger strand (miRNA*, star miRNA) (also named miR-3p/miR-5p). Whereas one of the two strands is selected as guide strand according to thermodynamic properties, the complementary one is usually subjected to degradation. The so called miRNA* was initially thought to be the strand subjected to degradation, instead more recent evidence suggests that it does not simply represent a non-functional bioproduct of miRNA biogenesis, but it can be selected as a functional strand and play significant biological roles (7).

This duplex is then loaded into the miRNA associated RNA-induced silencing complex (RISC), which preferentially includes the mature single-stranded miRNA molecule and AGO proteins, where they act as guiding molecules to deliver the complex to target mRNA. It seems that AGO is associated to Dicer in the dicing step as well as in the RISC assembly step. As a part of this complex, the mature miRNA is able to regulate gene expression at post-transcriptional level, binding for the most part through partial complementarity to target mRNAs, and mainly leading to mRNA degradation or translation inhibition, depending on the sequence complementarity between the small RNA and the target mRNA. The site-specific cleavage, commonly defined as RNA interference (RNAi), is actually restricted to miRNAs with a perfect or near-perfect match to the target RNA, and it is a very rare event in mammals. By contrast, in mammals the most frequent processes are enhanced mRNA degradation or translational inhibition, commonly associated respectively with higher or lower grade of mismatches in the miRNA/target sequences.

Considering the different rules regulating the interaction between a microRNA and its target mRNA, it is not surprising that each miRNA has the potential to target a large number of genes (8,9,10,11). On the other hand, approximately 60% of the mRNAs have one or more evolutionarily conserved sequences that are predicted to interact with miRNAs. Indeed, *in silico* analysis predicts that the 3′ UTR of a single gene is frequently targeted by several different miRNAs (8). Many of these predictions have been validated experimentally, suggesting that miRNAs might cooperate to regulate gene expression.

Overall, these data show the complexity and widespread regulation of gene expression by miRNAs, which should be taken into consideration when developing miRNA-based therapies.

microRNA expression regulation

Several mechanisms can control microRNA expression, and result to be altered in human diseases, including cancer (Figure 1).

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Chromosomal abnormalities, as first suggested by the discovery of two microRNA genes, miR-15a and 16-1, in a region of the Chr. 13 frequently deleted in CLL (12), supported by the evidence that microRNAs are frequently located in regions of the genome involved in alterations in cancer (13), and then confirmed by several studies (12,14,15); mutations, as the inherited mutations in the primary transcripts of miR-15a and miR-16-1 responsible for reduced expression of the two microRNAs *in vitro* and *in vivo* in CLL (16); polymorphisms (SNPs), as described in lung cancer (17).

In addition to structural genetic alterations, microRNA expression can be also modulated as a consequence of defects in the microRNA biogenesis machinery. Indeed, it has been reported that deregulation of different cofactors can affect miRNA expression with important biological implications: DGR8 knock-out mice arrested early in development and have defects in ES cell proliferation and differentiation (18); Lin28, originally discovered as a heterochronic gene regulating developmental timing in worms, is able to block let-7 biogenesis and it is activated in many human tumors (15%), being particularly associated with less differentiated cancers (19). Moreover, adenosine deaminases acting on RNA (ADARs) can affect the expression of microRNAs recognizing adenosine residues, as reported for miR-142: the editing of pri-miR-142 results in the suppression of its processing by Drosha, degradation by a component of the RISC complex and reduced levels of the mature product (20).

microRNA processing can be also affected by other microRNAs, thus creating a complex level of reciprocal interaction and regulation, as very recently reported by Tang R and colleagues (21), who demonstrated that mouse miR-709 is predominantly located in the nucleus, where it directly binds to a miR-709 recognition element on pri-miR-15a/16-1 preventing its processing into pre-miR-15a/16-1 and thus leading to a suppression of miR-15a/16-1 maturation.

Notably, changes in microRNA levels consequent an altered Drosha or Dicer activity have been reported in different tumor types (22,23,24). In particular, it seems that Dicer or Drosha silencing promotes cellular transformation and tumorigenesis in vivo: conditional loss of Dicer1 in the lung tissues of mice enhances the development of lung tumors in a K-ras mouse model (25). Finally, loss of Dicer and/or Drosha has also been inversely correlated with outcome in lung cancer (26), cancers of the ovarian epithelium (24), and more recently in other tumor types as nasopharyngeal carcinoma (27), neuroblastoma (28) and breast (29).

It may sound surprising that reprogramming of the whole ‘microRNome’, including both oncomiRNAs and tumor suppressor miRNAs, can lead to a specific anti-tumoral effect: how is the balance shifted in favor of a specific effect? This might be due to the possibility that most microRNAs seem to exert a role as oncosuppressors, and consequently are mostly dowregulated in human neoplasia (30).

A few reports, however, describe a positive correlation between Dicer expression and poor outcome in colorectal cancer (31) and in prostate cancer (32), or the overexpression of Drosha in cervical cancer (33), thus raising the important issue to validate this still debated question, and verify whether the effect of targeting the microRNA machinery might be tissue-related.

To complicate the scenario, a recent report by Piccolo’s group (34) describes a microRNA family, miR-103-107, able to empower the metastatic potential targeting Dicer and thus attenuating the global microRNA biosynthesis, with a particular effect mediated by the downregulation of miR-200 family, and the consequent switch to a more mesenchymal and aggressive phenotype.

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The deregulated microRNA expression in cancer can also be due to epigenetic changes, as altered DNA methylation, as suggested by an extensive analysis of genomic sequences of miRNA genes, which have shown that approximately half of them are associated with CpG islands (35). One of the first reports proving that an altered methylation status can be responsible for the deregulated expression of microRNAs in cancer, as the silencing of putative tumor suppressor microRNAs, was the study by Saito and colleagues (36), who observed a strong upregulation of miR-127 upon treatment of a bladder cancer cell lines and human fibroblasts with DNMT inhibitor 5-Aza-2'-deoxycytidine. As expected, miR-127, able to target the proto-oncogene BCL-6, is characterized by a CpG island promoter, and is silenced in several cancer cells. With the same approach of unmask epigenetically silenced microRNAs inducing chromatin-remodeling by drug treatment, it has been demonstrated that miR-9-1 is hypermethylated and consequently down-modulated in breast cancer (37), as well as the clustered miR-34b and miR-34c in colon cancer (38).

Analyzing miRNA profiling of DNMT1- and DNMT3b-deficient colorectal cancer cells Lujambio and colleagues (39) identified another oncosuppressor microRNA which results to be hypermethylated, and thus silenced, in tumor: miR-124a, embedded in a large CpG island and able to target cyclin D kinase 6, which mediates the phosphorylation of RB tumor suppressor gene.

Conversely, the upmodulation of putative oncogenic microRNAs in cancer can be due to DNA hypomethylation, as shown in lung adenocarcinoma for let-7a-3 (40) or in epithelial ovarian cancer for miR-21 (41).

Methylation is not the only epigenetic mechanism that can affect microRNAs expression: Scott and colleagues (42) showed for the first time that histone deacetylase inhibition is followed by the extensive and rapid alteration of microRNA levels in a breast cancer cell line. After this first evidence, several reports have shown that HDACis alter microRNA expression in several human carcinomas including colon (43,44), and gastric (45).Very recently, Rhodes LV and colleagues (46) described how HDACi Trichostatin A (TSA) alter the miRNA signature of an apoptosis-resistant breast cancer cell line and reduces its clonogenic potential.

To complicate the scenario connecting microRNAs and epigenetics, microRNAs themselves can regulate the expression of components of the epigenetic machinery, creating a highly controlled feedback mechanism: miR-29 family directly targets the *de novo* DNA methyltransferases DNMT-3A and -3B, while indirectly, through regulation of the transactivator Sp1, the maintenance DNA methyl transferase DNMT1. Interestingly, introduction of miR-29s into lung cancers and AMLs results in reactivation of silenced tumor suppressors and inhibition of tumorigenesis (47,48). Loss of miR-290 cluster in Dicer-deficient mouse ES cells leads to the downregulation of DNMT3a, DNMT3b and DNMT1 through upmodulation of their repressor, RBL-2, proven target of miR-290 (49,50). miR-1, involved in myogenesis and related diseases, directly targets HDAC4 (51) and miRNA-9* down-modulates HDAC4 and HDAC5 (52).

Considering the reported evidence of how microRNA expression can be affected by changes in the epigenetic program, a possible therapeutic approach might be represented by the modulation of microRNA expression by targeting components of these regulatory networks. The existence of epigenetic drugs, such as DNA demethylating agents and histone deacetylase inhibitors, able to reverse an aberrant methylation or acetylation status, raises indeed the intriguing possibility to regulate microRNA levels, for example to restore the expression of tumor suppressor microRNAs, thus reverting a tumoral phenotype.

Demethylating agents as Decitabine and 5-azacytidine, for example, are currently approved for the treatment of myelodysplastic syndrome, although they have shown activity in many other malignancies, including AML (53). These drugs are known to inhibit DNA methyltransferases, resulting in tumor suppressor gene re-expression. As previously described, miRNAs have also been shown to be actively re-expressed after treatment with these drugs and to largely contribute to the therapeutic effects of these compounds. Even though it is tempting to suggest that many of the biological effects of these drugs may be mediated by the re-expression of non-coding RNAs, this still needs to be verified. In particular, as previously underlined concerning the possibility to interfere with the biogenesis machinery, it is mandatory to verify the consequences of a global reprogramming of miRNA expression.

Finally, a deregulation of miRNA expression can be a result of increased or decreased transcription due to an altered transcription factor activity.

MiR-34a family of miRNAs, for instance, has been shown to be directly induced by the tumor suppressor p53, and to be partially responsible of the phenotype induced by this oncosuppressor (54,55).

Vice versa, oncogenes can also affect microRNA expression, and a clear example is represented by the oncoprotein MYC, which is able to both induce oncogenic microRNAs, as the miR-17-92 cluster, and negatively regulate transcription of tumor suppressor miRNAs, such as let-7 (56) and miR-29 family members (57).

An interesting regulatory loop has been demonstrated between ZEB1 transcription factor and miR-200 family: EMT inducers ZEB1 and ZEB2 are direct target of miR-200 family members (58), and in turn, ZEB1 has been shown to directly repress miR-200c and miR-141 transcription (59).

Another relevant example is represented by miR-221 and -222, negatively regulated by ERα and able, in turn, to directly target the receptor (60). Therefore, silencing ERα, for example by methylation or by dysregulating miR-221 and miR-222 through the activation of pathways, such as MET pathway, involved in oncogenesis, results in the constitutive activation of miR-221 and miR-222 and inhibition of the tumour suppressors p27, p57, PTEN and TIMP3, thus contributing to the development of the invasive phenotype characteristic of frankly malignant cells (61). This regulatory feedback loop seems to be involved in the development of ERα–breast cancers (62).

Nevertheless, despite the advances in our understanding of the mechanisms causing miRNA deregulation, the daunting task still remains the elucidation of the biological role of miRNAs in the initiation and in the development of cancer.

microRNA function: what is new under the sun

It is currently well recognized how microRNAs are not only deregulated in human cancer, but how they play a causal role, functioning as either tumor suppressors or oncogenes by targeting different steps of the tumorigenesis process, either occurrence, development or progression to a metastatic phenotype. Indeed, cancers develop sophisticated networks of biological activities that contribute to their ability to develop and, in some cases, evade treatment. This complex program relies on the communication between multiple cell types, including both the primary tumor as well as the stromal cells, and can be summarized in 6 essential characteristics of cancer progression: self–sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (63). microRNAs can affect any

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of these processes, as demonstrated by gain of-function and loss-of-function experiments, in combination with target prediction analyses.

miRNAs acting as tumor suppressors target oncoproteins with crucial roles in various cancer pathways, as the ones reported in the first pioneer studies: miR-15a-16-1, targeting BCL-2 (64) and let-7, targeting RAS (65) and MYC (66), whereas miRNAs with oncogenic properties negatively regulate tumor suppressor proteins, as the well described miR-21, which targets the tumor suppressors phosphatase and tensin homologue (PTEN) and programmed cell death 4 (67,68).

In addition to classical tumor suppressor or oncogene functions, miRNAs have been implicated also in cell migration and metastasis, either as promoters, as miR-10b (69) and miR-21 (70) or inhibitors, as miR-126 and miR-335 (71), miR-34a (72), miR-200 family (58) and miR-205 (73). Moreover, one of the crucial steps of the metastatic process is represented by neo-angiogenesis, which allows cells to reach and disseminate through the systemic circulation. microRNAs can control tumor progression also at this level, either promoting or inhibiting the proliferation of endothelial cells. miR-221 and miR-222 repress proliferative and angiogenetic properties of c-Kit in endothelial cells, whereas hypoxic reduction of miR-16, miR-15b, miR-20a and miR-20b, directly targeting VEGF, supports the angiogenic process (74). Angiogenesis can be also promoted by miR-210, activated by hypoxia and directly repressing endothelial ligand Ephrin A3 (75), and by miR-17-92 cluster, which sustains MYC angiogenic properties through repression of connective tissue growth factor (CTGF) and the anti-angiogenic adhesive glycoprotein thrombospondin 1 (TSP1) (76), also targeted by miR-27b and let-7f (77). Notably, the same group who identified a set of human microRNAs robustly suppressing breast cancer metastasis (71) has more recently revealed that endogenous miR-126 non-cell-autonomously regulates endothelial cell recruitment to metastatic breast cancer cells, *in vitro* and *in vivo*. It suppresses metastatic endothelial recruitment, metastatic angiogenesis and metastatic colonization through coordinate targeting of IGFBP2, PITPNC1 and MERTK, pro-angiogenic genes and biomarkers of human metastasis (78). Thus, miRNAs can exert their function influencing interaction between different cell types. Another example is represented by the acquirement of a metastatic phenotype following miR-320 loss in cancer-associated fibroblasts: miR-320 is indeed a crucial component of a PTEN-controlled tumor-suppressive axis in stromal fibroblasts, and loss of PTEN and miR-320 induces an oncogenic secretome that reprograms the tumor microenvironment to promote invasion and angiogenesis (79). However, despite the increasing body of *in vitro* and *in vivo* evidence supporting the involvement of microRNAs in cancer development or progression, to date just few microRNA engineered animal models have been developed: through knock out or transgene introduction, these animal models can provide the genetic demonstration of the causative involvement of a specific microRNA in a biological phenomenon. miR-17-92 cluster and miR-155, both overexpressed in lymphoproliferative disorders, including lymphomas and leukemia (80,81), were the first examples of miRNAs with oncogenic activity validated in engineered animal models. Infection of murine hematopoietic stem cells with a retrovirus carrying the miR-17-92 cluster accelerated the development of lymphomas in Myc transgenic mice (81). Transgenic mice overexpressing miR-17-92 cluster in B cells were discovered to develop lymphoproliferative disease and autoimmunity (82). The higher rate of proliferation and the lower rate of activation-induced cell death of lymphocytes in these mice were partially attributed to the direct targeting of the anti-apoptotic genes Bim and Pten by miR-17-92 cluster. Moreover, Ventura and colleagues showed that mice deficient for miR-17-92 cluster die shortly after birth with lung hypoplasia and a ventricular septal defect (83). Finally, Mu and colleagues determined that deletion of the complete miR-17-92 cluster slows Myc-induced oncogenesis (84).

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Notably, overexpression of miR-155 alone in the lymphoid compartment was sufficient to cause cancer and did not require any other cooperative mutation or oncogene expression. miR-155 transgenic mice developed polyclonal lymphoid proliferation followed by acute lymphocytic lymphoma or leukemia (85). This was the first report that the dysregulation of a single miRNA can lead to malignancy. More recently, Slack's group (86) has shown that mice conditionally expressing miR-21 develop a pre-B malignant lymphoid-like phenotype, thus demonstrating that miR-21 is a genuine oncogene.

Considering the different rules regulating miRNA/target interaction, and the evidence that microRNAs can target multiple molecules, it is unlikely that miRNAs will be responsible for a specific phenotype by aiming at a single target. Instead, it is thought that miRNAs engage in complex interactions with the machinery that controls the transcriptase and concurrently target multiple mRNAs. This is probably the most intriguing rational supporting the idea of using microRNAs as anticancer drugs.

However, recent reports have shed more light into the complex mechanisms regulating microRNA function on target mRNAs. Indeed, microRNAs mainly recognize complementary sequences in the 3' untraslated regions (UTRs) of their target mRNAs, however more recent studies have reported that they can also bind to the 5'UTR or the ORF (87,88,89,90) and, even more surprisingly, they can upregulate translation upon growth arrest conditions (91).

The discovery of other functional non-coding RNAs, interconnected with each other, has revealed a network of regulatory molecules definitely more complicated than expected.

One of the first studies reporting the existence of other non-coding RNAs involved in tumorigenesis and connected to microRNAs was reported by our group (92): more in detail, Calin and colleagues observed that a large fraction of genomic ultraconserved regions (UCRs) encode a particular set of ncRNAs whose expression is altered in human cancers, and which can be regulated by microRNAs.

A more recent report by Pandolfi's group (93) has introduced the revolutionary concept that miRNA effect on mRNA containing common miRNA recognition elements (MREs) can be affected by ceRNAs (competing endogenous RNAs): RNA transcripts, both protein coding and non-coding, can compete for miRNA binding, thus co-regulating each other.

Beside the existence of other RNAs able to interfere with miRNA function, other mechanisms can affect their regulatory action on target molecules: one example is represented by the evidence that mRNAs can present or develop specific alterations to escape miRNA control. Different studies have indeed reported the existence of oncogenic mRNAs carrying mutations or SNPs in their 3'UTR allowing them to avoid miRNA binding and consequent negative control, as demonstrated for example for let-7 and RAS interaction in lung cancer, where a SNP in the let-7 binding site on RAS 3'UTR alters RAS expression and is associated with higher occurrence risk (94). Another very interesting report is the study published by Sandberg R and colleagues (95), who discovered how proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites. It would be of extreme interest to evaluate the selection for oncogenes with shortened 3'UTRs in different tumor types.

From bench to clinics: how far are we with microRNAs?

The potential of miRNA signatures to differentiate tumors in comparison with their normal counterpart, to discriminate between different subgroups of tumors and to predict outcome

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or response to therapy have focused scientist attention on these small molecules as potential clinical biomarkers, either diagnostic, predictive or prognostic.

Interestingly, it has been observed that primary tumors and metastasis from the same tissue show a similar pattern of microRNAs expression (96). Being a more accurate classifier than mRNA expression studies, miRNA profiling has thus revealed the potential to solve one of the most demanding issues in cancer diagnostic: the origin of metastasis of unknown primary tumors.

Another major issue in clinics is clearly represented by the need of biomarkers for an early diagnosis, which is usually associated with the best prognosis. microRNAs have revealed a great potential as new potential early diagnosis biomarkers, as shown in ductal adenocarcinoma, where overexpression of miR-205 and miR-21 precede phenotypic changes in the ducts (97). Moreover, miRNAs can be reliably extracted and detected from different biological fluids, as blood (either total blood, plasma or serum) (98,99), from circulating exosomes (100), urine (101, saliva (102,103) and even sputum (104,105), and it has been reported that the profile of circulating miRNA of individuals affected by different neoplasias reflects the pattern observed in the tumor tissues (106). This evidence suggests the fascinating possibility of using circulating microRNAs as easily detectable tumor biomarkers, especially for early diagnosis (107,108,109,110).

Concerning the possibility to use miRNAs as prognostic markers to predict outcome, several groups have successfully addressed this issue: after the first evidence in CLL, where a unique microRNA signature was associated with prognostic factors and disease progression in CLL (111) and lung cancer, where miR-155 overexpression and let-7a downregulation were able to predict poor disease outcome (112), several other reports have supported the significance of microRNAs as prognostic biomarkers (113,114).

Even though outcome prediction is certainly relevant, the prediction of response to specific therapies is of even greater clinical value, since it might be useful for a more accurate selection of patients potentially responsive to a specific therapy. miR-21, for example, is sufficient to predict poor response to adjuvant chemotherapy in adenocarcinomas (115) and in pancreatic cancer patients treated with gemcitabine (116).

The correlation between microRNA expression and response to specific therapies has also suggested their promising potential as therapeutic adjuvant, even though this hypothesis mostly derives from *in vitro* studies of gain or loss of function, where candidate miRNAs are initially identified in tumor cell lines with different degrees of resistance to specific therapeutic drugs and then targeted in order to overcome drug resistance (117,118,119,120). Beside chemotherapy, microRNAs can also improve the responsiveness to targeted therapies, as anti-estrogenic therapies (121,122) to Tyrosin Kinase Inhibitors (123).

However, although significant advances have been made for the future role of miRNAs in diagnostics, there have been far fewer reported successes in the development of miRNAs for use in therapy. Indeed, even though a number of reports have described the possibility to reintroduce (124, 125) or inhibit (126,127,128,129,130,131,132) microRNAs (reviewed by Iorio and Croce) (133), there are still many issues that need to be addressed for an effective translation in clinics, as the development of efficient methods of a specific drug delivery, and the accurate prevision of putative unwanted off target effects.

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Conclusions and future perspectives

The past decade has witnessed an explosion of research focused on small non-coding RNAs: conserved among the species and involved in every biological process examined, these tiny RNA molecules have been demonstrated to be crucial regulators of gene expression.

Cancer is defined by abnormal and uncontrolled cell division, a phenotype that arises from the alteration of different mechanisms, leading not only to the misregulation of several protein coding genes, but also to a global change in miRNA profile. Being microRNAs major regulators of gene expression, with roles in nearly every area of cell behavior, development and survival, and able to regulate multiple targets acting as oncogenes or tumor suppressor genes, it is not surprising that their altered expression contributes to a substantial cell re-organization and is causally involved in so many different human tumors.

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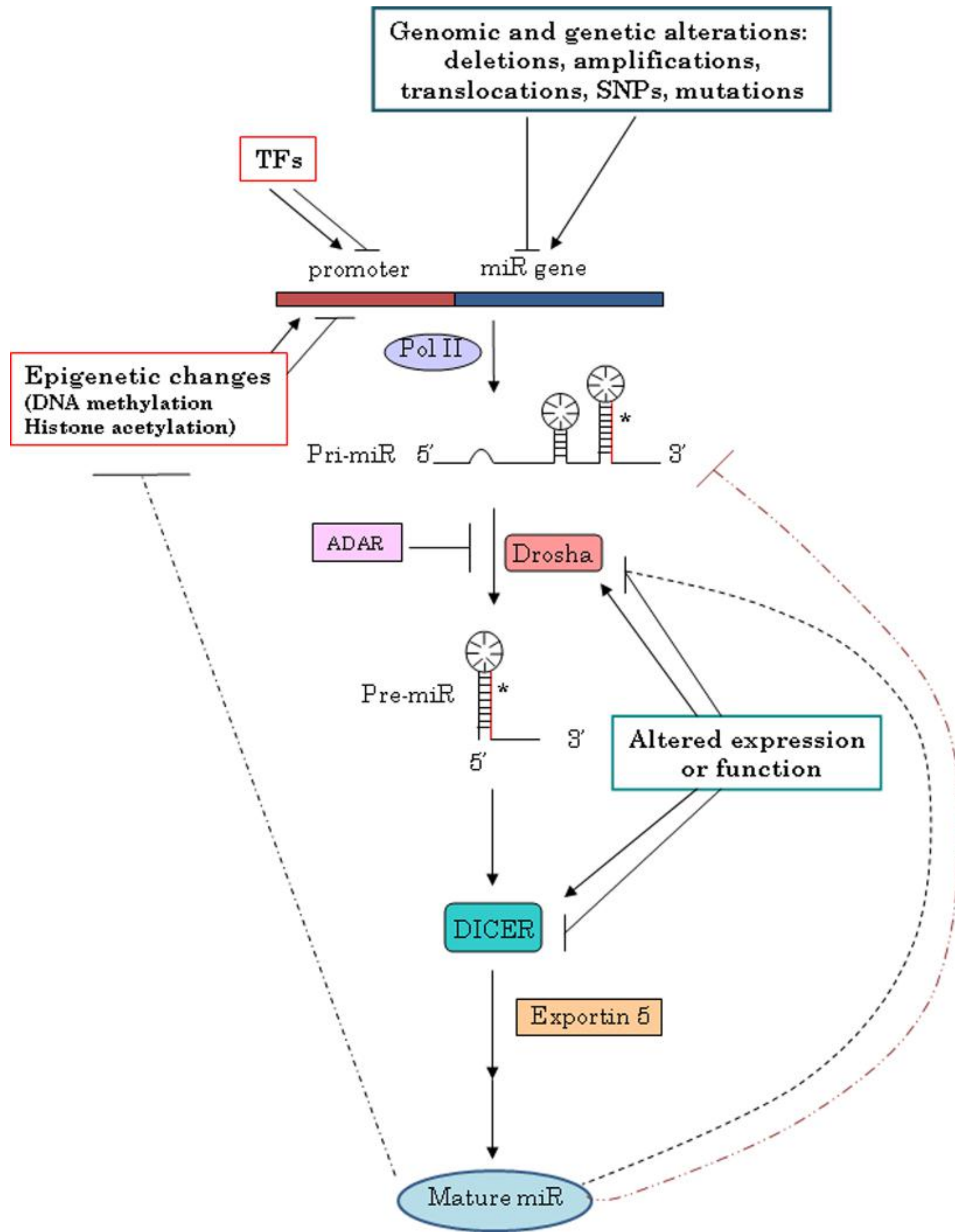

FIGURE 1.
MicroRNA expression regulation.